Perforin-Deficient CD8⁺ T Cells: In Vivo Priming and Antigen-Specific Immunity Against *Listeria monocytogenes*


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References

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Perforin-Deficient CD8⁺ T Cells: In Vivo Priming and Antigen-Specific Immunity Against Listeria monocytogenes

Douglas W. White,* Adam MacNeil,† Dirk H. Busch,‡ Ingrid M. Pilip,‡ Eric G. Pamer,‡ and John T. Harty*†

CD8⁺ T cells require perforin to mediate immunity against some, but not all, intracellular pathogens. Previous studies with H-2b MHC perforin gene knockout (PO) mice revealed both perforin-dependent and perforin-independent pathways of CD8⁺ T cell-mediated immunity to Listeria monocytogenes (LM). In this study, we address two previously unresolved issues regarding the requirement for perforin in antilisterial immunity: 1) Is CD8⁺ T cell-mediated, perforin-independent immunity specific for a single Ag or generalizable to multiple Ags? 2) Is there a deficiency in the priming of the CD8⁺ T cell compartment of PO mice following an immunizing challenge with LM? We used H-2b MHC PO mice to generate CD8⁺ T cell lines individually specific for three known Ags expressed by a recombinant strain of virulent LM. Adoptive transfer experiments into BALB/c host mice revealed that immunity can be mediated by PO CD8⁺ T cells specific for all Ags examined, indicating that perforin-independent immunity is not limited to CD8⁺ T cells that recognize listeriolysin O. Analysis of epitope-specific CD8⁺ T cell expansion by MHC class I tetramer staining and ELISPOT revealed no deficiency in either the primary or secondary response to LM infection in PO mice. These results demonstrate that the perforin-independent pathway of antilisterial resistance mediated by CD8⁺ T cells is generalizable to multiple epitopes. Furthermore, the results show that reduced antilisterial resistance observed with polyclonal PO CD8⁺ T cells is a consequence of a deficiency in effector function and not a result of suboptimal CD8⁺ T cell priming. The Journal of Immunology, 1999, 162: 980–988.

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3 Abbreviations used in this paper: LM, Listeria monocytogenes; ELISPOT, enzyme-linked immunospot assay; LCMV, lymphocytic choriomeningitis virus; LLO, listeriolysin O; NP, nucleoprotein; PEC, phycoerythrin; PO, perforin deficient.

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CD8+ T cells, to activate the caspase cascade of the target cell and induce apoptosis (21–23). The absolute requirement for this pathway in the clearance of lymphocytic choriomeningitis virus (LCMV) has been demonstrated in mice with targeted disruption of the gene for perforin (24, 25). Activated CD8+ T cells also express CD95 ligand, which can ligate CD95 (Fas, Apo-1) on a target cell and induce apoptosis via the caspase cascade. This pathway is probably most important in the elimination of self-reactive T cells that are repeatedly exposed to Ag (26). Evidence for a role of this pathway in CD8+ T cell-mediated immunity against viruses in vivo has been presented (27, 28), but its importance as an effector function against most infectious agents remains undemonstrated.

Two studies on the role of perforin in secondary resistance and CD8+ T cell-mediated immunity to LM have been reported previously (29, 30). The first report demonstrated that CD8+ splenocytes that lack perforin are deficient in their ability to transfer immunity (30). This finding indicates that perforin is required for an optimal CD8+ T cell response to LM. However, the data presented in this report did not rule out the possibility that the deficiency observed with PO splenocytes was due to suboptimal priming of the CD8+ compartment of PO donor mice.

The second report used H-2b PO CD8+ T cells that had been restimulated in vitro to identify a perforin-independent pathway by which CD8+ T cells are capable of mediating antilisterial immunity (30). However, this report concentrated on CD8+ T cells specific for a single LM Ag. It was therefore impossible to generalize the findings regarding perforin-independent immunity to CD8+ T cells specific for other LM-derived Ags.

Both studies were performed using PO mice of the H-2b haplotype in which precise LM-derived epitopes recognized by CD8+ T cells are unknown. The lack of known epitopes in the H-2b system prevented the analysis of CD8+ T cell priming in H-2b PO mice as well as the analysis of CD8+ T cells specific for more than one LM Ag. To address these issues, we generated H-2d MHC PO mice by backcross of H-2b PO mice with BALB/c. The CD8+ T cell response to LM is well characterized in the H-2d haplotype (31), allowing us to analyze multiple Ags as targets of PO CD8+ T cells, as well as the expansion of LM Ag-specific CD8+ T cells after immunization. We present data demonstrating that the perforin-independent pathway is not limited to CD8+ T cells specific for a single epitope, but rather functions in T cells specific for all Ags tested. We also present data that rule out suboptimal priming of the CD8+ T cell compartment in perforin-knockout mice as an explanation for the apparent deficiency of PO CD8+ splenocytes in mediating antilisterial immunity.

