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Adaptive Immunity and Enhanced CD8$^+$ T Cell Response to *Listeria monocytogenes* in the Absence of Perforin and IFN-γ[1]

Vladimir P. Badovinac* and John T. Harty[2]*†

Single Ag-specific CD8$^+$ T cells from IFN-γ-deficient (GKO) or perforin-deficient (PKO) mice provide substantial immunity against murine infection with *Listeria monocytogenes*. To address the potential for redundancy between perforin and IFN-γ as CD8$^+$ T cell effector mechanisms, we generated perforin/IFN-γ (PKO/GKO) double-deficient mice. PKO/GKO-derived CD8$^+$ T cells specific for the immunodominant listeriolysin O ($LLO_{91–99}$) epitope provide immunity to LM infection similar to that provided by Ag-matched wild-type (WT) CD8$^+$ T cells in the liver but reduced in the spleen. Strikingly, polyclonal CD8$^+$ T cells from immunized PKO/GKO mice were ~100-fold more potent in reducing bacterial numbers than the same number of polyclonal CD8$^+$ T cells from immunized WT mice. This result is probably quantitative, because the frequency of the CD8$^+$ T cell response against the immunodominant $LLO_{91–99}$ epitope is >4.5-fold higher in PKO/GKO mice than WT mice at 7 days after identical immunizations. Moreover, PKO/GKO mice can be immunized by a single infection with attenuated *Listeria* to resist >80,000-fold higher challenges with virulent organisms than naive PKO/GKO mice. These data demonstrate that neither perforin nor IFN-γ is required for the development or expression of adaptive immunity to LM. In addition, the results suggest the potential for perforin and IFN-γ to regulate the magnitude of the CD8$^+$ T cell response to infection. ***The Journal of Immunology*****, 2000, 164: 6444–6452.**

Murine infection with *Listeria monocytogenes* (LM),[3] a Gram-positive, facultative intracellular bacterium, is a widely used model for the analysis of cell-mediated immunity (1, 2). It is clear that both CD4$^+$ and CD8$^+$ T cells are activated in an Ag-specific fashion following infection with LM (3). However, a large body of evidence involving specific T cell depletion (4) and experiments performed in mice deficient in CD4$^+$ and/or CD8$^+$ T cells (5, 6), indicate that CD8$^+$ T cells are the most effective mediators of anti-listerial immunity. These observations are consistent with the life cycle of LM, which gains access to and multiplies within the cytoplasm of the host cell (7). Not only is the cytosol protected from the host's extracellular defenses, it is the initiation site of events that lead to the presentation of peptides bound to MHC class I molecules on the surface of infected cells. 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 such as LM.

Priming of naive CD8$^+$ T cells results from their encounter with professional APC, such as dendritic cells, that express pathogen peptides in the context of self MHC class I molecules and also express high levels of costimulatory molecules. Once a CD8$^+$ T cell is activated, it is capable of recognizing any infected cell and elaborating a number of effector functions that aid the immune system in the clearance of the pathogen. Previously activated effector or memory CD8$^+$ T cells readily produce IFN-γ and TNF in an Ag-specific fashion. Both of these cytokines, which are also produced by cells other than CD8$^+$ T cells, are known to be important in the normal immune response against LM and in other infectious disease models in which CD8$^+$ T cells are important mediators of the resistance (8). Moreover, efficient Ag-specific lysis of a target cell harboring an intracellular pathogen is a function largely limited to CD8$^+$ T cells. At least two molecular pathways have been identified by which CD8$^+$ T cells mediate cytolysis (9, 10). The granule exocytosis pathway requires the coordinated activity of perforin and granzymes, both of which are found in the granules of activated CD8$^+$ T cells, to activate the caspase cascade of the target cell and induce apoptosis (11). Activated CD8$^+$ T cells also express CD95 ligand, which can ligate CD95 (Fas, Apo-1) on a target cells and induce apoptosis via the caspase cascade.

Recent studies to address the roles of these effector mechanisms in resistance to infection rely primarily on two approaches: in vivo treatments with mAbs and application of specific gene knockout mice. These approaches are complicated by the fact that most, if not all, of the potential effector mechanisms are expressed by multiple cell types. In addition, it is important to note that many of these molecules have the potential to play regulatory roles in the immune response, ranging from T cell homeostasis to enhancement of Ag presentation. Thus, our current understanding of which effector mechanisms resist specific pathogens is lacking.

Early reports using perforin-deficient mice revealed that CD8$^+$ T cell immunity against LM was 10- to 100-fold reduced compared with that in wild-type (WT) mice (12), while IFN-γ-deficient mice developed CD8$^+$ T cell immunity to LM indistinguishable from that of WT mice (13). Although studies with Ab neutralization of IFN-γ have provided disparate results (14, 15), the demonstration by Unanue and coworkers (16) that IFN-γ-deficient CD8$^+$ T cells, but not IFN-γ-deficient CD4$^+$ T cells, are able to

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[3] Abbrevations used in this paper: LM, *Listeria monocytogenes*; PKO, perforin knockout mice; GKO, IFN-γ knockout mice; WT, wild type; PKO/GKO, perforin/IFN-γ double-deficient mice; LLO, listeriolysin O; LD_{50}, lethal dose for 50%; CRA, ^{51}Cr release assay; MIP-1α, macrophage inhibitory protein-1α.

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clear chronic LM infection of mice with severe combined immunodeficiency supports the contention that IFN-γ is not required for $CD8^+$ T cell immunity to LM (13). More recently, experiments with perforin-deficient $CD8^+$ T cells that are specific for single LM Ags demonstrated the existence of a pathway of resistance that is independent of both perforin- and CD95-mediated cytolysis (17).

The finding that no single effector mechanism studied to date can account for all the anti-listerial resistance mediated by $CD8^+$ T cells has led to the hypothesis that multiple mechanisms, each capable of functioning independently, may provide anti-listerial immunity (1). In the present study we address the potential for redundancy in major $CD8^+$ T cell effector mechanisms using the LM experimental model and perforin/IFN-γ (PKO/GKO) double-deficient mice. We present evidence that neither the major cytolytic pathway nor IFN-γ production is required for the development or expression of $CD8^+$ T cell-dependent adaptive immunity to LM. However, our results are consistent with a regulatory role for these molecules in control of the $CD8^+$ T cell response to infection.

Materials and Methods

Mice

BALB/c (H-2^d MHC) mice were obtained from the National Cancer Institute (Frederick, MD). H-2^d MHC perforin (PKO) and IFN-γ (GKO) double-deficient (PKO/GKO) mice were generated by crossing H-2^d MHC PKO (18) and GKO (13) mice (both on the BALB/c background). Because perforin and IFN-γ are both on chromosome 10, F_1 mice were backcrossed to H-2^d PKO mice, and $PKO^{-/-}/GKO^{+/-}$ progeny, resulting from meiotic recombination, were identified by screening for perforin and IFN-γ deficiency as previously described (13, 18) and mated to generate PKO/GKO double-deficient mice. PKO/GKO 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 until the initiation of experiments with LM, at which point the mice were transferred to standard housing. All mice were used at 8–16 wk of age.

