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Perforin-Deficient CD8$^+$ T Cells Provide Immunity to *Listeria monocytogenes* by a Mechanism That Is Independent of CD95 and IFN-γ but Requires TNF-α[1]

Douglas W. White* and John T. Harty[2]*†

CD8$^+$ T cells are effective mediators of immunity against *Listeria monocytogenes*, but the mechanisms by which they provide antilisterial immunity are poorly understood. CD8$^+$ T cells efficiently lyse target cells in vitro by at least two independent pathways. To test the hypothesis that CD8$^+$ T cell-mediated immunity to *L. monocytogenes* is dependent on perforin or CD95 (Fas, Apo-1), we used C57Bl/6 (B6) and perforin-deficient (PO) mice to generate CD8$^+$ T cell lines specific for the *L. monocytogenes*-encoded Ag listeriolysin O (LLO). Both lines specifically produce IFN-γ and TNF-α, and mediate target cell lysis in vitro. Cytolysis mediated by the PO-derived CD8$^+$ T cell line is delayed relative to the B6-derived line and is completely inhibited by anti-CD95 Abs. In vivo, PO-derived CD8$^+$ T cells provide specific antilisterial immunity in B6 hosts, CD95-deficient hosts, and IFN-γ-depleted hosts. However, PO-derived CD8$^+$ T cells fail to provide antilisterial immunity in hosts depleted of TNF-α. These results indicate that single Ag-specific CD8$^+$ T cells derived from PO mice can mediate antilisterial immunity by a mechanism that is independent of CD95 or IFN-γ, but requires TNF-α. ***The Journal of Immunology,* 1998, 160: 898–905.**

Murine listeriosis is a widely used model of cell-mediated immunity (1). It is clear that both CD4$^+$ and CD8$^+$ T cells are activated in an Ag-specific fashion following infection with *L. monocytogenes* (2). However, a large body of evidence involving specific T cell subset depletion (3) and experiments performed in mice deficient in CD4$^+$ and/or CD8$^+$ T cells (4–6), indicates that CD8$^+$ T cells are the most effective mediators of antilisterial immunity. These observations are consistent with the life cycle of *L. monocytogenes*, an intracellular bacterium that gains access to and multiplies within the cytoplasm of the host cell (7). Not only is the cytosol protected from the host's humoral immune system, it is the initiation site of events that lead to the presentation of peptides bound to MHC class I molecules on the surface of the infected cell. Recognition of pathogen-derived peptides bound to MHC class I molecules is a required event in specific CD8$^+$ T cell-mediated immunity to intracellular microbes.

Activated CD8$^+$ T cells are capable of elaborating a diverse array of effector functions. Following Ag-specific stimulation, CD8$^+$ T cells produce a broad range of cytokines including IFN-γ and TNF-α. These cytokines, which are also produced by other cell types, have been shown to play critical roles in innate resistance of mice to *L. monocytogenes* infection (2). In contrast, we have used IFN-γ gene knockout mice to provide evidence that CD8$^+$ T cell immunity to *L. monocytogenes* can occur in the absence of IFN-γ (8). These experiments suggested that CD8$^+$ T cell-mediated cytolysis may be important in specific immunity to *L. monocytogenes*.

The ability of activated CD8$^+$ T cells to carry out in vitro cytolysis is well documented. Following ligation of the TCR-$\alpha\beta$ by the appropriate MHC class I-peptide complex, the CD8$^+$ T cell induces its target to undergo programmed cell death (PCD).[3] Two independent pathways account for the majority of in vitro target cell lysis by CD8$^+$ T cells (9). One is a perforin-dependent pathway, mediated by granzymes, which are serine proteases found in the cytoplasmic granules of activated CD8$^+$ T cells that gain access to the cytoplasm of the target cell and induce PCD by activation of the caspase cascade. Although the mechanism(s) by which perforin and granzymes are involved in cytolysis remain controversial (10, 11), the dependence of some cytolytic activity on perforin is clear (12–15).

The other pathway by which CD8$^+$ T cells are capable of efficient in vitro cytolysis is dependent upon interactions between CD95 ligand (CD95L, Fas ligand) on the activated CD8$^+$ T cell and CD95 on the target cell. Ligation of CD95, in some cell types, leads to intracellular signaling events that also activate the caspase cascade and induce PCD (16).

The relevance of perforin-dependent killing in CD8$^+$ T cell-mediated resistance to infectious disease was demonstrated by experiments using mice with a targeted disruption of the perforin gene (PO mice). These animals are susceptible to infection with lymphocytic choriomeningitis virus (LCMV) in spite of the ability of their CD8$^+$ T cells to be specifically activated during the infection (12, 15). The role of perforin in CD8$^+$ T cell-dependent immunity to other viruses has been subsequently explored (17–20) and remains a topic of current investigation.

The role of perforin in resistance to *L. monocytogenes* was addressed by Kagi and colleagues using PO mice (21). In these experiments, it was demonstrated that CD8$^+$ T cells from immune

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[3] Abbreviations used in this paper: PCD, programmed cell death; PO, perforin deficient; LLO, listeriolysin O; LCMV, lymphocytic choriomeningitis virus.

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H-2^b MHC PO mice are deficient, relative to CD8$^+$ T cells derived from control mice, in their ability to transfer antilisterial immunity to naive hosts. These experiments suggest that perforin-dependent cytotoxicity plays an important role in antilisterial immunity mediated by CD8$^+$ T cells. Unfortunately, little information exists regarding *L. monocytogenes*-derived Ags presented by H-2^b MHC molecules. Thus, the activation status of the CD8$^+$ T cell compartment of the PO donor mice could not be easily evaluated. Therefore, an alternative interpretation of these data is that the doses of *L. monocytogenes* used to immunize donor mice did not activate the CD8$^+$ T cell compartment equivalently in the PO donors and the B6 donors. The finding that PO mice exhibit more resistance than B6 mice to primary challenge with *L. monocytogenes* is consistent with this alternative interpretation (21).

In the present study, we confirm the findings of Kagi and colleagues that splenocytes from immunized PO mice are deficient relative to splenocytes from B6 mice in the transfer of antilisterial immunity. We then make use of single Ag-specific CD8$^+$ T cell lines that are uniformly restimulated in vitro to test the hypothesis that perforin is required for CD8$^+$ T cell-mediated immunity to *L. monocytogenes*.