Materials and Methods

Mice

BALB/c (H-2d MHC) mice were obtained from the National Cancer Institute (Frederick, MD) and crossed with PO (H-2b MHC) mice kindly provided by Dr. W. R. Clark (25). F1 mice were backcrossed to BALB/c and H-2b/220 perforin+/− mice were identified by flow-cytometric analysis of PBL using Abs specific for H-2d (SF1-1.1 and H-2b (Y-3) and Southern blot analysis for perforin genotype, as described (25). H-2b/220 perforin+/− mice were backcrossed to BALB/c three additional times and then intercrossed to generate H-2b/220 perforin−/− (H-2b PO) mice. H-2b PO mice were maintained by brother-sister mating and housed under specific pathogen-free conditions at the University of Iowa (Iowa City, IA) animal care unit. All mice were used at 8–16 wk of age in an age- and sex-matched fashion.

Bacteria

LM strain 10403s (32) and recombinant strain XFL-303 (derived from 10403s), which expresses the LCMV NP 118–126 epitope as a secreted fusion protein (33), are both resistant to streptomycin and were used as previously described (33–36). Briefly, bacteria were grown in tryptic soy broth to an OD600 of approximately 0.1, diluted in pyrogen-free 0.9% sodium chloride (Abbott Laboratories, North Chicago, IL), and injected i.v. in 0.2 ml per animal. Aliquots were plated onto tryptic soy agar containing 50 μg/ml 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-2b MHC) (American Type Culture Collection (ATCC), Manassas, VA; ATCC TIB-64); P815-LLO refers to P815 cells stably transfected with a plasmid construct expressing the LM Ag LLO and neo-resistance (37); P815-p60 refers to P815 cells stably transfected with a plasmid construct expressing the LM Ag p60 and neo-resistance (34). P815-Fas is a derivative of P815 that expresses 10-fold more surface CD95 than P815 (38); L1210/Fas (25) and L1210+Fas (39) are derivatives of the lymphoblastic cell line L1210 (ATCC CCL-219) that have been transfected with Fas antisense and sense cDNA, respectively. Cell lines were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS, antibiotics, 1-glutamine, HEPEs buffer, and 2-ME (RP10 (34)). Transfected cells were maintained in G418 at 400 μg/ml.

mAbs, which were purified from culture supernatants and quantitated as previously described (17), were: rat anti-mouse TNF IgG (XT22 and XT3 (40) used in combination at a mass ratio of 1:1), rat anti-mouse IFN-γ IgG (37, 41), rat anti-mouse IFN-γ (37). Briefly, bacteria were grown in tryptic soy broth to an OD600 of approximately 0.1, diluted in pyrogen-free 0.9% sodium chloride (Abbott Laboratories, North Chicago, IL), and injected i.v. in 0.2 ml per animal. Aliquots were plated onto tryptic soy agar containing 50 μg/ml streptomycin to verify the number of CFU injected.

Generation and maintenance of CD8+ T cell lines

CD8+ T cell lines specific for LLO 91–99, p60 217–225, or NP 118–126 were derived from BALB/c and H-2b PO mice using methods previously described (37). Briefly, 2–4 × 106 splenocytes from mice injected 7–10 days previously with 104 CFU of virulent LM strain 10403s or XFL303 were incubated in RP10 with 109 irradiated (150 Gy) syngeneic stimulator cells (P815-LLO cells or P815-p60 cells). Subsequent weekly restimulations were conducted by combining 1–3 × 106 responder cells with 3 × 106 irradiated stimulator cells and approximately 4 × 106 irradiated (30 Gy) syngeneic splenocytes in RP10 supplemented with 5% supernatant from Con A-stimulated rat spleen cells and 30 mM e-methyl mannoside. In the case of T cell lines specific for NP 118–126, P815-derived stimulator cells were left out and 3 × 106 irradiated syngeneic splenocytes were incubated with 100 nM synthetic NP 118–126 peptide for 1 h at 37°C and rinsed three times before their addition to the T cell culture.