Cell lines and Abs

P815 cells are a DBA/2 (H-2^d)-derived mastocytoma tumor cell line (American Type Culture Collection, Manassas, VA; ATCC TIB-64); P815-LLO refers to P815 cells stably transfected with a plasmid construct expressing the LM Ag listeriolysin O (LLO) (19) and the G-418 resistance gene (20). P815-p60 refers to P815 cells stably transfected with a plasmid construct expressing the LM Ag p60 (21) and the G-418 resistance gene (22). Cells were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS, antibiotics, L-glutamine, HEPES buffer, and 2-ME (RP10) (13). Transfected cells were maintained in RP10 supplemented with G-418 at 400 μg/ml.

In vivo $CD8^+$ T cell subset depletion was performed with anti-CD8 mAb 2.43 (23), which was purified from culture supernatants using protein G affinity chromatography as recommended by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ). Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). In vivo $CD8^+$ T cell depletion was conducted by i.p. injection of 0.3 mg of mAb 2.43 on days −1, −2, and −3 relative to infection as previously described (13). In vitro T cell subset depletion was conducted with IgM mAbs RL-172 (anti-CD4) or 3.168 (anti-CD8) and rabbit complement as previously described (24). Depletion of T cell subsets was monitored by staining with FITC-conjugated anti-CD8 mAb 53.6–7 (PharMingen, San Diego, CA) and PE-conjugated anti-CD4 mAb H129.19 (PharMingen). Cells were analyzed on a FACScan (Becton Dickinson, Franklin Lakes, NJ) using Flow Jo (Tree Star, San Carlos, CA) software. Armenian hamster anti-mouse CD95 IgG (Jo2) and control Armenian hamster polyclonal IgG were purchased from PharMingen.

Bacteria and immunizations

Virulent LM strain 10403s (25), attenuated LM strain DP-L1942 (ActA deficient) (26), and Y92F LM mutant strain DP-L2528 (27) are resistant to streptomycin and were used as previously described (13). Briefly, frozen stocks of bacteria were grown in a bacterial shaker at 37°C in tryptic soy broth to an OD_{600} of ~0.1 (~10^8 CFU/ml), diluted in pyrogen-free 0.9% sodium chloride (Abbott Laboratories, North Chicago, IL), and injected i.v. in a 0.2-ml volume/animal. Aliquots were plated onto tryptic soy agar containing 50 μg/ml of streptomycin (TSB-Strep) to verify the number of CFU injected.

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

Several H-2^d MHC $CD8^+$ T cell lines specific for $LLO_{91–99}$ or $p60_{217–225}$ were derived from individual LM-infected WT (BALB/c) and PKO/GKO mice and were restimulated with P815-LLO or P815-p60 cells as previously described (13). Briefly, 2–4 × 10^7 splenocytes from mice injected 7 days previously with 10^3 CFU of virulent LM strain 10403s i.v. or 10^6 attenuated LM DP-L1942 (in the case of PKO/GKO mice) i.v. were incubated in RP10 at 37°C in 7% CO_2 with 3 × 10^6 irradiated (150 Gy) P815-LLO or P815-p60 stimulator cells. Subsequent weekly restimulations were conducted by combining 1–3 × 10^6 responder cells with 3 × 10^6 irradiated stimulator cells and ~5 × 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.

^{51}Cr release assays (CRA)

CRA were performed as previously described (13, 28). Briefly, 10^4 labeled target cells were combined with effector cells at the indicated ratios in 200 μl of RP10/well in round-bottom 96-well plates. Following a 3- to 9.5-h incubation (as indicated), 100 μl/well of supernatant was harvested and assayed for ^{51}Cr release. The percent specific release of ^{51}Cr was calculated by the formula: 100 × [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)]. Spontaneous release was <20% of the total in all assays. Armenian hamster anti-mouse CD95 IgG (Jo2) and control Armenian hamster polyclonal IgG (PharMingen) were used in the CRA as indicated.

Adoptive transfer experiments

The capacity of splenocytes derived from immunized animals and $CD8^+$ T cell lines to mediate anti-listerial immunity in vivo was quantitated using adoptive transfer assays as described previously (13, 28). Briefly, RBC-depleted splenocytes from donor mice immunized 7 days previously with ~10^6 ActA-deficient LM strain DP-L1942 or $CD8^+$ T cells restimulated in vitro 7 days previously were harvested, washed, and resuspended in pyrogen-free 0.9% sodium chloride. Cells were delivered i.v. in a 0.3-ml volume into naive BALB/c host mice. Within 2 h host mice, including saline-injected naive controls, were challenged i.v. with the indicated dose of virulent LM strain 10403s. CFU per spleen and gram of liver were determined 3 days postchallenge by homogenizing the spleens and livers in 0.2% IGEPAL (Sigma, St. Louis, MO), plating 10-fold serial dilutions onto TSB-Strep, and calculating colony count averages after overnight incubation at 37°C.

Intracellular cytokine staining

Intracellular cytokine staining was performed using the Cytofix/Cytoperm plus (with GolgiPlug) kit (PharMingen) as previously described (29). Briefly, 20 × 10^6 splenocytes from an infected mouse were treated with ACK lysis buffer for 5 min at room temperature to remove RBC. Splenocytes were washed twice in RP10 and resuspended in the same medium in 1 ml. Cells (200 μl) were incubated for 6 h at 37°C with medium alone or with a synthetic peptide epitope consisting of aa 91–99 from the LM LLO molecule ($LLO_{91–99}$, 200 nM), all in the presence of 1 μl/ml GolgiPlug (Brefeldin A). Cells were washed twice in FACS buffer (PBS supplemented with 1% FCS and NaN_3) and were incubated with Ab directed against the FcγII/III receptors (2.4G2) and FITC-labeled anti-CD8 (53-6.7) on ice for 30 min. The cells were washed twice with FACS buffer, then fixed and permeabilized by incubating for 15 min in 250 μl of Cytofix/Cytoperm solution. Then, cells were washed twice in Perm/wash solution and stained with PE-conjugated anti-TNF (MP6-XT22, PharMingen) for 30 min on ice. Cells were washed twice in Perm/wash solution and resuspended in 350 μl of FACS buffer before flow cytometry analysis.

The same procedure was followed for detection of intracellular TNF and IFN-γ (XMG1.2, PharMingen) production by $CD8^+$ T cell lines, except that the $CD8^+$ T cells were incubated for 4 h with P815 cells in the presence or the absence of synthetic $LLO_{91–99}$ (200 nM) peptide at the indicated E:T cell ratios.