Materials and Methods

Mice

C57Bl/6 (H-2^b MHC) mice were obtained from the National Cancer Institute (Frederick, MD). PO (H-2^b MHC) mice were kindly provided by Dr. W. R. Clark (15) and then maintained by brother-sister mating. MRL *lpr/lpr* (H-2^b MHC) mice were the kind gift of Dr. M. O. Dailey at the University of Iowa. All mice were housed under specific pathogen-free conditions at the University of Iowa and were generally used at 8 to 12 wk of age in an age- and sex-matched fashion.

Bacteria

L. monocytogenes strain 10403s (22) and *Salmonella typhimurium* strain SL1344 (23) are both resistant to streptomycin and were used as previously described (24). Briefly, bacteria were grown in tryptic soy broth to an OD_{600} of approximately 0.1, diluted in pyrogen-free 0.9% sodium chloride (Abbott Laboratories, North Chicago, IL) and injected i.v. in 0.2- to 0.5-ml vol per animal. Aliquots were plated onto tryptic soy agar containing 50 μg/ml of streptomycin to verify the number of CFU injected.

Cell lines, Abs, flow cytometry, and T cell depletion

P815 is a DBA/2-derived mastocytoma (H-2^d MHC) (American Type Culture Collection (ATCC), Rockville, MD; TIB-64); EL4 is a B6-derived thymoma cell line (H-2^b MHC) (ATCC TIB-39); EL4-LLO refers to EL4 cells stably transfected with a plasmid construct expressing the *L. monocytogenes* Ag listeriolysin O (LLO) and neo-resistance (25); L-929 is a C3H/An-derived fibroblast cell line (H-2^k MHC) (ATCC CCL-1); L-Kb refers to L-929 cells stably transfected with the gene for H-2K^b (26) and neo-resistance; L-Kb-LLO refers to L-929 cells stably transfected with the LLO expression construct (27) and the H-2K^b gene. Electroporation and selection of transfectants with G-418 were performed as described (24). Parental cell lines were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS, antibiotics, L-glutamine, HEPES buffer, and 2-ME (RP10 (24)). Transfected cells were maintained in RP10 supplemented with G-418 at 400 μg/ml.

Expression of H-2K^b by L-Kb-LLO cells was confirmed in ^{51}Cr release assays in which CD8$^+$ T cells specific for the OVA peptide SIINFEKL bound to H-2K^b (28, 29) were observed to specifically lyse L-Kb-LLO cells in the presence (but not in the absence) of 2 nM of SIINFEKL.

mAbs, which were purified from culture supernatants and quantitated as previously described (8), were: rat anti-mouse TNF-α IgG1 (XT22 and XT3 (30) used in combination at a mass ratio of 1:1) and rat anti-mouse IFN-γ IgG1 (XMG1.2 (31)). Control polyclonal rat IgG was purchased from Sigma (St. Louis, MO). Armenian hamster anti-mouse CD95 IgG (Jo2) and control Armenian hamster polyclonal IgG were purchased from PharMingen (San Diego, CA). Flow cytometric analysis of T cell subsets was performed as previously described (8) using FITC-conjugated anti-CD8 (53.6–7; Sigma) and phycoerythrin-conjugated anti-CD4 (H129.19; Sigma). T cell subset depletion by mAb and complement was performed using rat anti-mouse CD4 (RL172) and rat anti-mouse CD8 (3.168) as previously described (8).

Generation and maintenance of CD8$^+$ T cell lines

CD8$^+$ T cell lines specific for LLO were derived from B6 and PO mice using methods previously described (25). Briefly, 2 to 4 $\times$ 10^7 splenocytes from mice injected 7 to 10 days previously with 10^4 CFU of virulent *L. monocytogenes* were incubated with 3 $\times$ 10^6 irradiated (150 Gy) EL4-LLO cells in RP10. Subsequent weekly restimulations were conducted by combining 1 to 3 $\times$ 10^6 responder cells with 3 $\times$ 10^6 irradiated stimulator cells and approximately 4 $\times$ 10^7 irradiated (30 Gy) syngeneic splenocytes in RP10 supplemented with 5% supernatant from Con A-stimulated rat spleen cells and 50 mM α-methyl mannoside.

In vitro characterization of CD8$^+$ T cell lines

^{51}Cr release assays were performed as previously described (32, 8). Briefly, 10^4 labeled target cells were combined with effector cells at the indicated ratios in 200 μl of RP10 per well in round-bottom 96-well plates. Following a 4- to 8.5-h incubation (as indicated), 100 μl per well of supernatant was harvested and assayed for ^{51}Cr release. Percent specific release of ^{51}Cr was calculated by the formula:

$$100 \times \frac{(\text{experimental cpm} - \text{spontaneous cpm})}{(\text{total cpm} - \text{spontaneous cpm}).}$$

Spontaneous release was less than 20% of total in all experiments.

TNF-α was quantitated using WEHI 164 clone 13 cells, which die in the presence of TNF (33). Supernatants (50 μl from each well) from the indicated ^{51}Cr release assays or analogous (nonradioactive) co-incubations of CD8$^+$ T cells and target cells were added to 50 μl of WEHI 164 cells (6 $\times$ 10^5/ml in RP10 supplemented with 2 μg/ml of actinomycin D and 40 mM $LiCl_2$) in flat-bottom 96-well plates. Following overnight incubation at 37°C and 7% CO_2, 10 μl/well of Alamar blue (Acumed, West Lake, OH) was added. The percent death of the indicator cells, a relative measure of TNF production, was determined 2 to 6 h after addition of Alamar blue from the OD_{570} and OD_{600} according to the manufacturer's protocol. Production of TNF by target cells in the absence of CD8$^+$ T cells was not detected. In some experiments, mAb specific for TNF-α (a 1:1 mix of XT22 and XT3 at a final concentration of 50 μg/ml) or control rat IgG were added to the target cells before addition of the CD8$^+$ T cells. Recombinant murine TNF-α was used as a control and to determine the detection limits of the WEHI bioassay. Concentrations $<$1 pg/ml of rTNF-α were routinely detected using this assay.