In vitro characterization of CD8+ T cell lines

51Cr release assays were performed as previously described (44, 17, 30). Briefly, labeled target cells were combined with effector cells at the indicated ratios in RP10 in round-bottom 96-well plates. Following a 4–7.5-h incubation (as indicated), supernatant was harvested and assayed for % specific release of 51Cr. The formula: 100 × (spontaneous cpm) / (total cpm − spontaneous cpm). Spontaneous release was less than 20% of total in all experiments.

TNF was quantitated using a WEHI 164 clone 13 bioassay (45), as previously described (30). Briefly, supernatants from coinoculations of CD8+ T cells and target cells were added to WEHI 164 cells in flat-bottom 96-well plates. Following overnight incubation, survival of incubator cells was assayed by the addition of alamar blue (Acumed, West Lake, OH). Death of the indicator cells, a relative measure of TNF production, was determined 2–6 h after addition of alamar blue by measuring OD50 of OD600. Production of TNF by target cells in the absence of CD8+ T cells was not detected. Murine rTNF (Boehringer Mannheim, Indianapolis, IN) was used as a control and to determine the detection limits of the WEHI bioassay. Concentrations of 1–10 pg/ml of rTNF were routinely detected using this assay.

IFN-γ was quantitated by ELISA, as previously described (30). Briefly, supernatants from overnight coinoculations of effector cells and target cells and IFN-γ controls were added to 96-well plates that had been previously coated with rat anti-mouse IFN-γ (XMG1.2) mAb. Rabbit anti-mouse IFN-γ (a gift from J. Cowdery at the University of Iowa), alkaline phosphatase-conjugated goat anti-rabbit Ig, and alkaline phosphatase

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substrate (Sigma) were added sequentially according to the manufacturer’s protocol. OD was measured at 405 nm. Limit of detection was less than 50 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 determined using adoptive transfer assays, as described previously (44, 17, 30). Briefly, B6C-depleted splenocytes from donor mice immunized 7–10 days previously with 10³–10⁴ virulent LM strain 10403s or XFL303 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 0.5 ml into naive BALB/c host mice. Within 2 h, host mice, including un.injected controls, were challenged i.v. with 5 x 10⁵ indicated dose of virulent LM strain 10403s or XFL303. CFU/spleen and liver were determined 3 days postchallenge by homogenizing the spleens and livers in 0.2% IGE-PAL (Sigma), plating 10-fold serial dilutions onto tryptic soy agar containing 50 µg/ml streptomycin, and calculating colony count averages after overnight incubation at 37°C.

ELISPOT

The prevalence of activated Ag-specific CD8⁺ T cells in the spleens of BALB/c and PO mice was determined by ELISPOT analysis, as previously described (46–49). Briefly, splenocytes (5 x 10⁵/ml) were cocultured with P815-LL0 target cells (10⁶/well) for 24–48 h in flat-bottom 96-well plates that had been previously coated with rat anti-mouse IFN-γ mAb (R4-6A2; PharMingen). Following lysis of the cells with distilled water and rinses with PBS containing 0.2% Tween-20, the plates were incubated with rabbit anti-IFN-γ polysera. After rinsing, the plates were incubated with donkey anti-rabbit Ig conjugated to alkaline phosphatase (Jackson). Further rinsing was followed by the addition of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Sigma) in AMP buffer impregnated with 0.75% agarose. The reaction was developed at 37°C and spots were counted using a dissection microscope. The average frequency of responders from triplicate determinations was multiplied by the total number of splenocytes to calculate responders per spleen.

Analysis of CD8⁺ T cells using Kd-peptide tetramer complexes

Kd-peptide tetramer complexes were generated and used as previously described (50), with minor modifications. For staining, approximately 5 x 10⁶ lympholyte-M (Cedarlane) separated splenocytes were blocked with anti-FcR mAb (2.4G2, gift of T. Waldschmidt at the University of Iowa) before staining with FITC-conjugated anti-CD8 (53.6-7; Sigma) and PE-conjugated Kd-peptide tetramer complexes for 1 h at 4°C. After washing, the cells were resuspended in PBS containing 0.01% sodium azide, 1% BSA, and 1 µg/ml propidium iodide, and then analyzed on a FACSan using CyCLOPS software (Cytomation, Fort Collins, CO). Lymphocytes (determined by forward scatter and side scatter) that excluded propidium iodide were analyzed for CD8 and Kd-peptide tetramer-specific staining. The frequency of CD8⁺ cells and CD8⁺, tetramer⁻ cells was used with the total splenocyte count to calculate the responders per spleen.