Acquisition and analysis

List mode data were acquired on a FACScan flow cytometer (Becton Dickinson) using CYCLOPS software. Dead cells, monocytes, and tumor cells from coculture experiments were excluded by forward and side scatter gating. Typically, 25,000 events for CD8 T cell lines or 100,000 events for splenocytes were acquired for analysis with Flow Jo software (Tree Star).

Table I. *PKO/GKO mice exhibit increased susceptibility to virulent LM compared to WT mice but similar resistance to ActA-deficient LM*[a]

LM Strain	WT (H-2^d), log 10 LD_{50}	PKO/GKO (H-2^d), log 10 LD_{50}
10403s (virulent)	4.4	1.0
DP-L1942 (ActA$^-$)	6.9	6.6

[a] WT BALB/c and PKO/GKO mice were infected with the indicated virulent (10403s) or attenuated (DP-L1942) LM strains and LD_{50}s were estimated as described (13).

The gate for TNF$^+$ cells was selected such that the percentage of TNF$^+$ cells in the unstimulated sample for each mouse was 0.5 ± 0.05% of the CD8$^+$ splenocytes; this level has been subtracted from the peptide-stimulated splenocytes to determine the frequency of the response above the background.

Results

PKO/GKO double-deficient mice are extremely susceptible to infection with virulent LM, but exhibit WT resistance to ActA-deficient LM

It has been determined previously that IFN-γ-deficient (GKO) mice and IFN-γ receptor-deficient mice are extremely susceptible to primary infection with virulent LM (13, 30). In contrast, perforin-deficient (PKO) mice and WT mice are equally resistant to primary infection with virulent LM (12, 17, 18). To quantify the resistance of PKO/GKO double-deficient mice to primary infection with virulent LM, groups of PKO/GKO mice were injected i.v. with serially diluted aliquots of virulent LM 10403s and monitored for survival. The WT mice were injected as controls. The estimated LD_{50} of virulent LM 10403s in each strain of mouse is displayed in Table I. As expected, based on their lack of IFN-γ, PKO/GKO mice were highly susceptible to primary infection with virulent LM (LD_{50}, ~10 organisms) and died 4–6 days postchallenge at all lethal doses.

On the other hand, we have previously shown that GKO mice, despite extreme susceptibility to primary infection with virulent LM, are equally resistant as WT mice to primary infection with attenuated LM strain DP-L1942 (13). To determine the feasibility of immunizing PKO/GKO mice by the same strategy, we measured the LD_{50} of the attenuated LM strain DP-L1942 in naive WT and PKO/GKO mice. LM strain DP-L1942 exhibits attenuated virulence in mice due to an engineered in-frame deletion of the gene coding for ActA, a molecule that is required for cell-to-cell spread (26). The WT and PKO/GKO mice were injected with graded doses of DP-L1942 and monitored for survival. Estimates of the LD_{50} of the attenuated strain in each strain of mouse (Table I) show that PKO/GKO and WT mice exhibit similar resistance to high dose challenge ($>10^6$ organisms) with attenuated LM DP-L1942.

PKO/GKO-derived LLO-specific CD8$^+$ T cells mediate delayed, CD95-dependent cytotoxicity in vitro, and produce TNF in an Ag-specific fashion

LM Ag-specific CD8$^+$ T cells from H-2^d MHC GKO (13) or PKO (17, 18) single-knockout mice provide significant immunity to LM infection. To address the issue of redundancy in these effector mechanisms, we generated CD8$^+$ T cell lines specific for the LM Ags LLO and p60 from DP-L1942-immunized PKO/GKO double-deficient mice by in vitro restimulation with P815-LLO or P815-p60 APC.

After several in vitro restimulations all lines were >95% CD8$^+$CD4$^-$, as measured by flow cytometry (data not shown). To verify the Ag specificity and cytolytic activity of the CD8$^+$ T cells

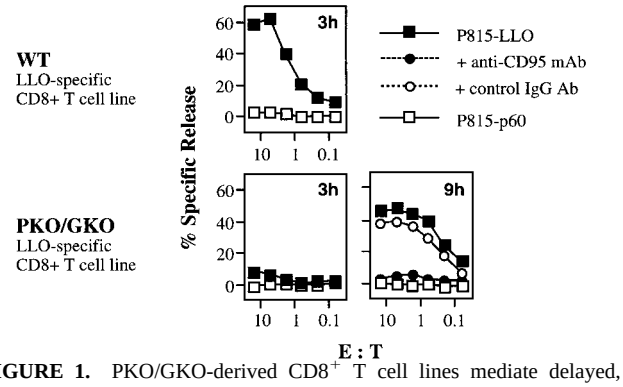

FIGURE 1. PKO/GKO-derived CD8$^+$ T cell lines mediate delayed, CD95-dependent cytotoxicity in vitro. The WT and PKO/GKO mice were infected with DP-L1942, and 7 days later their spleen cells were restimulated in vitro with an LLO transfectant of P815 tumor cells (P815-LLO) (19). The WT and PKO/GKO LLO-specific CD8$^+$ T cells were incubated in standard (3-h) and extended (9-h) CRAs with P815-LLO or P815-p60 (22) cells at the indicated E:T cell ratio. All data are representative of at least three independent experiments. Anti-CD95 mAb (Jo2) or control IgG were added at 5 μg/ml.

we performed CRA. Whereas WT-derived LLO-specific CD8$^+$ T cells were capable of high levels of specific lysis of LLO-expressing target cells (Fig. 1) (13, 18)), PKO/GKO-derived LLO-specific CD8$^+$ T cells, similar to LLO-specific CD8$^+$ T cells from PKO single-knockout mice (17, 18), mediated minimal levels of specific lysis of the same target cells in a short term (3-h) assay (Fig. 1). Similar results were obtained with WT- and PKO/GKO-derived p60-specific CD8$^+$ T cell lines (data not shown).

To test the ability of PKO/GKO-derived LLO-specific CD8$^+$ T cells to mediate perforin-independent cytolysis, we performed extended CRA as previously described (17, 18). The same PKO/GKO LLO-specific CD8$^+$ T cell line was able to carry out Ag-specific target cell lysis in a 9-h CRA. Moreover, perforin-independent killing was CD95L/CD95 dependent, because mAb against CD95 completely inhibits the cytotoxic response (Fig. 1). Thus, we have generated LLO-specific CD8$^+$ T cell lines from perforin and IFN-γ double-deficient mice that can mediate Ag-specific cytolysis through CD95-CD95L interactions.