IFN-γ was quantitated by ELISA. The 96-well flat-bottom plates were coated with 600 ng/ml of XMG1.2 in bicarbonate buffer (pH 9.6) at 4°C for 24 h, blocked with 1% BSA in PBS at 37°C for 1 h, and washed with 0.05% Tween-20 in PBS. Supernatants (100 μl from each well) from co-incubations of effector cells and target cells and rIFN-γ controls were added and incubated at room temperature for 2 to 3 h. Following three rinses, 100 μl per well of rabbit anti-mouse IFN-γ (a kind gift of Dr. John Cowdery, University of Iowa; diluted 1:1500 in PBS-Tween) was added and incubated at room temperature for 1 to 2 h. Following three rinses, 100 μl per well of alkaline phosphatase-conjugated goat anti-rabbit Ig (Sigma; diluted 1:20,000 in PBS-Tween) was added and incubated at 37°C for 1 h. Following three rinses, alkaline phosphatase substrate (Sigma) in 1 M diethanolamine, 0.5 mM $MgCl_2$ (pH 9.8) was added and developed according to the manufacturer's protocol. OD was measured at 405 nm. Limit of detection was 10 U/ml.

Adoptive transfer experiments

The capacity of splenocytes derived from immunized animals and CD8$^+$ T cell lines to mediate antilisterial immunity in vivo was quantitated using adoptive transfer assays as described previously (32, 8). Briefly, RBC-depleted splenocytes from donor mice immunized 7 to 10 days previously with approximately 10^4 virulent *L. monocytogenes*, or CD8$^+$ T cells restimulated in vitro 7 to 9 days previously, were harvested, washed in antibiotic-free buffer, and resuspended in pyrogen-free 0.9% sodium chloride. Cells were delivered i.v. in 0.2 to 0.5 ml vol into naive B6 or MRL *lpr*/*lpr* host mice. Within 2 h, host mice, including uninjected or saline-injected naive controls, were challenged i.v. with the indicated dose of 10403s or SL1344. CFU/spleen and liver were determined 3 days postchallenge by homogenizing the spleens and livers in 0.2% IGEPAL (Sigma), plating 10-fold serial dilutions onto tryptic soy agar containing 50 μg/ml of streptomycin, and calculating colony count averages after overnight incubation at 37°C.

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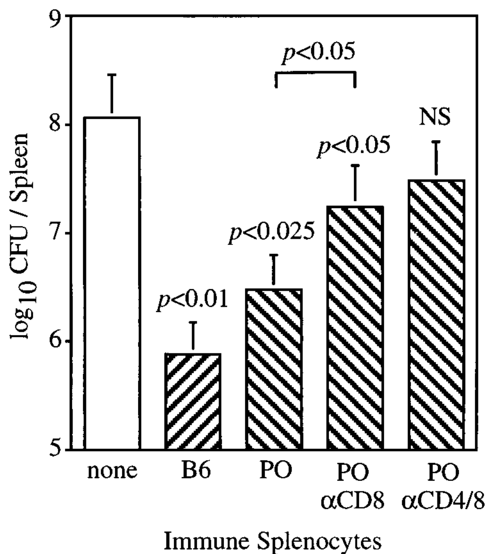

FIGURE 1. Immune splenocytes from perforin knockout (PO) mice are deficient compared with immune splenocytes from C57Bl/6 (B6) mice but mediate $CD8^+$ T cell-dependent immunity against *L. monocytogenes*. Naive B6 mice were injected i.v. with 20 to 50 × 10^6 splenocytes from PO or B6 mice that had been immunized 7 days previously with approximately 10^4 CFU of *L. monocytogenes*. As indicated, immune splenocytes were depleted of $CD4^+$ (αCD4) and/or $CD8^+$ (αCD8) T cells with mAbs and complement. Splenocyte-injected mice and noninjected controls were then challenged with 1.4 to 1.7 × 10^5 *L. monocytogenes*, and CFU/spleen were determined 3 days later. Similar results were obtained in the spleen and liver (data not shown). These data are pooled from two independent experiments, each with three mice per group, since two of three control mice died in one experiment. Efficiency of depletion averaged 90% for αCD8-treated cells and 73% for αCD4/8-treated cells as determined by flow cytometric analysis (data not shown). Data are presented as mean $\log_{10}$ CFU + S.D. Student's *t* test was used in statistical analysis; *P* values are shown for each group compared with the control group in the same experiment, which did not receive splenocytes; an additional *P* value comparing PO and $CD8^+$ T cell-depleted PO splenocytes is also indicated; NS, not significant.

Results

PO splenocytes from L. monocytogenes*-infected mice provide immunity to infection that is partially dependent on $CD8^+$ T cells*

Perforin is a vital component in the protective $CD8^+$ T cell response to some but not all viruses (17–20, 34–36). Previous studies with perforin gene knockout (PO) mice suggest a role for perforin-mediated cytolysis in $CD8^+$ T cell-mediated immunity to *L. monocytogenes* (21). Consistent with these studies, we found that splenocytes from immunized B6 (H-2^b MHC) mice were more effective than splenocytes from immunized PO (H-2^b MHC) mice at transferring antilisterial immunity to B6 recipients as measured by the number of *L. monocytogenes* recovered at 3 days postinfection from control groups and splenocyte recipients (Fig. 1). Depletion of the $CD8^+$ T cells from the immune PO splenocytes further reduced their ability to transfer antilisterial immunity (Fig. 1), suggesting the presence of a perforin-independent, $CD8^+$ T cell-mediated pathway of antilisterial immunity.

PO-derived LLO-specific $CD8^+$ T cells produce IFN-γ and TNF-α but are deficient in target cell lysis in vitro

To further address the impact of perforin deficiency on $CD8^+$ T cell immunity to *L. monocytogenes*, we generated $CD8^+$ T cell lines, specific for the same *L. monocytogenes* Ag, from MHC-matched PO and B6 mice. Splenocytes from B6 and PO mice, immunized 7 days previously with approximately 10^4 CFU of virulent *L. monocytogenes*, were cultured in vitro with syngeneic irradiated stimulator cells that express the *L. monocytogenes* $CD8^+$ T cell Ag, LLO (EL4-LLO cells) (25). Following 4 wk of in vitro restimulation, flow cytometric analysis demonstrated that >95% of the cells displayed a $CD4^-$, $CD8^+$ phenotype (data not shown).