Results

CD8⁺ splenocytes derived from H-2b PO mice appear to be deficient, compared with wild-type CD8⁺ splenocytes, in mediating antilisterial immunity

We bred the PO mutation onto the BALB/c (H-2d) background in which multiple LM Ags have been described (31). Experiments in which we compared naive H-2b PO mice with BALB/c mice did not reveal a significant difference in the LD₅₀ of virulent LM (data not shown). This result is consistent with studies that compared H-2b PO mice with wild-type C57BL/6 mice (29, and data not shown) and verifies that perforin is not required for resistance to primary LM infection.

We next compared the antilisterial activity of immune splenocytes derived from H-2b PO mice with those from BALB/c mice. Since the resistance of naive PO and BALB/c mice to primary LM infection was similar, we used standard sublethal doses of virulent LM (~0.1 LD₅₀) to immunize H-2b PO and BALB/c donor mice. Seven days postimmunization, equivalent numbers of BALB/c or PO donor splenocytes were transferred into naive BALB/c host mice that were subsequently challenged with a high dose of virulent LM. Bacterial counts in the spleens and livers of splenocyte recipient and control mice 3 days postchallenge demonstrated that BALB/c-derived splenocytes provided dramatic antilisterial protection in both the spleen (Fig. 1A) and the liver (Fig. 1B), reducing bacterial recovery >10,000-fold, respectively, compared with mice that did not receive splenocytes. This activity was primarily mediated by CD8⁺ cells since depletion of the CD8⁺ compartment with Ab and complement before transfer eliminated the majority of the protection (Fig. 1, A and B). Immunity mediated by splenocytes derived from H-2b PO mice was comparable with that provided by wild-type splenocytes in the liver (8,000-fold reduction), but somewhat less than that provided by wild-type splenocytes in the spleen (1,000-fold reduction). Depletion of CD8⁺ cells from H-2b PO-derived splenocytes had a modest effect on the level of immunity transferred, but did not eliminate immunity mediated by these cells (Fig. 1, A and B). These results recapitulate those from H-2b mice (29, 30) and further support the model that perforin plays a role in the normal immune response to LM, especially in the spleen, and that splenocytes other than CD8⁺ T cells may play a significant role in adaptive immunity to LM in the PO mouse.

Splenocytes from immunized PO mice also transferred immunity into naive PO host mice at levels that were indistinguishable from that observed in BALB/c hosts (data not shown). This result argues against a model in which perforin, derived from host cells, plays a role in antilisterial immunity observed in adoptive transfer experiments.
Generation and characterization in vitro of CD8<sup>+</sup> T cell lines specific for three known antigenic peptides expressed by recombinant LM XFL303.

Experiments with LLO-specific CD8<sup>+</sup> T cell lines from H-2<sup>b</sup> PO mice previously identified a perforin-independent pathway by which CD8<sup>+</sup> T cells mediate antilisterial immunity (30). To determine whether this result was restricted to LLO-specific CD8<sup>+</sup> T cells, we generated and characterized CD8<sup>+</sup> T cell lines specific for multiple Ags from H-2<sup>b</sup> PO and syngeneic control (BALB/c) mice, which were incubated with P815 target cells (open symbols) or P815 target cells coated with the appropriate peptide (closed symbols) in a 7.5-h (A–C) or 4-h (D) 51Cr release assay. These data are representative of at least three independent experiments with similar results.

*FIGURE 2.* In vitro characterization of PO CD8<sup>+</sup> T cell lines. A–D, CD8<sup>+</sup> T cell lines, specific for LLO 91–99 (circles), p60 216–225 (squares), or NP 118–126 (triangles), derived from PO (circles, squares, and upward triangles) or BALB/c (downward triangles) mice, were incubated with P815 target cells (open symbols) or P815 target cells coated with the appropriate peptide (closed symbols) in a 7.5-h (A–C) or 4-h (D) 51Cr release assay. These data are representative of at least three independent experiments with similar results. E–H, Supernatants from overnight coincubations of CD8<sup>+</sup> T cell lines and target cells (as in A–D) were assayed for IFN-γ by ELISA. These data represent the mean of triplicate determinations made in four independent experiments. SD did not exceed 21% for any data point. I–L, Supernatants from 10-h coincubations of CD8<sup>+</sup> T cell lines and target cells (as in A–D) were assayed for TNF. TNF was quantitated in a bioassay using WEHI 164 clone 13 cells (45), which die in the presence of TNF. These data represent at least three independent experiments with similar results for each CD8<sup>+</sup> T cell line.