To measure cytokine production and verify Ag specificity, LLO-specific CD8$^+$ T cells from WT and PKO/GKO mice were assayed for Ag-specific IFN-γ and TNF synthesis using intracellular cytokine staining. Previous studies from our laboratory demonstrated that detection of Ag-specific CD8$^+$ T cells with intracellular cytokine staining is clearly dependent on the E:T cell ratio as well as the incubation time in the presence of the protein transport inhibitor Brefeldin A (29). Under stimulation conditions where LLO-specific WT CD8$^+$ T cells produce both TNF and IFN-γ, the PKO/GKO-derived LLO-specific CD8$^+$ T cell line produced TNF, but not IFN-γ (Fig. 2). The response is specific, because intracellular TNF was not detected after incubation with P815 cells in the absence of $LLO_{91–99}$ peptide. Similar results were obtained using a bioassay with WEHI 164 clone 13 cells that die in the presence of TNF (data not shown). Finally, comparable results were obtained with the p60-specific PKO/GKO CD8$^+$ T cell line (data not shown).

Taken together, these experiments demonstrate that infection of PKO/GKO mice with an ActA-deficient LM strain primed the CD8$^+$ T cell compartment and allowed the generation of Ag-specific CD8$^+$ T cells that are incapable of mediating perforin-dependent cytotoxicity or producing IFN-γ.

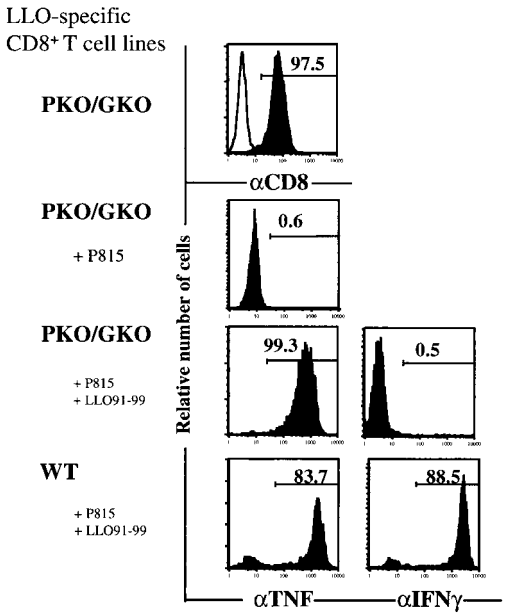

FIGURE 2. Intracellular cytokine staining of the PKO/GKO LLO-specific $CD8^+$ T cell line reveals Ag-specific TNF, but not IFN-γ, production. The LLO-specific $CD8^+$ T cell lines derived from WT and PKO/GKO mice were incubated at an E:T cell ratio of 1:1 with P815 cells in the absence or the presence of $LLO_{91–99}$ peptide (200 nM). After 4 h of incubation in the presence of Brefeldin A, $CD8^+$ T cell lines were stained for CD8 expression, then fixed and separated before the addition of anti-TNF or anti-IFN-γ mAbs. Data are presented as the percentage of $CD8^+$ T cells or as the percentage of $CD8^+$/cytokine-positive cells. All data are representative of three independent experiments with similar results.

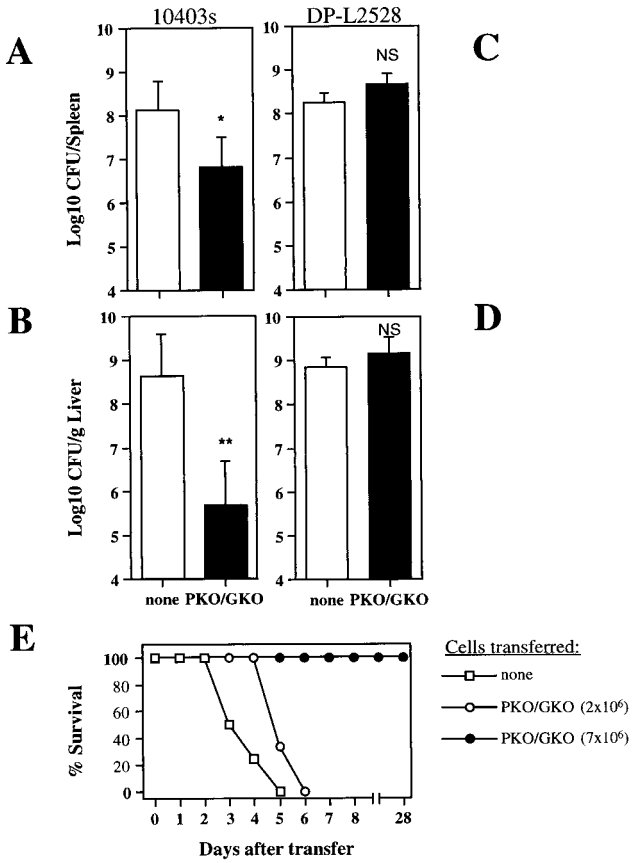

FIGURE 3. PKO/GKO-derived LLO-specific $CD8^+$ T cells can provide specific immunity against LM. Naive WT mice were injected i.v. with LLO-specific $CD8^+$ T cells derived from PKO/GKO mice and within hours were challenged with ~10 LD_{50} of virulent LM strain 10403s (25) or Y92F LM mutant strain DP-L2528 (27). CFU from the spleen (*A* and *C*) and liver (*B* and *D*) were quantitated 3 days postchallenge. Data are presented as the mean ± SD for six (*A* and *B*; two pooled experiments) or three (*C* and *D*) mice per group. Student's *t* test was used in the statistical analysis; *p* values are shown for each group compared with the control group in the same experiment, which did not receive T cells (*, $p < 0.05$; **, $p < 0.01$; NS, not significant). *E*, Naive WT mice were injected i.v. with LLO-specific $CD8^+$ T cells derived from PKO/GKO mice and within hours were challenged with ~10 LD_{50} of virulent LM strain 10403s. Survival was monitored through 28 days postchallenge for three or four mice per group. LLO-specific $CD8^+$ T cells transferred: *A* and *B*, 7 or 10×10^6; *C* and *D*, 7×10^6; *E*, ○, 2×10^6; and *E*, ●, 7×10^6.

PKO/GKO-derived LLO-specific $CD8^+$ T cells provide specific immunity against LM infection

To determine the requirement for $CD8^+$ T cell-derived perforin and IFN-γ in adoptive transfer of anti-listerial immunity, we analyzed the ability of LLO-specific $CD8^+$ T cell lines from PKO/GKO mice to transfer immunity to naive WT host mice. The degree of immunity transferred was estimated by infecting groups of mice with >10 LD_{50} of virulent LM strain 10403s in the presence or the absence of LLO-specific $CD8^+$ T cells and determining the number of LM in organ homogenates at 3 days postinfection.

PKO/GKO-derived $CD8^+$ T cell lines specific for LLO transferred significant immunity against LM infection. Immunity, as estimated by CFU reduction, was more potent in the liver (~3 log10) than in the spleen (~1.5 log10; Fig. 3, *A* and *B*). We have shown previously that Ag-specific $CD8^+$ T cells from WT and GKO mice reduced CFU to a similar degree in spleen and liver (13). Similarly, PKO-derived $CD8^+$ T cells reduced CFU in the liver to the same extent as Ag-matched WT $CD8^+$ T cells (17, 18). In contrast, the degree of immunity in the spleen mediated by PKO-derived $CD8^+$ T cells was typically less than that usually observed with WT- or GKO-derived $CD8^+$ T cells. Thus, perforin-dependent organ-specific differences in immunity are also observed with PKO/GKO-derived $CD8^+$ T cells.