To measure cytokine production and verify Ag-specificity, LLO-specific $CD8^+$ T cells were assayed for IFN-γ and TNF-α release in response to Ag-expressing and control target cells. Both B6- and PO-derived LLO-specific $CD8^+$ T cells produced IFN-γ when incubated with EL4-LLO cells, but not in response to the parental, nontransfected EL4 cells (Fig. 2*A*). Similarly, TNF-α was produced by both B6- and PO-derived LLO-specific $CD8^+$ T cells in response to EL4-LLO but not EL4 cells (Fig. 2*B*). Nontransfected EL4 cells, incubated with HPLC-purified peptides derived from *L. monocytogenes*-infected B6 spleens (27), also induced TNF-α production by both B6- and PO-derived LLO-specific $CD8^+$ T cells (data not shown). The same peptide fraction sensitized EL4 cells for lysis by B6-derived LLO-specific $CD8^+$ T cells (data not shown).

Cytolysis mediated by LLO-specific $CD8^+$ T cells derived from PO and B6 mice was compared using chromium release assays. Whereas B6-derived LLO-specific $CD8^+$ T cells were capable of high levels of specific lysis of LLO-expressing target cells, PO-derived LLO-specific $CD8^+$ T cells mediated minimal levels of specific lysis of the same target cells in a short-term (4 h) assay (Fig. 2*C*). Short-term assays also revealed that target cell lysis by B6-derived LLO-specific $CD8^+$ T cells was inhibited by EGTA, consistent with the Ca^{2+} dependency of perforin-mediated cytolysis (37) (data not shown). These results are consistent with a lack of perforin-dependent cytolysis by PO-derived $CD8^+$ T cells and a functional perforin-dependent pathway in $CD8^+$ T cells derived from B6 mice. Thus, we have generated LLO-specific $CD8^+$ T cell lines from B6 and PO mice that produce IFN-γ and TNF-α in an Ag-specific fashion but that differ in their ability to perform perforin-dependent cytolysis.

PO-derived LLO-specific $CD8^+$ T cells mediate delayed target cell lysis and possess a functional CD95-dependent cytolytic pathway

$CD8^+$ T cells can also kill target cells via the CD95L/CD95 pathway (38). To test the ability of PO-derived LLO-specific $CD8^+$ T cells to mediate perforin-independent cytolysis, we performed extended chromium release assays (Fig. 3*A*). This approach revealed that PO-derived LLO-specific $CD8^+$ T cells were able to carry out Ag-specific target cell lysis that is delayed compared with cytolysis mediated by B6-derived $CD8^+$ T cells. We then tested whether in vitro lytic activity mediated by PO-derived LLO-specific $CD8^+$ T cells was dependent upon CD95L or TNF-α. Extended assays revealed that specific target cell lysis by PO-derived LLO-specific $CD8^+$ T cells was not inhibited by neutralizing mAb against TNF-α (Fig. 3*A*). Control assays for TNF-α, performed on supernatants derived from the cytotoxicity assays in Figure 3*A*, verified the complete neutralization of soluble TNF-α by the anti-TNF-α mAb (Fig. 3*B*). To test for CD95L/CD95-dependent killing, we used CD95-expressing (data not shown) murine fibroblasts as targets. PO-derived LLO-specific $CD8^+$ T cell-mediated cytolysis of L929 murine fibroblasts (H-2^k MHC) transfected with expression constructs for H-$2K^b$ and LLO (L-Kb-LLO), but not L929 cells transfected with H-$2K^b$ alone (L-Kb) (Fig. 3*C*). Ag-specific lysis by PO-derived LLO-specific $CD8^+$ T cells was completely

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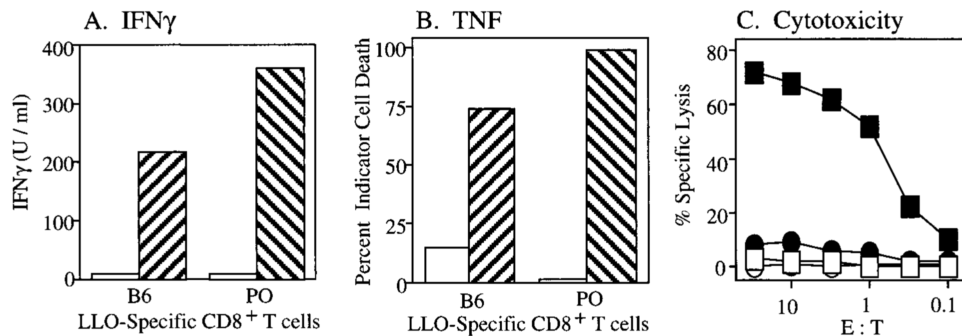

FIGURE 2. PO-derived LLO-specific CD8$^+$ T cells produce TNF-α and IFN-γ in an Ag-specific fashion, but are deficient in short-term cytolysis. *A*, LLO-specific CD8$^+$ T cells derived from B6 or PO mice were incubated for 48 h with EL4 cells (open bars) or EL4-LLO cells (hatched bars) at an E:T of 0.3:1. IFN-γ in the supernatants was quantitated by ELISA. *B*, LLO-specific CD8$^+$ T cells were incubated overnight with the same target cells as in *A* at an E:T of 3:1. TNF in the supernatant was quantitated in a bioassay using WEHI 164 clone 13 cells (33), which die in the presence of TNF. *C*, LLO-specific CD8$^+$ T cells derived from B6 (squares) or PO (circles) mice were incubated at the indicated E:T with EL4 (open symbols) or EL4-LLO (closed symbols) cells. Cytolysis was measured in a standard 4-h ^{51}Cr release assay. All data are representative of at least three independent experiments.