After several in vitro restimulations, all lines were >95% CD8<sup>+</sup>CD4<sup>-</sup>, as measured by flow cytometry (data not shown). To verify Ag specificity and cytolytic activity, we performed 51Cr release assays. All CD8<sup>+</sup> T cell lines mediated cytolyis of H-2<sup>d</sup> MHC target cells in the presence, but not in the absence, of the appropriate Ag (Fig. 2, A–D). As expected, CD8<sup>+</sup> T cells derived from control mice (Fig. 2D) mediated higher levels of specific lysis in shorter time period than did CD8<sup>+</sup> T cells derived from PO mice (Fig. 2, A–C). Delayed cytosis in the absence of perforin is consistent with previous studies that have documented the importance of perforin in cytolytic assays in vitro (24, 25, 51, 52).

To verify that PO CD8<sup>+</sup> T cells mediate cytosis via CD95, cells of the H-2<sup>d</sup> haplotype that vary in their expression of CD95 were utilized as target cells in 51Cr release assays (Fig. 3). Whereas peptide-coated, CD95-expressing (P815-Fas (38)) target cells were lysed at high levels in a 51Cr release assay by PO-derived CD8<sup>+</sup> T cells, peptide-coated P815 cells that express very low levels of CD95 (P815) were not lysed (Fig. 3A). PO CD8<sup>+</sup> T cell lines specific for p60 and NP also exhibited increased lysis of target cells expressing CD95 compared with target cells that express minimal CD95 (data not shown). Similar results were obtained with another pair of target cells that differ in expression of CD95, namely L1210F<sup>+</sup> and L1210F<sup>-</sup> (39, 25). We observed specific lysis of CD95-expressing targets (Fig. 3B) and background levels of lysis of targets that do not express CD95 (data not shown). Finally, Ag-specific lysis of L1210F<sup>+</sup> cells by PO CD8<sup>+</sup> T cells was inhibited by a mAb specific for CD95, but not by
control rat IgG (Fig. 3B). Complete inhibition was observed at most E:T ratios using 5 μg/ml of anti-CD95 mAb, a concentration that did not kill target cells nor the CD8+ T cells in the time frame of the 51Cr release assay (data not shown). While these results do not rigorously rule out perforin-independent, CD95-independent mechanisms of lysis by PO-derived CD8+ T cells, they do establish the presence of a CD95-dependent pathway.

All CD8+ T cell lines that were generated also produced IFN-γ (Fig. 2, E–H) and TNF (Fig. 2, I–L) in an Ag-specific fashion. IFN-γ production by H-2d PO CD8+ T cells specific for LLO and p60 was also verified by ELISPOT analysis (data not shown).

In general, we observed higher levels of IFN-γ production by PO-derived CD8+ T cells compared with wild-type cells (Fig. 2), and that did not kill target cells nor the CD8+ T cells in the time frame of the 51Cr release assay (data not shown).

We previously hypothesized that splenocytes from PO mice might be sufficient in mediating antilisterial immunity due to inefficient priming of LM-specific CD8+ T cells in these mice (30). To address this issue, we quantitated LLO-specific CD8+ T cells in PO-derived CD8+ T cells specific for multiple LM-derived peptides. Naïve BALB/c mice were injected i.v. with CD8+ T cells (hatched bars) derived from PO (A–C and E–G) or BALB/c (D and H) mice, and within hours challenged with virulent LM. Control mice did not receive any T cells (open bars). CFU from the spleen (A–D) and liver (E–H) were quantitated 3 days post-challenge. Data are presented as mean log10 CFU ± SD for five to nine animals per group. These data are pooled from two to three independent experiments for each CD8+ T cell line. 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 that did not receive T cells. T cells transferred: 1–1.6 × 106 per mouse. Challenge with XFL303 (in the case of NP-specific CD8+ T cells) or 10403s (in the case of LLO- or p60-specific CD8+ T cells): 0.5–1.7 × 105 CFU per mouse.

Table I. CD8+ T cell lines derived from H-2d PO mice provide Ag-specific immunity as measured by survival*

<table>
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<td>1/6</td>
</tr>
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<td>10403s (wild type)</td>
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* BALB/c- or PO-derived CD8+ T cells (5–10 × 105) specific for LLO 91–99 (LLO) or NP 118–126 (NP) were injected i.v. into BALB/c host mice that were subsequently challenged with 1–2 × 106 NP 118–126-expressing virulent rLM (XFL303) or the parental strain 10403s that does not express NP 118–126. Survival was monitored for 12 days. All animals that died succumbed within 5 days of challenge.