Previous adoptive transfer experiments demonstrated 1) that $CD8^+$ T cells specific for non-LM-derived Ags provide no protection against infection with LM; 2) that $CD8^+$ T cells specific for LM-derived Ags provide no protection against infection with an unrelated intracellular bacterial pathogen; and 3) that 10-fold or greater reductions in the level of infection 3 days postchallenge directly correlate with the survival of animals injected with an otherwise lethal dose of LM (17, 18). To verify the specificity of in vivo immunity mediated by PKO/GKO-derived $CD8^+$ T cells, we transferred these cells into naive WT mice that were subsequently challenged with >10 LD_{50} of virulent LM strain DP-L2528. This strain expresses a mutant LLO molecule containing a conservative Y-to-F (Y92F) substitution for the anchor residue at a position 2 within the 91–99 epitope (27). This mutation abolishes the $LLO_{91–99}$ response in immunized mice and prevents protective immunity mediated by LLO-specific WT $CD8^+$ T cells (J. T. Harty, unpublished observation). Consistent with these data, the Y92F mutation completely abolished the capacity of PKO/GKO-derived $LLO_{91–99}$-specific $CD8^+$ T cells to provide anti-listerial immunity (Fig. 3, *C* and *D*). Thus, the immunity provided by PKO/GKO-derived $CD8^+$ T cells is Ag specific.

Finally, the CFU reductions mediated by PKO/GKO-derived-LLO-specific $CD8^+$ T cells are biologically significant, as these

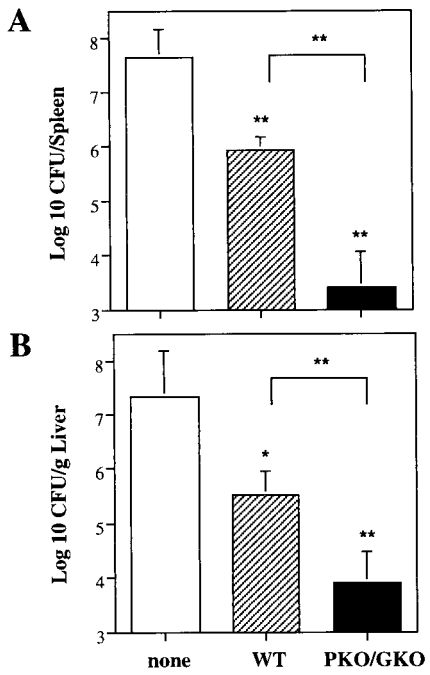

FIGURE 4. Enhanced anti-listerial immunity mediated by splenocytes derived from PKO/GKO mice compared with WT splenocytes. Naive WT mice were injected i.v. with 5×10^7 splenocytes from WT or PKO/GKO mice that had been immunized i.v. with 1×10^6 ActA-deficient LM strain DP-L1942 7 days previously. Following the transfer of immune splenocytes, recipient mice were challenged i.v. with $1.4 \pm 0.2 \times 10^5$ virulent LM strain 10403s (~15 LD_{50}). CFU analysis was performed on day 3 postchallenge. Data are presented as the mean log10 CFU ± SD for four animals per group. Student's *t* test was used in statistical analysis; statistical significance is shown for each group compared with the control group in the same experiments that did not receive immune splenocytes; an additional *p* value comparing groups that received WT and PKO/GKO immune splenocytes is also indicated. Data are representative of two independent experiments with similar results. *, $p < 0.05$; **, $p < 0.01$.

cells confer dose-dependent survival against lethal LM infections (Fig. 3*E*).

These experiments demonstrate that CD8$^+$ T cell-derived perforin and IFN-γ are not required for transfer of anti-listerial resistance by single Ag-specific CD8$^+$ T cells.

PKO/GKO CD8$^+$ T cells from LM-infected mice provide increased immunity to LM infection compared with WT CD8$^+$ T cells

Perforin- and IFN-γ-independent transfer of anti-listerial immunity by relatively large numbers of in vitro-propagated single Ag-specific CD8$^+$ T cells provides evidence that CD8$^+$ T cell-derived perforin and IFN-γ are not required for expression of immunity. However, this scenario does not accurately reflect the in vivo situation, where CD8$^+$ T cell responses to multiple LM Ags would be expected. To address this issue, we infected WT or PKO/GKO mice with LM DP-L1942, and 7 days later equivalent numbers (5×10^7) of WT or PKO/GKO donor splenocytes were transferred into naive WT host mice that were subsequently challenged with a high dose (~10 LD_{50}) of virulent LM. Bacterial counts in splenocyte recipients and control mice 3 days postchallenge demonstrated that PKO/GKO-derived splenocytes provided dramatic anti-listerial immunity in both spleens (Fig. 4*A*) and livers (Fig. 4*B*), reducing bacterial recovery >10,000-fold compared with mice that did not receive immune splenocytes. Moreover, the degree of immunity provided by the PKO/GKO immune splenocytes was significantly (50- and 100-fold in the liver and spleen, respectively) higher than provided by the same number of WT immune splenocytes (Fig. 4).

Anti-listerial immunity mediated by LM-immune WT splenocytes is dependent on CD8$^+$ T cells (13, 17, 18). To determine the effectors of anti-listerial immunity in the splenocyte populations from PKO/GKO mice we depleted CD4$^+$ or CD8$^+$ T cells before transfer (Fig. 5). Depletion of the majority of CD4$^+$ T cells from immune PKO/GKO splenocytes (>95% depletion as measured by CD8 vs CD4 staining; data not shown) with Ab and complement did not significantly affect their capacity to provide anti-listerial immunity compared with mock-depleted splenocytes (Fig. 5, *A* and *B*). On the other hand, depletion of >95% of the CD8$^+$ T cells (data not shown) eliminated >99.8%, based on CFU reduction, of the protective immunity seen in mock-depleted splenocytes (Fig. 5, *C* and *D*). Immunity mediated by CD4-depleted PKO/GKO splenocytes was dose dependent (Fig. 5, *A* and *B*). Interestingly, immunity mediated by 2.5×10^7 PKO/GKO CD4$^+$-depleted immune splenocytes was still higher than that observed by twice as many WT cells. Transfer of 5×10^6 CD4-depleted PKO/GKO splenocytes did not result in significant immunity. Thus, immunity provided by CD4-depleted immune PKO/GKO splenocytes is >2-fold, but <10-fold, better than that of WT immune splenocytes on a per cell basis. Together, these data are consistent with the idea that elevated immunity provided by LM-immune splenocytes from PKO/GKO mice is CD8$^+$ T cell dependent.