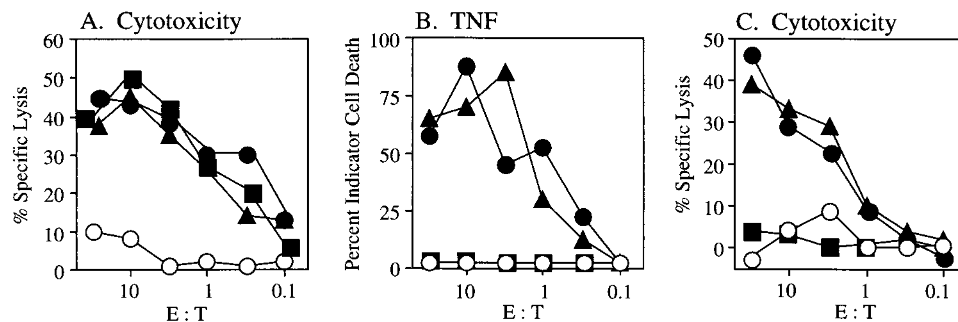

FIGURE 3. PO-derived LLO-specific CD8$^+$ T cells mediate delayed, CD95-dependent cytotoxicity in vitro, which is not inhibited by Abs that neutralize TNF-α. *A*, Cytolysis of EL4 (open symbols) or EL4-LLO (closed symbols) target cells by PO-derived LLO-specific CD8$^+$ T cells was measured in an extended (8.5-h) ^{51}Cr release assay in the absence (circles) or presence of control IgG (triangles) or mAb against TNF-α (squares) at 50 μg/ml. *B*, Supernatants from the extended ^{51}Cr release assay in *A* were assayed for TNF. Same symbols apply as in *A*. *C*, PO-derived LLO-specific CD8$^+$ T cells were incubated for 6 h with L929 cells (H-2^k MHC) stably expressing H-2K^b (L-Kb) (open symbols) or H-2K^b and LLO (L-Kb-LLO) (closed symbols) in the absence (circles) or presence of control IgG (triangles) or anti-CD95 mAb (squares) at 5 μg/ml. All data are representative of at least three independent experiments.

inhibited in the presence of a mAb that binds CD95 (Fig. 3*C*). Six hours of exposure to the same concentration of anti-CD95 mAb did not kill the targets nor the CD8$^+$ T cells (data not shown). These experiments demonstrate that LLO-specific CD8$^+$ T cells from PO mice are capable of CD95-dependent target cell lysis in vitro.

LLO-specific CD8$^+$ T cells from PO mice transfer immunity against L. monocytogenes *in the presence and absence of CD95 (Fas)*

B6-derived LLO-specific CD8$^+$ T cells provide antilisterial immunity in adoptive transfer assays (25). To determine the requirement for perforin in CD8$^+$ T cell-mediated immunity against *L. monocytogenes*, LLO-specific CD8$^+$ T cells were transferred into naive B6 host mice, which were subsequently challenged with approximately 10 LD$_{50}$ of *L. monocytogenes*. B6- and PO-derived LLO-specific CD8$^+$ T cells provide antilisterial immunity in both the spleen and liver (Fig. 4). In the liver, B6- and PO-derived LLO-specific CD8$^+$ T cells transferred similar degrees of immunity, as measured by reduction in CFU per organ (Fig. 4*C*). Over multiple experiments, the degree of immunity in the spleen transferred by PO-derived LLO-specific CD8$^+$ T cells varied from intermediate (approximately 2 log$_{10}$ CFU reduction as seen in Fig. 4*A*) to equivalent (3–4 log$_{10}$ CFU reduction) to that typically seen with B6-derived LLO-specific CD8$^+$ T cells (Fig. 6*A*). In a survival study, 100% of mice that received PO-derived LLO-specific CD8$^+$ T cells survived 1 wk postchallenge, while all mice that did not receive T cells died within 4 days. These results demonstrate that PO CD8$^+$ T cells can mediate significant antilisterial immunity in both the spleen and liver.

LLO-specific CD8$^+$ T cells mediate CD95-dependent target cell lysis in vitro (Fig. 3). To determine whether B6- and PO-derived LLO specific CD8$^+$ T cells provide antilisterial immunity via a CD95-dependent pathway, B6- and PO-derived LLO-specific CD8$^+$ T cells were transferred into naive H-2^b MHC MRL *lpr/lpr* host mice (genetically deficient in CD95 expression (39)). T cell recipients and control MRL *lpr/lpr* mice were then challenged with virulent *L. monocytogenes*, and CFU/organ were determined 3 days later. The results revealed that both B6- and PO-derived LLO-specific CD8$^+$ T cells provide antilisterial immunity in the spleen (Fig. 4*B*) and liver (Fig. 4*D*) in the absence of CD95. In these experiments, the degree of immunity transferred by PO-derived LLO-specific CD8$^+$ T cells was consistently equivalent to the degree of immunity transferred by B6-derived LLO-specific CD8$^+$ T cells. Thus, in the absence of both pathways required for efficient in vitro cytolysis (perforin-dependent and CD95-dependent (9)), LLO-specific CD8$^+$ T cells are capable of providing antilisterial immunity.

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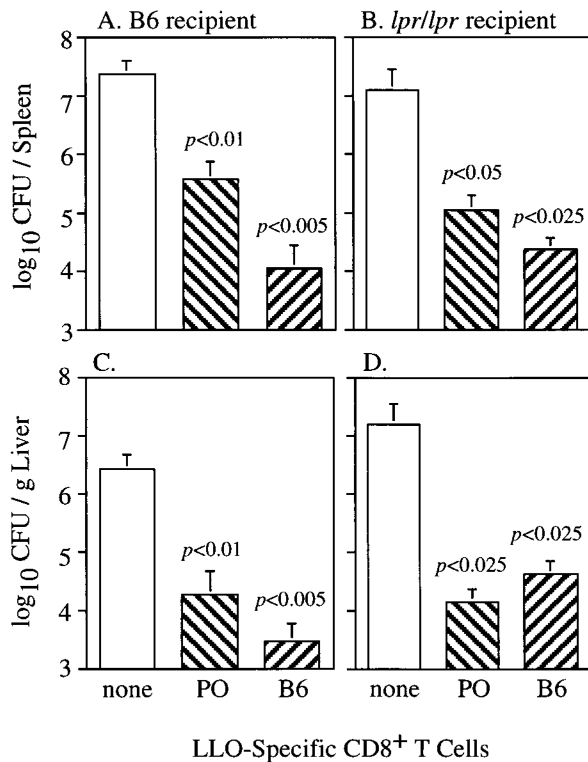

