Cutting Edge: Antilisterial Activity of CD8+ T Cells Derived from TNF-Deficient and TNF/Perforin Double-Deficient Mice

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The mechanisms by which CD8+ T cells mediate immunity against bacterial pathogens remain largely unknown. Perforin-dependent cytolysis plays a role, but is not required for CD8+ T cell-mediated immunity against *Listeria monocytogenes*. TNF is essential for CD8+ T cell immunity to *L. monocytogenes*, but the cellular source of TNF is undefined. TNF-deficient and TNF/perforin double-deficient mice were used to generate CD8+ T cells specific for an *L. monocytogenes*-derived Ag. Wild-type and TNF-deficient CD8+ T cells mediated antilisterial immunity in wild-type but not TNF-deficient host mice, revealing that CD8+ T cell-derived TNF is not required for CD8+ T cell-mediated antilisterial immunity, but demonstrating a role for TNF derived from other cell types. TNF/perforin double-deficient CD8+ T cells mediated antilisterial immunity in the liver, but not in the spleen, of wild-type recipient mice, suggesting that perforin-independent immunity in the spleen requires CD8+ T cell-derived TNF. *The Journal of Immunology*, 2000, 165: 5–9.

CD8+ T cells are potent mediators of immunity against the intracellular bacterial pathogen *Listeria monocytogenes* (LM)\(^4\) (1). In vitro, Ag-stimulated CD8+ T cells express an array of effector functions including cytokinesis and elaboration of cytokines such as IFN-γ and TNF. However, the mechanisms by which CD8+ T cells provide antilisterial immunity in vivo remain poorly understood. Studies by Kagi et al. indicated that a perforin-dependent pathway, presumably cytolytic, participates in optimal CD8+ T cell-mediated immunity against LM, especially in the spleen (2). However, while studies with LM Ag-specific CD8+ T cell lines derived from perforin-deficient (P\(^0\)) mice confirmed a role for perforin in the spleen, they also revealed a perforin-independent pathway for antilisterial immunity (3, 4).

Numerous studies have demonstrated a role for TNF in resistance to primary listeriosis (5–8). Neutralization of TNF with mAbs in WT mice also inhibited secondary responses against LM, while neutralization of IFN-γ did not (9). Additionally, antilisterial immunity mediated by P\(^0\)-derived CD8+ T cell lines is inhibited by pretreatment of host animals with anti-TNF mAbs (3). Because mAb-mediated neutralization cannot identify the cellular source of the required TNF, it has not been clear whether TNF derived from LM Ag-specific CD8+ T cells, or some other cell type, is required for effective CD8+ T cell-mediated immunity in vivo. To address this issue, we analyzed the capacity of TNF-deficient (T\(^0\)) and TNF/perforin double-deficient (T\(^0\)P\(^0\)) CD8+ T cells to provide immunity against LM.

**Materials and Methods**

**Mice**

C57BL/6 (B6) (H-2b MHC) mice were obtained from the National Cancer Institute (Frederick, MD). T\(^0\) (H-2b MHC) mice on the B6/129 background and B6/129 controls have been described (10). T\(^0\)P\(^0\) (H-2b MHC) mice were generated by appropriate cross and backcross of T\(^0\) mice with P\(^0\) (H-2b MHC) mice (11).

**Bacteria and cell lines**

LM strain 10403s (12) and attenuated LM strain DP-L1942 (ActA\(^-\)) (13) were maintained and used for animal injection as described (14). Recombinant LM XFL204 was kindly provided by Dr. H. Shen (University of Pennsylvania, Philadelphia, PA). XFL204 is derived from 10403s and was engineered using previously described strategies (15) to secrete a fusion protein consisting of dihydrofolate reductase and amino acids 396–404 of the nucleoprotein (NP) of lymphocytic choriomeningitis virus (LCMV). NP\(_{396-404}\) is a well characterized H-2D\(^b\)-restricted CD8+ T cell epitope from LCMV (16). EL4, a B6-derived thymoma cell line, and EL4 expressing the LM listeriolysin O (LLO) gene (EL4-LLO) were maintained as described (17).

**Generation of CD8+ T cell lines**

CD8+ T cell lines specific for LLO were derived from LM immune B6 (wild type; WT), P\(^0\), T\(^0\), and T\(^0\)P\(^0\) mice by in vitro restimulation with EL4-LLO cells as described (17). WT and P\(^0\) mice were immunized by i.v. injection with 10\(^3\) to 10\(^4\) CFU of virulent LM strain 10403s, while T\(^0\) and T\(^0\)P\(^0\) mice were immunized with 10\(^3\) to 10\(^4\) attenuated LM DP-L1942. In the case of T cell lines specific for NP\(_{396-404}\), T\(^0\) mice were immunized...
with XFL204 and EL4 cells supplemented with 100 nM synthetic NP396–404 peptide were used as stimulators as described (18).