Thus, although they are deficient in two major CD8$^+$ T cell effector pathways, PKO/GKO immune splenocytes provide an unexpectedly high degree of CD8$^+$ T cell-dependent anti-listerial immunity.

LLO-specific CD8$^+$ T cell priming is significantly elevated in PKO/GKO double-deficient mice compared with WT mice

The increased efficiency of anti-listerial immunity provided by PKO/GKO CD8$^+$ T cells compared with WT cells could result from quantitative or qualitative differences. To address this issue we analyzed the level of expansion of Ag-specific CD8$^+$ T cells in WT and double-deficient PKO/GKO mice.

The WT and PKO/GKO mice were infected with DP-L1942, and at the peak of the primary response (day 7), the CD8$^+$ T cell response against the immunodominant $LLO_{91–99}$ epitope (31) was determined by intracellular TNF staining. Spleen cells freshly explanted from infected mice were cultured in vitro for 6 h either without stimulation (i.e., no peptide) or with $LLO_{91–99}$ peptide. In some experiments the frequency of LLO-specific CD8$^+$ T cell was determined in the same populations used in adoptive transfer assays.

Although intracellular IFN-γ staining appears to consistently detect a higher frequency of Ag-specific CD8$^+$ T cells than detection of intracellular TNF (32, 33), intracellular staining for TNF can be used to reliably detect Ag-specific CD8$^+$ T cell responses (29), particularly in IFN-γ deficient mice. Intracellular cytokine staining detects ~2% (1 of 52) $LLO_{91–99}$-specific CD8$^+$ T cells on day 7 after immunization of WT BALB/c mice (Fig. 6, *A* and *B*). This number is in good agreement with estimates of the frequency of LLO-specific CD8$^+$ T cells at the peak of the primary response in WT mice, detected either by phenotypic (MHC class I-peptide tetramer staining) or functional (IFN-γ enzyme-linked immunospot) measures, after virulent LM infection (18, 34–36).

Strikingly, priming of LLO-specific CD8$^+$ T cells in PKO/GKO double-deficient mice was substantially higher than that in MHC-matched WT mice. More than 9% of CD8$^+$ T cells (1 of 11) on day 7 responded with specific TNF production after stimulation

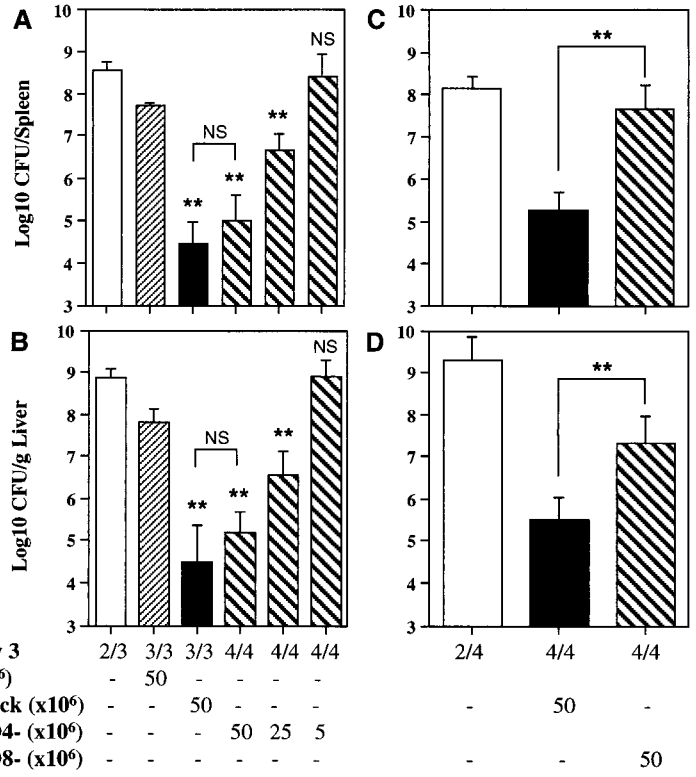

FIGURE 5. Anti-listerial immunity mediated by PKO/GKO immune splenocytes is $CD8^+$ T cell dependent. Naive WT mice were injected i.v. with the indicated number of splenocytes from the donor WT or PKO/GKO mice that had been immunized with i.v. with 1×10^6 ActA-deficient LM strain DP-L1942 7 days previously. As indicated, immune splenocytes were depleted of $CD4^+$ (αCD4; *A* and *B*) or $CD8^+$ (αCD8) T cells (*C* and *D*) with mAbs and complement in two independent experiments. The efficiency of depletion was >95% in both experiments as determined by flow cytometric analysis (data not shown). Splenocyte-injected mice and controls were than challenged with 1.2×10^5 virulent LM strain 10403s. CFU analysis was performed on day 3 postchallenge. Data are presented as the mean log10 CFU ± SD for the indicated numbers of surviving animals per group. Student's *t* test was used in statistical analysis. *, $p < 0.05$; **, $p < 0.01$; NS, not significant.

with the $LLO_{91–99}$ peptide (Fig. 6, *A* and *B*). This increased Ag-specific response was not completely dependent on delayed bacterial clearance, as only three of six PKO/GKO mice had evidence of low levels (<2000 CFU) of bacteria on day 7 postinfection (Fig. 6*B*).

Thus, in the absence of perforin and IFN-γ, priming of $LLO_{91–99}$-specific $CD8^+$ T cells is enhanced compared with that in WT mice and may account for the increased immunity observed in adoptive transfer assays.

Vaccination of PKO/GKO mice with attenuated LM induces specific resistance to virulent LM that is dependent on $CD8^+$ T cells

The immunity mediated by PKO/GKO-derived $CD8^+$ T cells in the transfer experiments described above occurred in WT recipients that are capable of expressing IFN-γ in response to LM infection. To determine whether secondary resistance to virulent LM challenge could be expressed in the absence of perforin and IFN-γ we vaccinated PKO/GKO mice by infection with 1×10^6 LM DP-L1942, and 35 days later challenged the immunized mice with various doses of virulent LM 10403s. Vaccinated PKO/GKO mice exhibited ~80,000 times higher resistance (LD_{50}, $10^{5.8}$ CFU; Table II) compared with naive PKO/GKO mice (LD_{50}, 10 CFU; Table I). Again, depletion of $CD8^+$ T cells before secondary challenge with virulent LM revealed that $CD8^+$ T cells are the major effectors of acquired anti-listerial immunity in the PKO/GKO mice (Table II).

Thus, substantial adaptive immunity to LM can be generated and expressed in the absence of perforin and IFN-γ. In addition, the difference in resistance in naive PKO/GKO mice and PKO/GKO mice receiving immunization with an attenuated LM strain demonstrates that vaccination-induced immunity can overcome the lack of a major cytolytic pathway and the lack of a cytokine that is critical for resistance to acute infection.