FIGURE 4. PO-derived LLO-specific $CD8^+$ T cells transfer antilisterial immunity to wild-type B6 mice and CD95-deficient mice. Naive B6 (*A* and *C*) and MRL *lpr/lpr* mice (*B* and *D*) were injected i.v. with LLO-specific $CD8^+$ T cells derived from B6 or PO mice and within hours challenged with wild-type *L. monocytogenes*. CFU from the spleen (*A* and *B*) and liver (*C* and *D*) were quantitated 3 days postchallenge. Data are presented as mean $\log_{10}$ CFU + S.D. for three animals per group. Each experiment was performed independently at least twice with similar results. Student's *t* test was used in statistical analysis; *P* values are shown for each group compared with the control group in the same experiment, which did not receive T cells; NS, not significant. T cells transferred: 1.5×10^7 (*A* and *C*); 7×10^6 (*B* and *D*). Challenge with *L. monocytogenes*: 8×10^4 (*A* and *C*); 2×10^5 (*B* and *D*) CFU.

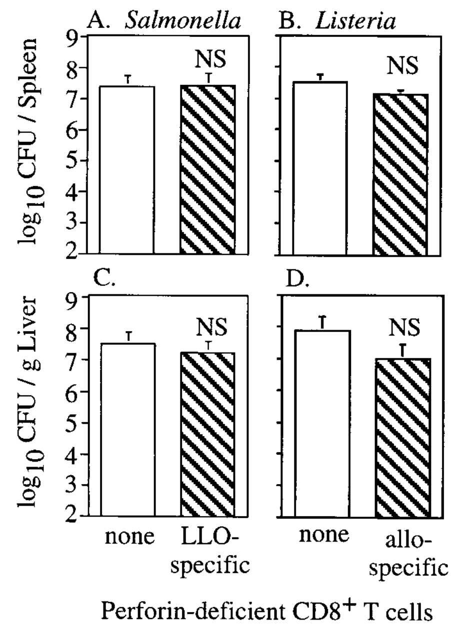

FIGURE 5. In vivo immunity mediated by PO-derived LLO-specific $CD8^+$ T cells is Ag-specific. Naive B6 mice were injected i.v. with $CD8^+$ T cells derived from PO mice specific for LLO (*A* and *C*) or allospecific (anti-H-2^d) (*B* and *D*) and within hours challenged with wild-type *S. typhimurium* (*A* and *C*) or *L. monocytogenes* (*B* and *D*). CFU from the spleen (*A* and *B*) and liver (*C* and *D*) were quantitated 3 days postchallenge. Data are presented as mean $\log_{10}$ CFU + S.D. for two to three animals per group. Data are representative of at least three independent experiments with each T cell line. Student's *t* test was used in statistical analysis; NS, not significant. T cells transferred: 5×10^6 (*A* and *C*); 1×10^7 (*B* and *D*). Challenge with *S. typhimurium*: 7×10^2 CFU. Challenge with *L. monocytogenes*: 1.6×10^5 CFU.

In vivo immunity mediated by PO-derived $CD8^+$ T cells is Ag-specific

The in vivo antilisterial immunity conducted by PO-derived LLO-specific $CD8^+$ T cells was Ag-specific. PO-derived LLO-specific $CD8^+$ T cells did not cause a reduction in CFU in B6 mice challenged with the unrelated bacterium *S. typhimurium* (Fig. 5, *A* and *C*). Likewise, transfer of a large number of PO-derived alloreactive (H-2^b anti-H-2^d) $CD8^+$ T cells into naive B6 mice did not result in a significant reduction in CFU following challenge with *L. monocytogenes* (Fig. 5, *B* and *D*). Specificity of PO allospecific $CD8^+$ T cells for H-2^d was verified in extended chromium release assays using P815 target cells (data not shown). These results confirm previous findings that $CD8^+$ T cells provide Ag-specific immunity in transfer assays (32) and extend this finding to immunity mediated by PO-derived LLO-specific $CD8^+$ T cells.

LLO-specific $CD8^+$ T cells from PO mice transfer antilisterial immunity to hosts depleted of IFN-γ

Previous studies with in vivo Ab neutralization (40) and IFN-γ knockout mice (8) revealed that $CD8^+$ T cell-derived IFN-γ was not required for antilisterial immunity but did not rule out the possibility that $CD8^+$ T cell-derived IFN-γ normally contributes to antilisterial immunity or that it plays a major role in the absence of perforin-dependent cytotoxicity (21). PO-derived LLO-specific $CD8^+$ T cells clearly produce IFN-γ in response to specific target cells (Fig. 2). To determine whether LLO-specific $CD8^+$ T cells from PO mice require IFN-γ to mediate antilisterial immunity, we performed transfer experiments into naive B6 mice that had been injected 1 day previously with a high dose (1 mg/animal) of neutralizing anti-IFN-γ mAb. Since IFN-γ plays an important role in the innate response to *L. monocytogenes* (8, 25), pretreatment of mice with neutralizing mAb to IFN-γ results in a severe exacerbation of infection. Preliminary studies revealed that doses of mAb as low as 500 μg/animal resulted in maximal exacerbation of infection at 3 days postchallenge (data not shown). Previous studies have shown that mAb neutralization of IFN-γ in normal mice (40) exacerbates *L. monocytogenes* infection to a similar degree as seen in IFN-γ gene knockout mice (8). To prevent an increased bacterial load in IFN-γ-depleted animals, which would make it difficult to compare T cell function in depleted vs control animals, the dose of *L. monocytogenes* used to challenge IFN-γ-depleted animals was reduced by 50% relative to the dose used to challenge rat IgG-treated animals. Even with the reduced challenge dose, *L. monocytogenes* infection in mice that received no T cells was exacerbated in the spleens (>30-fold) and the livers (>100-fold) by pretreatment with anti-IFN-γ mAb compared with rat IgG-pretreated mice (Fig. 6). These results, combined with studies of *L. monocytogenes* infection in IFN-γ gene knockout mice (8) and mice injected with neutralizing mAb against IFN-γ (40), indicate that the mAb treatment resulted in effective neutralization of IFN-γ