In vivo immunity can be mediated by PO CD8+ T cells specific for a range of LM-derived peptides

Our previous studies demonstrated that LLO-specific CD8+ T cells from H-2d PO mice can provide significant immunity to LM. To test whether perforin-independent CD8+ T cell-mediated immunity is generalizable to other Ags, we tested the ability of H-2d PO CD8+ T cells specific for LLO 91–99, p60 217–225, and NP 118–126 to mediate immunity against rLM XFL303 in vivo. CD8+ T cells were transferred into naïve BALB/c host mice that were subsequently challenged with approximately 10 LD50s of virulent LM XFL303. PO-derived CD8+ T cells specific for LLO 91–99, p60 217–225, and NP 118–126 all provided antilisterial immunity in the liver (Fig. 4, A–C), albeit to a lesser degree. BALB/c-derived CD8+ T cells specific for NP 118–126 provided high levels of antilisterial immunity in both the spleen (Fig. 4D) and the liver (Fig. 4H). Immunity mediated by BALB/c-derived CD8+ T cells specific for LLO 91–99 and p60 217–225 has been described previously (44, 34). Consistent with our previous studies, the degree of immunity in the spleen mediated by PO-derived CD8+ T cells was typically less compared with that which is usually observed with BALB/c-derived CD8+ T cells (Fig. 4, A–C versus D).

Studies were performed to confirm that the reduction in CFUs provided by PO CD8+ T cells correlated with survival of the animals to an otherwise lethal challenge. One hundred percent of mice that received PO-derived CD8+ T cells (specific for LLO 91–99 or NP 118–126) survived at least 12 days without any overt signs of illness following a challenge of XFL303 that killed all mice that did not receive any CD8+ T cells (Table I). Similarly, all mice that received PO CD8+ T cells specific for LLO survived a lethal challenge with virulent LM 10403s (Table I).

The Ag specificity of immunity mediated by PO CD8+ T cell lines was confirmed in experiments in which PO CD8+ T cells specific for NP 118–126 mediated immunity, as measured by survival, against virulent LM XFL303, which expresses the NP epitope, but failed to protect against the parental LM strain 10403s that lacks the NP epitope (Table I).

These results demonstrate that perforin-independent mechanisms of antilisterial immunity mediated by CD8+ T cells are not restricted to CD8+ T cells that recognize LLO.

Analysis of priming and expansion of the CD8+ T cell compartment in PO versus syngeneic BALB/c mice

We previously hypothesized that splenocytes from PO mice might be sufficient in mediating antilisterial immunity due to inefficient priming of LM-specific CD8+ T cells in these mice (30). To address this issue, we quantitated LLO-specific CD8+ T cells in PO mice that were subsequently challenged with 1–2 × 106 NP 118–126-expressing virulent rLM (XFL303) or the parental strain 10403s that does not express NP 118–126. Survival was monitored for 12 days. All animals that died succumbed within 5 days of challenge.
Phenotypic as well as functional assays are now available to identify and quantitate Ag-specific CD8+ T cells ex vivo (54). The utility of Kd-peptide tetramer complexes in the phenotypic analysis of Ag-specific CD8+ T cells in wild-type mice undergoing a response to LM has been demonstrated previously (50). We applied this technique to address the issue of priming in the PO mouse. The specificity of these reagents in the PO system was verified using H-2d PO CD8+ T cell lines. Kd tetramers folded with the peptide...
LLO 91–99 (K\(^d\)LLO) stained LLO 91–99-specific CD8\(^{+}\) T cells from the H-2\(^d\) PO mouse, but failed to stain H-2\(^d\) PO CD8\(^{+}\) T cells specific for p60 217–225 (Fig. 5A). A control reagent generated by folding K\(^d\) tetramers with a self peptide (50), K\(^d\)JAK1) did not stain PO-derived CD8\(^{+}\) T cells specific for LLO 91–99 (Fig. 5A).