Discussion

Although $CD8^+$ T cells possess many weapons with the potential to resist infection, recent experiments with gene knockout mice indicate that components of this arsenal are differentially effective against specific pathogens (8). Given the complexity of both the $CD8^+$ T cell arsenal and the strategies employed by pathogens to circumvent host immunity, understanding which mechanisms are important in $CD8^+$ T cell mediated resistance to infection is a substantial challenge. To address this issue in the murine listeriosis model, we have taken the approach of systematic elimination of known $CD8^+$ T cell effector mechanisms through combinations of specific gene knockout mice. Our results with PKO/GKO mice demonstrate that neither perforin nor IFN-γ is required for the development or expression of $CD8^+$ T cell immunity to LM infection. However, these molecules have a substantial quantitative impact on the magnitude of the $CD8^+$ T cell response to the immunodominant $LLO_{91–99}$ epitope.

Lethal infection of $H\text{-}2^d$ PKO/GKO double-deficient mice requires 1000-fold fewer LM than the number required for lethal infection of mice with an intact IFN-γ structural gene. These data are in line with data obtained with GKO single-deficient mice (13) and confirm that IFN-γ is a critical mediator of innate immunity to acute LM infection. In contrast, PKO/GKO mice resist infection with an ActA-deficient LM strain at a level comparable to that in WT mice. Although they are attenuated for virulence, ActA-deficient LM enter the cytoplasm of infected cells and are accessible to the endogenous MHC class I Ag presentation pathway. Indeed, infection of WT and GKO mice with ActA-deficient LM elicits a $CD8^+$ T cell response that can transfer anti-listerial immunity to naive WT mice (13, 37). Because PKO/GKO mice survive infection with ActA-deficient LM, we used this strain to elicit LM-specific $CD8^+$ T cells that are incapable of perforin-mediated cytolysis or IFN-γ production.

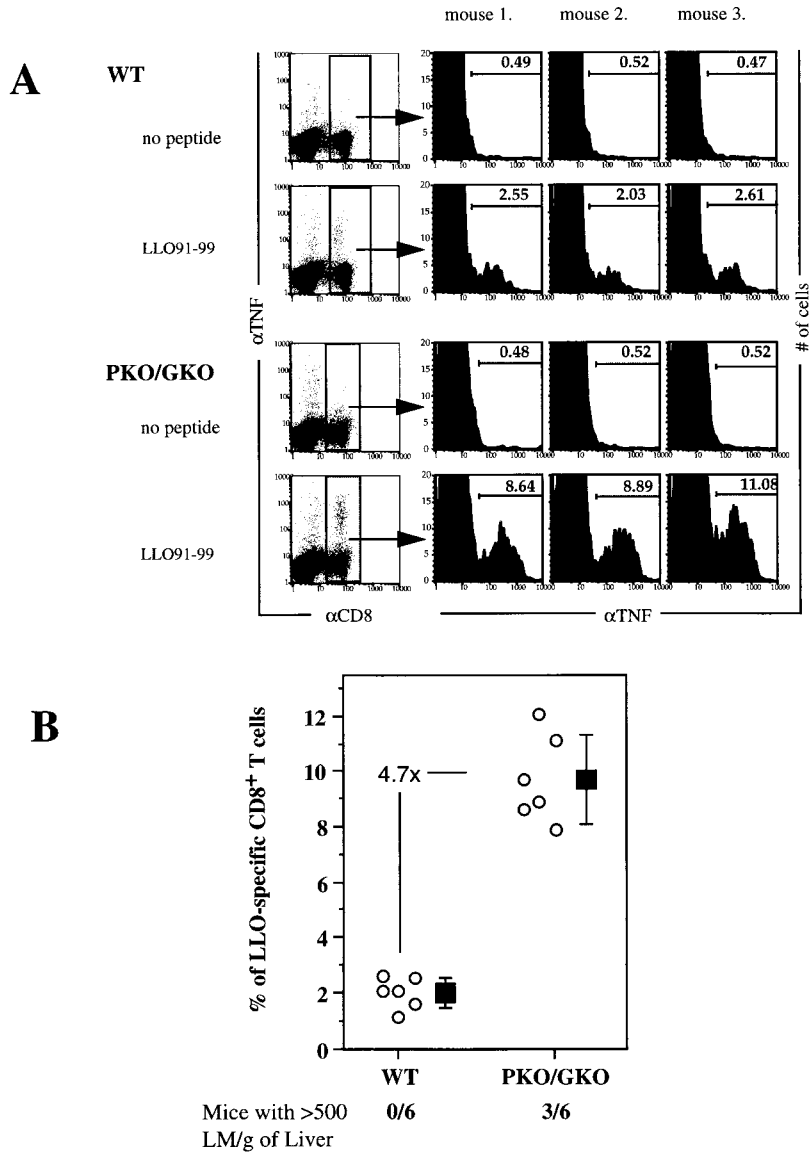

FIGURE 6. PKO/GKO double-deficient mice have higher frequencies of LLO-specific CD8$^+$ T cells after primary LM infection. The WT and PKO/GKO mice were infected with $1.3 \pm 0.4 \times 10^6$ LM strain DP-L1942, and 7 days later the frequency of LLO-specific CD8$^+$ T cells in the spleen was measured by intracellular TNF staining. Spleen cells from LM-infected WT and PKO/GKO mice were cultured in vitro for 6 h in the presence of Brefeldin A, either with or without the $LLO_{91–99}$ peptide (200 nM), and stained for surface CD8 and intracellular TNF expression. *A*, Data are presented for individual WT or PKO/GKO mice in one representative experiment. The numbers inside each histogram represent the percentage of CD8$^+$ T cells that responded in the absence (background) or the presence of $LLO_{91–99}$ peptide. *B*, Data are presented as the percentage of CD8$^+$ T cells that make TNF upon stimulation with the LLO epitope. ○, An individual mouse; ■, average value ± SD. Data are pooled from two independent experiments with three mice per group in each experiment. CFU analyses were performed on livers with a limit of detection of 500 LM/g of tissue. PKO/GKO mice with detectable infection had <2000 LM/g of liver.

PKO/GKO-derived CD8$^+$ T cell lines specific for the single epitope derived from the secreted LLO molecule transferred Ag-specific anti-listerial immunity to naive WT mice. Similar to the results we obtained with CD8$^+$ T cells from PKO single-deficient mice (17, 18), the level of immunity provided by the PKO/GKO-derived LLO-specific CD8$^+$ T cell lines is greater in the liver than in the spleen. This organ-specific immunity is probably due to perforin deficiency, because LM-specific CD8$^+$ T cell lines derived from GKO and WT mice transfer indistinguishable levels of immunity in both organs (13). On the other hand, adoptive transfer of polyclonal immune splenocytes from PKO/GKO mice reveals an unexpectedly high degree of CD8$^+$ T cell-dependent anti-listerial immunity compared with immune splenocytes from WT mice.