Intracellular cytokine staining and 51Cr release assays

Intracellular cytokine staining was performed as described (19). Briefly, CD8+ T cells specific for LLO were incubated for 4 h in the presence of EL4 or EL4-LLO cells and stained with FITC-labeled anti-CD8 (53-6.7; PharMingen, San Diego, CA). The cells were then fixed, permeabilized, and stained with PE-conjugated anti-TNF (MP6-XT22; PharMingen) or PE-conjugated anti-IFN-γ (XMG1.2; PharMingen). List mode data was acquired on a FACScan (Becton Dickinson, Mountain View, CA) using Cytomax software (Cytomation, Fort Collins, CO) and analyzed with FlowJo software (Tree Star, San Carlos, CA). 

Adoptive transfer and survival assays

LLO- or NP396–404-specific CD8+ T cells were washed in antibiotic-free buffer and resuspended in pyrogen-free normal saline. Cells were delivered i.v. in 0.2- to 0.5-ml volumes into naive host mice. Within 2 h, host mice were challenged i.v. with the indicated dose of bacteria. CFU per spleen and liver were determined 3 days postchallenge as described (14). Data are presented as mean log10 CFU ± SD per spleen or per gram of liver. Student’s t test was used for statistical analysis; values of p are shown for each group compared with the control group that did not receive T cells. The susceptibility of B6, B6/129, T0, and T0 P0 mice to infection with LM was estimated as described (14).

Results and Discussion

Resistance of T0 and T0P0 mice to primary infection with virulent and ActA−LM

While P0 and WT mice exhibit similar resistance to primary infection with virulent LM (2–4), T0 (8) and TNF receptor type I (p55)-deficient (20–22) mice are extremely susceptible to infection. Consistent with these studies, the estimated LD50 of virulent LM 10403s in WT, T0, and T0P0 mice was 106.7, 107.9, and 102.3, respectively. This result confirms the critical role played by TNF in resistance to primary infection with virulent LM (8). Furthermore, this result indicates that a lack of perforin does not increase the susceptibility of mice already profoundly immunocompromised due to a lack of TNF.

We have previously elicited potent CD8+ T cell responses in immunocompromised mice (18, 23) by immunizing with attenuated LM DP-L1942 (13). Both T0 and T0P0 mice survived at least 4 wk following high-dose challenges with ActA−LM (>106 CFU), suggesting that this strategy might be used to generate CD8+ T cell responses in the absence of TNF.

Generation and characterization of CD8+ T cells from T0 and T0P0 mice

Adaptive immunity to LM in WT mice involves CD8+ T cells (24). To address the effector functions involved in CD8+ T cell immunity to LM, we analyzed the protective capacity of CD8+ T cell lines, specific for known LM Ags, from WT and various gene knockout mice (25). To this end, we immunized T0 and T0P0 mice with DP-L1942 and generated CD8+ T cell lines by in vitro restimulation with EL4 cells that express the LM Ag LLO (3, 17). LLO-specific CD8+ T cell lines from WT and P0 mice were analyzed as controls. T cell lines derived from all mice were ≥93% CD8+ (Fig. 1). Restimulation in vitro followed by intracellular staining for IFN-γ and TNF revealed that all CD8+ T cell lines produced IFN-γ in an Ag-specific fashion, but that only CD8+ T cells derived from WT and P0 mice produced TNF (Fig. 1). These results demonstrate that the antilisterial response in T0 and T0P0 mice involves the activation of LLO-specific CD8+ T cells that produce IFN-γ, but not TNF, when restimulated in vitro.

Interestingly, perforin deficiency resulted in faster (not shown) and more uniform production of cytokines. Similar results were obtained when cytokine secretion by P0 CD8+ T cells was measured, suggesting that the presence of a perforin-dependent mechanism of cytolysis slows the production of cytokines by CD8+ T cells in vitro (4, 26).

T0 CD8+ T cell lines mediated Ag-specific cytolyis of EL4-LLO cells that was comparable to WT CD8+ T cells (Fig. 2). CD8+ T cells from T0P0 mice mediated Ag-specific cytolyis of LLO-expressing target cells, which was delayed compared with WT and T0-derived CD8+ T cells. Consistent with previous in vitro studies of P0 CD8+ T cells (3, 4), this cytolyis was inhibited by anti-CD95 mAbs (Fig. 2). These results demonstrate Ag-specific, CD95-dependent cytolyis in vitro by CD8+ T cells derived from LM-immune T0P0 mice.