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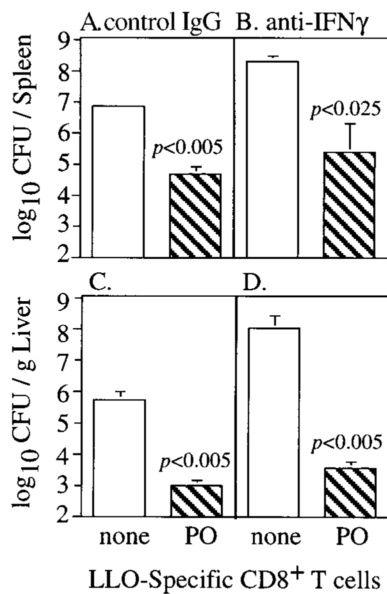

FIGURE 6. PO-derived LLO-specific CD8$^+$ T cells transfer antilisterial immunity to IFN-γ-depleted mice. Naive B6 host mice were injected i.p. with 1 mg/animal control rat IgG (*A* and *C*) or mAb against IFN-γ (*B* and *D*) 1 day before challenge. Host mice were then injected i.v. with LLO-specific CD8$^+$ T cells derived from PO mice and within hours challenged with wild-type *L. monocytogenes*. CFU from the spleen (*A* and *B*) and liver (*C* and *D*) were quantitated 3 days postchallenge. Data are presented as mean $\log_{10}$ CFU + S.D. for three animals per group. Data are representative of three independent experiments. Student's *t* test was used in statistical analysis; *P* values are shown for each group compared with the control group in the same experiment, which did not receive T cells; T cells transferred: 6×10^6. Challenge with *L. monocytogenes*: 3.6×10^4 (*A* and *C*); 1.8×10^4 (*B* and *D*) CFU.

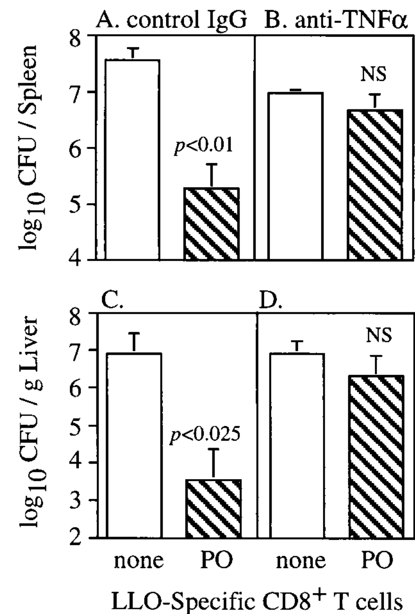

FIGURE 7. PO-derived LLO-specific CD8$^+$ T cells fail to transfer antilisterial immunity to TNF-α-depleted host mice. Naive B6 host mice were injected i.p. with 0.5 mg/animal control rat IgG (*A* and *C*) or mAb against TNF-α (*B* and *D*) 1 day before challenge. Host mice were then injected i.v. with LLO-specific CD8$^+$ T cells derived from PO mice and within hour challenged with wild-type *L. monocytogenes*. CFU from the spleen (*A* and *B*) and liver (*C* and *D*) were quantitated 3 days postchallenge. Data are presented as mean $\log_{10}$ CFU + S.D. for three animals per group. Data are representative of two independent experiments with varying challenge doses. Student's *t* test was used in statistical analysis; *P* values are shown for each group compared with the control group in the same experiment, which did not receive T cells; NS, not significant. T cells transferred: 5×10^6. Challenge with *L. monocytogenes*: 8.7×10^4 (*A* and *C*); 8.7×10^2 (*B* and *D*) CFU.

in vivo. Despite the severity of the infection, PO-derived LLO-specific CD8$^+$ T cells provided immunity to *L. monocytogenes* infection in anti-IFN-γ-treated mice (Fig. 6, *B* and *D*) that is at least equivalent to that seen in mice treated with rat IgG (Fig. 6, *A* and *C*). Thus, antilisterial immunity mediated by PO-derived LLO-specific CD8$^+$ T cells is not inhibited by neutralization of IFN-γ.

LLO-specific CD8$^+$ T cells from PO mice fail to transfer antilisterial immunity to hosts depleted of TNF-α

Besides IFN-γ, PO-derived LLO-specific CD8$^+$ T cells produce TNF-α in response to specific target cells (Fig. 2). To determine whether LLO-specific CD8$^+$ T cells from PO mice require TNF-α to mediate antilisterial immunity, we performed transfer experiments into naive B6 mice that had been injected 1 day previously with mAb that neutralize TNF-α. In contrast to the result observed following depletion of IFN-γ, depletion of TNF-α severely abrogated the ability of PO-derived CD8$^+$ T cells to mediate antilisterial immunity in vivo (Fig. 7). Polyclonal splenocytes from previously immunized PO mice also failed to transfer immunity to naive animals that had been treated with anti-TNF-α mAb, but provided normal levels of protection in hosts preinjected with control IgG (data not shown). As is the case with IFN-γ, TNF-α plays an important role in the innate response to *L. monocytogenes* (8, 25). Consequently, the dose of *L. monocytogenes* used to challenge TNF-α-depleted animals was reduced relative to the dose used to challenge rat IgG-treated animals. Reduction of the challenge dose by 100-fold resulted in approximately equal degrees of infection in TNF-α-depleted animals compared with rat IgG-treated mice that received no T cells (Fig. 7). Despite the lower challenge dose, PO-derived LLO-specific CD8$^+$ T cells failed to mediate immunity to *L. monocytogenes* in anti-TNF-α-treated mice (Fig. 7, *B* and *D*), whereas the same T cells provided significant immunity in mice treated with control rat IgG (Fig. 7, *A* and *C*). In additional experiments, in which TNF-α-depleted and control animals were challenged with equivalent high doses of *L. monocytogenes*, PO-derived CD8$^+$ T cells also failed to provide protection (data not shown). Thus, antilisterial immunity mediated by PO-derived LLO-specific CD8$^+$ T cells is dependent on TNF-α.