LLO 91–99-specific CD8\(^{+}\) T cells from H-2\(^d\) PO and BALB/c mice were quantified by staining with anti-CD8 mAb and K\(^d\)LLO. During the primary response to LM infection, approximately 2–3% of CD8\(^{+}\) T cells from both BALB/c and H-2\(^d\) PO mice stained with K\(^d\)LLO (Fig. 5, B and C). This value for BALB/c mice is slightly higher than that previously reported (50), due to higher background staining in naive mice (0.53 ± 0.24% CD8\(^{+}\) K\(^d\)LLO\(^{+}\) cells in the BALB/c and H-2\(^d\) PO mice (n = 9) and 0.49 ± 0.28% in the H-2\(^d\) PO (n = 7)) and the fact that, in this study, we did not differentiate between CD62L\(^{high}\) (naive) and CD62L\(^{low}\) (activated) CD8\(^{+}\) T cells. Expansion of LLO 91–99-specific CD8\(^{+}\) T cells could clearly be observed compared with background staining of naive splenocytes (Fig. 5B). Reinfection of immune BALB/c and H-2\(^d\) PO mice resulted in further expansion of the LLO 91–99-specific T cell population (Fig. 5, B and C). As was the case with the primary response, the secondary response to LLO 91–99 in BALB/c and H-2\(^d\) PO mice was similar (Fig. 5C), accounting for 11 and 14%, respectively, of all CD8\(^{+}\) T cells in the representative mice shown in Fig. 5B.

Every analysis using K\(^d\)LLO was accomplished by an analysis on the same cells with K\(^d\)JAK1. We did not observe a significant increase in the percentage of CD8\(^{+}\) K\(^d\)JAK1\(^{+}\) cells in animals undergoing a response to LM compared with naive animals (data not shown). The percentage of cells that stained positive using the control reagent was similar to the percentage of cells that stained positive with K\(^d\)LLO from naive animals (data not shown).

ELISPOT analysis was also performed on splenocytes from H-2\(^d\) PO and control BALB/c animals undergoing a primary and secondary response to LM (48). The number of cells that specifically produce IFN-\(\gamma\) in response to LLO 91–99 was similar to the number of CD8\(^{+}\) cells specific for LLO 91–99, as determined by tetramer analysis (data not shown). In sum, we do not find any evidence, using functional as well as phenotypic analyses, that the CD8\(^{+}\) T cell compartment in PO mice is deficient in Ag-specific expansion in response to LM. Thus, deficiencies in CD8\(^{+}\) T cell immunity in PO mice are a consequence of the effector and not the afferent immune response.

Discussion

The CD8\(^{+}\) T cell response of BALB/c (H-2\(^d\) MHC) mice to four endogenous LM epitopes, LLO 91–99, p60 217–225, p60 440–448, and mpl 84–92, has been analyzed extensively after primary and secondary infection (50). The recently described rLM strain XFL303, which expresses a secreted fusion protein containing the H-2L\(^d\)-restricted NP 118–126 epitope from LCMV (33), provides an additional well-characterized epitope for analysis in LM-infected H-2\(^d\) MHC mice. A hierarchy of responses, LLO 91–99 > NP 118–126 > p60 217–225, is observed with the p60-specific response 5–10-fold lower than LLO-specific response in both the primary and secondary immune response to LM (50, 33). Still lower responses have been observed against two subdominant LM epitopes, mpl 84–92 and p60 440–448 (50). We have shown previously that BALB/c or IFN-\(\gamma\) gene knockout mouse-derived CD8\(^{+}\) T cells specific for either LLO 91–99 (44) or p60 217–225 (34) provide immunity to LM infection. In this study, we extend this finding to BALB/c-derived CD8\(^{+}\) T cells specific for the NP epitope expressed by rLM XFL303, further demonstrating that the recombinant Ag functions as a target for protective CD8\(^{+}\) T cells in a similar fashion to endogenous LM Ags.

Activated CD8\(^{+}\) T cells elaborate a number of effector functions following Ag-specific stimulation in vitro. A current challenge is to understand the relative importance of these effector mechanisms in CD8\(^{+}\) T cell-mediated immunity against different pathogens in vivo. Two previous reports addressed the role of perforin in CD8\(^{+}\) T cell-mediated immunity to LM (29, 30). The first by Kagi and colleagues showed that the CD8\(^{+}\) compartment in the spleens of PO mice is deficient, compared with wild-type mice, in antilisterial activity. This result, which has since been independently corroborated (30), and Fig. 1), suggests that perforin plays an important role in the normal antilisterial response.