T0 CD8+ T cells mediate immunity against LM in WT, but not T0, host mice

WT and P0 LLO-specific CD8+ T cells mediate antilisterial immunity in adoptive transfer assays (3, 17). Immunity mediated by P0 CD8+ T cells is abrogated by treatment of host mice with neutralizing anti-TNF mAbs (3). Although this approach does not identify the cellular source of the biologically relevant TNF, these experiments suggest that TNF is required for CD8+ T cell-mediated immunity against LM. To address this hypothesis, we transferred T0-derived LLO-specific CD8+ T cells into WT host mice, which were subsequently challenged with virulent LM. LLO-specific CD8+ T cells from T0 mice provided antilisterial immunity as measured by a ≥100-fold decrease in LM CFUs in the spleen (Fig. 3A) and liver (Fig. 3B). These results demonstrate that CD8+ T cell-derived TNF is not required for antilisterial immunity.

In the same experiment, LLO-specific CD8+ T cells from T0 donors were transferred into T0 hosts. Due to the importance of TNF in the innate response to LM, the dose of LM administered to T0 hosts was reduced by 100-fold. This challenge resulted in comparable levels of infection in control T0 and WT hosts (3). However, even with these lower challenge doses, T0 hosts were not significantly protected by T0 CD8+ T cells (Fig. 3, C and D).
suggests that antilisterial immunity mediated by LLO-specific T CD8 T cells requires host cell-derived TNF.

To determine whether host cell-derived TNF is also required for immunity mediated by WT CD8 T cells, T 0 or WT hosts were injected with WT CD8 T cells and then challenged with the low dose or high dose, respectively, of virulent LM. WT CD8 T cells mediated dramatic reductions in bacterial numbers in the spleen (>100-fold) and liver (>1000-fold) of WT hosts (Fig. 4, A and B) but were unable to provide significant protection in T 0 hosts (Fig. 4, C and D).

While both strains of mice are H-2 b , the possibility exists that minor Ag differences between B6 and T 0 (B6/129 background) strains could stimulate host responses that nonspecifically decrease the in vivo bacterial counts. This is unlikely because the degree of

**FIGURE 2.**  Ag-specific cytolysis by T 0 and T 0 P 0 CD8 T cell lines. LLO-specific CD8 T cells derived from WT, T 0 , or T 0 P 0 mice were incubated for the indicated time at various E:T ratios with 51Cr-labeled EL4 or EL4-LLO target cells in the absence (■) or presence of control IgG (○) or anti-CD95 mAb (●). Data are representative of at least three independent experiments.

**FIGURE 3.**  T 0 -derived LLO-specific CD8 T cells transfer antilisterial immunity to WT, but not T 0 , host mice. Naive B6 (A and B) and T 0 (C and D) mice were injected i.v. with 1–1.6 × 10 7 LLO-specific CD8 T cells derived from WT or T 0 mice and then challenged with 0.8–1.7 × 10 5 (A and B) or 0.8–1.7 × 10 3 (C and D) virulent LM 10403s. CFU from the spleen (A and C) and liver (B and D) were quantitated 3 days postchallenge. Data are presented as mean log 10 CFU ± SD for six animals per group pooled from two independent experiments. Student’s t test was used in statistical analysis; values of p are shown for each group compared with the control group in the same experiment that did not receive T cells.

**FIGURE 4.**  B6-derived LLO-specific CD8 T cells transfer antilisterial immunity to WT, but not T 0 , host mice. Naive B6 (A and B) and T 0 (C and D) mice were injected i.v. with 1.4 × 10 7 LLO-specific CD8 T cells derived from WT mice and then challenged with 1.6 × 10 5 (A and B) or 1.6 × 10 3 (C and D) virulent LM 10403s. Data from four mice/group are presented as described in Fig. 3. The asterisk indicates that one animal died before the CFU assay and is therefore not included.
mice were injected i.v. with $10^7$ LLO-specific CD8 T cells. Naive B6 T cells provided significant antilisterial immunity in the liver, but not in the spleen (Fig. 5, E and F). These results suggest that CD8$^+$ T cells provide immunity in the liver by a pathway that is independent of both CD8$^+$ T cell-derived perforin and TNF. This result also indicates that perforin-independent CD8$^+$ T cell immunity in the spleen requires CD8$^+$ T cell-derived TNF.