Discussion

Our experiments demonstrate that PO CD8$^+$ T cells, specific for a single *L. monocytogenes* Ag, provide significant immunity to *L. monocytogenes* infection in vivo. Immunity mediated by PO-derived, LLO-specific CD8$^+$ T cells was equivalent in wild-type and CD95-deficient host mice. Thus, CD8$^+$ T cell-mediated immunity to an intracellular bacterial pathogen can function independently of perforin and CD95, which define the two major pathways of cell-mediated cytolysis (9). Furthermore, these results uncover a perforin-independent pathway of CD8$^+$ T cell immunity that does not require IFN-γ but does require TNF-α.

The use of Ag-specific CD8$^+$ T cell lines from wild-type and perforin gene knockout mice allowed us to extensively characterize the effector functions of these cells in vitro before their analysis in vivo. While the perforin-dependent pathway accounts for most

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short-term in vitro cytolysis, CD8^{+} T cells can also kill target cells via the CD95L/CD95 pathway (38) and perhaps by production of TNF-α (41, 42). Our in vitro studies demonstrated that both B6-derived and PO-derived LLO-specific CD8^{+} T cells lyse target cells and produce IFN-γ and TNF-α in an Ag-specific fashion. Their cytolytic mechanism, however, is clearly different. Whereas B6-derived LLO-specific CD8^{+} T cells mediate rapid, Ca^{2+}-dependent cytolysis, PO-derived LLO-specific CD8^{+} T cells mediate delayed cytolysis, which is independent of soluble TNF-α and dependent on CD95. These results suggest that the only major difference between the wild-type and PO-derived CD8^{+} T cells is the lack of perforin-dependent cytolysis.

The level of immunity provided by PO-derived CD8^{+} T cells, at least in the liver, is indistinguishable from that provided by wild-type CD8^{+} T cells. These results are consistent with experiments using immune polyclonal splenocytes from PO mice (Fig. 1 and (21)), which do not rule out the existence of a perforin-independent CD8^{+} T cell-mediated pathway of antilisterial immunity. However, while the lack of perforin has little impact on immunity mediated by single Ag-specific PO-derived CD8^{+} T cells, perforin deficiency dramatically impairs antilisterial immunity mediated by polyclonal CD8^{+} T cells from immunized PO mice (21). This difference may be a consequence of the uniform activation of CD8^{+} T cells provided by in vitro restimulation compared with the complex in vivo setting where other immunologic parameters may decrease priming of *L. monocytogenes* specific CD8^{+} T cells in PO mice. This possibility is consistent with the finding that CD8^{+} T cell-mediated antilisterial immunity is at least partially functional in the absence of perforin (Fig. 1 and (21)). In addition, this notion is consistent with the observation that the innate immune response in PO animals is hyperactive, resulting in increased resistance to primary *L. monocytogenes* infection (21). Since the innate immune response functions to inhibit early bacterial replication (43), a heightened innate response might decrease Ag levels and result in suboptimal CD8^{+} T cell priming in PO mice. Thus, the lack of perforin-dependent cytotoxicity may not be the sole reason for the partial loss of CD8^{+} T cell-mediated antilisterial immunity observed with PO-derived splenocytes. Our single Ag-specific CD8^{+} T cell lines, which are uniformly restimulated in vitro, provide a method to overcome the potential differences in priming of specific CD8^{+} T cells which may occur in mice with different levels of innate immunity to *L. monocytogenes*. Alternatively, the results may be specific for CD8^{+} T cell responses to LLO, an immunodominant *L. monocytogenes* Ag (44), as a target for protective CD8^{+} T cells.

The observation that PO-deficient CD8^{+} T cells provide antilisterial immunity in the presence of an IFN-γ-specific neutralizing mAb is consistent with our previous studies demonstrating that perforin-expressing CD8^{+} T cells can provide immunity to *L. monocytogenes* infection in the absence of IFN-γ (8). It should be pointed out that our results do not rule out a contribution from perforin or IFN-γ in CD8^{+} T cell-mediated antilisterial immunity in wild-type mice. However, our results do suggest that these effector functions are not required for antilisterial immunity mediated by single Ag-specific CD8^{+} T cells. In addition, the current results suggest that IFN-γ expression is not the only effector function that can compensate for perforin deficiency, and vice versa. Despite the ability of the neutralizing anti-IFN-γ mAb to dramatically exacerbate *L. monocytogenes* infection, it is possible that this treatment is insufficient to completely eliminate the contribution of CD8^{+} T cell-derived IFN-γ.

Our experiments suggest a critical role for TNF-α in PO CD8^{+} T cell immunity to *L. monocytogenes*. These results are consistent with the results of Samsom and colleagues (45), who reported that the secondary response to *L. monocytogenes* in wild-type mice is inhibited by depletion of TNF-α but not IFN-γ. Neither of these systems, however, addresses the cellular source of the required TNF-α. CD8^{+} T cell-derived TNF-α could mediate antilisterial immunity by activating macrophages (46), recruiting accessory phagocytes to sites of infection (47), or by directly inducing the death of infected target cells (41, 42). Whether CD8^{+} T cells produce the required TNF-α, or it is elaborated by another cell type and is simply required for the expression of immunity by CD8^{+} T cells, requires further investigation.

A number of experiments have addressed the mechanisms by which CD8^{+} T cells provide immunity to various pathogens. These include *L. monocytogenes* (21), vaccinia virus (48, 17), LCMV, vesicular stomatitis virus, Semliki forest virus (17), murine rotavirus (19), hepatitis B virus (18, 49), ectromelia virus (50), mouse hepatitis virus (20), *Mycobacterium tuberculosis* (34, 35, 51), and *Plasmodium berghei* (36). While perforin is a vital component in the protective CD8^{+} T cell response to some viruses, such as LCMV (12, 15), it is not required for clearance of all intracellular pathogens (17–20, 34–36). Collectively, these studies suggest that CD8^{+} T cells can mediate resistance in vivo to some pathogens that is independent of efficient cytolytic pathways. The mechanisms utilized by CD8^{+} T cells to mediate antimicrobial resistance may vary from pathogen to pathogen and vary in effectiveness. Understanding the character of an effective CD8^{+} T cell response against specific pathogens may improve our ability to immunize healthy as well as immunocompromised patients against infectious agents.

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