Table II. *Vaccination of PKO/GKO mice with DP-L1942 induces specific CD8$^+$ T cell-dependent immunity*[a]

PKO/GKO	10403s	Survival	
		− Anti-CD8	+ Anti-CD8
Immune	10^7	1/3	ND
	10^6	3/6	0/6
	10^5	3/6	0/3
	10^4	6/6	0/3
Naive	10^3	0/3	ND

[a] PKO/GKO mice were infected with 1×10^6 CFU of DP-L1942. Mice were subsequently depleted of CD8$^+$ T cells or not and were challenged at 35 days postimmunization with the indicated number of virulent LM 10403s. Survival was monitored for 28 days. Data are pooled from two independent experiments. The LD_{50} in the absence of CD8$^+$ T cell depletion was $10^{5.8}$ CFU. ND, not determined.

The increased efficiency of anti-listerial immunity observed in adoptive transfer assays probably results from quantitative differences, because the PKO/GKO-derived immune splenocytes contain >4.5-fold more CD8$^+$ T cells specific for the immunodominant $LLO_{91–99}$ epitope than the WT populations. Further studies with ex vivo separated Ag-specific CD8$^+$ T cells will be required to demonstrate whether qualitative differences in providing anti-listerial immunity exist between double-deficient and WT CD8$^+$ T cells.

Taken together, the adoptive transfer experiments with single-Ag-specific as well as polyclonal CD8$^+$ T cells showed that neither perforin nor IFN-γ is required as an effector mechanism for

$CD8^+$ T cells to provide substantial anti-listerial immunity. Thus, the capacity of PKO- or GKO-derived single knockout $CD8^+$ T cells to provide anti-listerial immunity does not result from a paired redundancy between these effector mechanisms. These findings raise several important questions.

If $CD8^+$ T cells can provide anti-listerial immunity in the absence of two major effector mechanisms, what $CD8^+$ T cell effector mechanism(s) is responsible for $CD8^+$ T cell-mediated protection? PKO/GKO $CD8^+$ T cells produce TNF after specific Ag recognition and TNF derived from $CD8^+$ T cells could participate in anti-listerial immunity by at least three independent mechanisms. $CD8^+$ T cell-derived TNF might contribute to the maximal activation of listericidal capacities in macrophages (38). Besides activation of proinflammatory genes, signaling through TNFRI can activate the caspase cascade and induce apoptosis (39), suggesting that $CD8^+$ T cells might deliver a TNF-dependent signal resulting in the death of the infected cell. This would release LM into the extracellular space, where they are susceptible to phagocytosis by listericidal macrophages and neutrophils. Finally, TNF has been demonstrated to up-regulate adhesion molecule expression on endothelial cells, which could aid in the recruitment of accessory phagocytes to the site of infection (40–42). Consistent with a role for TNF in adaptive immunity against LM are our results showing that Ab-mediated neutralization of TNF prevents anti-listerial immunity mediated by PKO-derived $CD8^+$ T cells (17) and that similar treatments inhibited secondary resistance to challenge infection of immune WT mice (14). However, $CD8^+$ T cells derived from LM immune TNF-deficient (52) and TNF receptor I-deficient[4] animals were cytolytic, produced IFN-γ in an Ag-specific fashion, and mediated substantial anti-listerial immunity when transferred to WT mice. Although these results do not support an essential role for $CD8^+$ T cell-derived TNF in anti-listerial immunity, they do not rule out the possibility that TNF is required as a $CD8^+$ T cell effector mechanisms in the absence of perforin and IFN-γ.

Besides Ag-specific TNF production, PKO/GKO $CD8^+$ T cells are also capable of mediating CD95L-CD95-dependent lysis of APCs. The importance of CD95-dependent cytolysis in $CD8^+$ T cell control of LM was previously addressed by determining the ability of LM Ag-specific $CD8^+$ T cells, from WT and PKO mice to provide anti-listerial immunity to CD95-deficient ($B6.MRL^{lpr/lpr}$) hosts. It was shown that LM Ag-specific $CD8^+$ T cells, regardless of whether they express perforin, are capable of providing anti-listerial immunity that is independent of both cytolytic pathways and is even independent of IFN-γ production (17); however, immunity mediated by these $CD8^+$ T cells was inhibited by in vivo neutralization of TNF, pointing out the possible requisite role for TNF in settings completely devoid of perforin and IFN-γ.

Interestingly, recent work suggests that $CD8^+$ T cell-derived macrophage inhibitory protein-1α (MIP-1α), a chemokine produced by multiple cell types, including $CD8^+$ T lymphocytes, may be required for adaptive immunity to LM (43). $CD8^+$ T cells, derived from LM-infected MIP-1α-deficient mice, were capable of LM-specific cytolysis in vitro, but failed to transfer anti-listerial immunity to naive mice. These results suggest that MIP-1α and chemokine production, in general, may represent another mechanism for $CD8^+$ T cell-mediated anti-listerial immunity. Finally, $CD8^+$ T cells may elaborate molecules with direct microbicidal activity (44, 45). Purified recombinant granulysin, a protein normally found in the granules of activated human $CD8^+$ T cells and NK cells, mediates direct microbicidal activity against a range of pathogens, including LM (45). It has been shown that the presence of perforin is necessary for granulysin to inhibit the growth of intracellular *Mycobacterium tuberculosis* (44). Extension of these studies into the LM model is currently limited by the absence of an identified mouse homologue of human granulysin.

Priming of $LLO_{91–99}$-specific $CD8^+$ T cells in the absence of perforin and IFN-γ is substantially greater than that in WT mice. In addition to their potential as $CD8^+$ T cell effector mechanisms, most, if not all, of these molecules play regulatory roles ranging from T cell homeostasis to enhancement of Ag presentation. The expansion of LM-specific $CD8^+$ T cells depends on the infecting dose (our unpublished observation) (46). PKO/GKO mice show a moderate delay in the clearance of LM in both spleen and liver compared with WT mice, and the increased duration of the infection may account for the increased Ag-specific $CD8^+$ T cell response seen in double-deficient mice. Also, recent studies have shown a role for perforin in down-regulating Ag-specific T cell responses during chronic viral infection (47) and in response to superantigen stimulation (48). In addition, the antiproliferative and cytotoxic effects of IFN-γ could participate in the regulation of T cell expansion (49–51). Further analysis of single- and double-deficient mice should clarify these issues.

Finally, the ability of PKO/GKO mice to develop $CD8^+$ T cell-mediated resistance to virulent LM after infection with an attenuated LM strain demonstrates that vaccination-induced immunity can overcome the lack of a major cytolytic pathway and a cytokine that is critical for resistance to acute infection. These studies suggest that specific gene knockout mice can be used as models of immune deficiency to learn whether an immune response can be generated that overcomes the specific dysfunction. Such studies may impact vaccine strategies for immunocompromised individuals.

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