The second report, which used Ag-specific CD8\(^{+}\) T cell lines, revealed a perforin-independent pathway that results in significant CD8\(^{+}\) T cell-mediated immunity against LM. The relative importance of perforin-dependent versus perforin-independent pathways of antilisterial resistance in immunocompetent hosts is not known. Furthermore, the precise mechanisms involved in perforin-independent resistance to LM mediated by CD8\(^{+}\) T cells remain undefined. Our previous studies demonstrated that both PO and wild-type Ag-specific CD8\(^{+}\) T cells provide antilisterial immunity in vivo in MRL lpr/lpr host mice (30). Thus, although PO CD8\(^{+}\) T cells conducted Ag-specific cytosis in vitro, which was dependent on CD95 (30), we found no evidence for a CD95-dependent pathway of antilisterial resistance in vivo. The role of CD95 in immunity to LM in vivo has been further addressed in a recent report by Jensen and colleagues (55). They have shown that mice deficient in both perforin and CD95 are more susceptible than mice deficient in perforin alone to primary and secondary listeriosis. While this study did not specifically examine CD8\(^{+}\) T cells in vivo, these results suggest a role for CD95 in the development or expression of antilisterial resistance in the absence of perforin. In the present study, we confirm the presence of a CD95-dependent pathway of Ag-specific cytosis in vitro mediated by PO CD8\(^{+}\) T cells of the H-2\(^d\) haplotype (Fig. 3). The ability of H-2\(^d\) CD8\(^{+}\) T cells to mediate antilisterial immunity in H-2\(^d\) lpr/lpr host mice has not been tested.

By performing the present experiments with PO mice of the H-2\(^d\) haplotype, we were able to address two previously unresolved issues. First, we show that PO CD8\(^{+}\) T cells specific for three epitopes, LLO 91–99, NP 118–126, and p60 217–225, can provide immunity to LM infection. This result demonstrates that perforin-independent mechanisms are not limited to CD8\(^{+}\) T cells specific for a single Ag and suggests the possibility that CD8\(^{+}\) T cells, regardless of Ag specificity, may mediate antilisterial resistance in a perforin-independent fashion in the normal mouse. This result argues against the previously untested possibility that CD8\(^{+}\) T cell lines from PO mice mediate efficient antilisterial immunity based on their oligoclonal specificity for LLO (30).

While our data demonstrate potent, perforin-independent, Ag-specific resistance to LM mediated by CD8\(^{+}\) T cells (Table I), our data also confirm a deficiency due to the absence of perforin, which is especially notable in the spleen (29, 30, 55, and Fig. 4). These results suggest that optimal antilisterial immunity mediated by CD8\(^{+}\) T cells may involve distinct effector functions in the liver and spleen. A model by which CD8\(^{+}\) T cells mediate resistance to LM infection in the liver in the absence of cytosis is consistent with data from a model of hepatitis (56, 16), and may imply a greater role for cytokines versus cytolytic activity in this target organ. Elucidating the mechanisms by which distinct effector functions are triggered in vivo, or the factors that determine the susceptibility of an infected cell to perforin-dependent cytosis, remains a topic of current interest.
The second issue, whether priming of CD8+ T cells is deficient in PO mice, was addressed by functional as well as phenotypic analysis. Three current methods have replaced limiting dilution assays and subsequent CTL precursor frequency analysis as techniques to quantitate the expansion of Ag-specific CD8+ T cells in response to pathogens. ELISPOT analysis, intracellular staining for cytokines, and flow-cytometric analysis using MHC class I tetramers have all provided equivalent estimates of the degree of expansion of CD8+ T cells in response to individual pathogens (48, 50, 57, 58). Neither ELISPOT analysis for IFN-γ-producing CD8+ T cells, nor flow-cytometric analysis for CD8+ T cells specific for H-2Kb bound to L91-99 (Fig. 5), showed a defect in priming of LM-specific CD8+ T cells in PO mice relative to BALB/c mice. This result indicates that the deficiency of PO-derived CD8+ splenocytes in mediating antilisterial immunity cannot be attributed to a failure in priming after immunization, as was previously suggested (30).

Our findings to date do not account for the relative deficiency of polyclonal CD8+ splenocytes versus in vitro restimulated, oligo- clonal CD8+ T cell lines to mediate antilisterial immunity. Possible explanations that remain to be tested include differences in homing following i.v. injection between splenocytes and restimulated cells, and the impact of cells other than CD8+ T cells, which are present in a dose of splenocytes, but missing from a dose of restimulated CD8+ T cells. In addition, while we now have the tools to estimate the number of Ag-specific CD8+ T cells typically injected into a recipient mouse during an adoptive transfer experiment, we do not know the proportion of those Ag-specific CD8+ T cells that actually mediates antilisterial immunity in vivo following transfer. In vitro manipulations might increase or decrease the fraction of cells that are capable of mediating antilisterial immunity in vivo. The possibility that in vitro restimulation might enhance alternative pathways of antimi crobial resistance by CD8+ T cells could have important implications for adoptive immunotherapy against pathogens as well as tumors (59).

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