Together, these studies demonstrate an in vivo pathway of CD8$^+$ T cell-mediated immunity against LM that is independent of CD8$^+$ T cell-derived TNF. This result underscores the apparent versatility of activated CD8$^+$ T cells in terms of their ability to fight listeriosis. TNF joins the arsenal of perforin, CD95, and IFN-$\gamma$ (3, 4, 23) as the fourth effector function that is individually expendable for effective CD8$^+$ T cell immunity to LM. However, CD8$^+$ T cell immunity is severely hampered when two effector functions (perforin and TNF) are simultaneously deactivated. The relevance of CD8$^+$ T cell-derived TNF in this process is underscored by our recent results demonstrating that deletion of IFN-$\gamma$ and perforin does not additionally compromise CD8$^+$ T cell-mediated antilisterial immunity compared with perforin deficiency alone (27). The present results also uncover a pathway of CD8$^+$ T cell-mediated antilisterial immunity in the liver that is independent of both perforin and CD8$^+$ T cell-derived TNF. While it is well appreciated that the molecular mechanisms of effective CD8$^+$ T cell responses are pathogen specific (1), the present results emphasize that effective CD8$^+$ T cell responses may be expressed in a tissue- or organ-specific fashion as well. Finally, while the role of a number of CD8$^+$ T cell effector functions have been examined, the importance of as yet untested (28) or unknown effector functions should not be overlooked.

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References


antilisterial immunity provided by T$^0$ LLO-specific CD8$^+$ T cells in adoptive transfer experiments was indistinguishable in B6 and B6/129 host mice (data not shown). Second, a T$^0$-derived CD8$^+$ T cell line specific for a non-LM Ag (LCMV NP$_{396-404}$) did not protect when transferred into B6 mice that were subsequently challenged with virulent LM. B6 mice that received $8 \times 10^6$ NP$_{396-404}$-specific T$^0$-derived CD8$^+$ T cells had similar levels of infection in their spleen ($10^{4.7\pm0.5}$ CFU) as mice that received no T cells ($10^{4.8\pm0.5}$ CFU). Similar results were obtained in the liver (not shown). These results are also consistent with the absence of non-specific immunity observed in similar experiments with B6/129-derived P$^0$ CD8$^+$ T cell lines (3). Thus, minor Ag differences cannot account for the antilisterial immunity provided by T$^0$ CD8$^+$ T cell lines in these adoptive transfer experiments.

These results demonstrate that CD8$^+$ T cell-derived TNF is not required for antilisterial immunity in WT hosts. However, consistent with the TNF neutralization experiments, CD8$^+$ T cells from both WT and T$^0$ mice fail to transfer significant antilisterial immunity to T$^0$ hosts. This finding suggests a requisite role for TNF produced by host cells in antilisterial immunity mediated by LLO-specific CD8$^+$ T cells in adoptive transfer assays. The underpinnings of such a requirement are not clear, but could involve deficient homing of CD8$^+$ T cells to the spleen and liver in T$^0$ hosts, or a failure in the activation of CD8$^+$ T cells at the site of infection.

T$^0$ P$^0$ CD8$^+$ T cells mediate antilisterial immunity in the liver, but not the spleen, of WT host mice

Previous experiments have demonstrated that P$^0$ CD8$^+$ T cells mediate significant antilisterial immunity in the livers, but reduced immunity in the spleen, of WT hosts (3, 4). To address the influence of TNF on perforin-independent antilisterial immunity, LLO-specific CD8$^+$ T cells from T$^0$P$^0$ mice were transferred into WT hosts that were subsequently challenged with virulent LM. T$^0$P$^0$-derived CD8$^+$ T cells provided significant antilisterial immunity in the liver, but not in the spleen (Fig. 5, E and F). These results suggest that CD8$^+$ T cells provide immunity in the liver by a pathway that is independent of both CD8$^+$ T cell-derived perforin and TNF. This result also indicates that perforin-independent CD8$^+$ T cell immunity in the spleen requires CD8$^+$ T cell-derived TNF.

FIGURE 5. T$^0$P$^0$-derived LLO-specific CD8$^+$ T cells mediate antilisterial immunity in the liver, but not the spleen, of WT host mice. Naive B6 mice were injected i.v. with $10^7$ LLO-specific CD8$^+$ T cells derived from T$^0$P$^0$ mice and then challenged with $10^5$ virulent LM 10403s. CFU from the spleen (A) and liver (B) were quantitated 3 days postchallenge. Data from two independent experiments, six mice/group, are presented as described in Fig. 3.


