Viral Infection Results in Massive CD8+ T Cell Expansion and Mortality in Vaccinated Perforin-Deficient Mice

Vladimir P. Badovinac,[1] Sara E. Hamilton,[2] and John T. Harty[1,2,*]
[1]Department of Microbiology and
[2]Interdisciplinary Graduate Program in Immunology
University of Iowa
Iowa City, Iowa 52242

Summary

Perforin-mediated cytotoxicity is essential for clearance of primary LCMV infection. BALB/c-perforin-deficient (PKO) mice survived LCMV infection by deleting NP$_{118}$-specific CD8+ T cells whereas vaccination of PKO mice with *Listeria* expressing NP$_{118}$ generated a stable memory CD8+ T cell population. However, >85% of vaccinated BALB/c-PKO mice died after LCMV infection. Mortality was associated with enormous expansion of NP$_{118}$-specific CD8+ T cells in both lymphoid and nonlymphoid tissues and aberrant CD8+ T cell cytokine production. Depletion of CD8+ T cells or treatment with anti-IFNγ antibody rescued vaccinated mice from mortality. Thus, perforin was essential for resistance to secondary LCMV infection, and, in the absence of perforin, vaccination resulted in lethal disease mediated by dysregulated CD8+ T cell expansion and cytokine production.

Introduction

Early events after primary infection or vaccination program antigen (Ag)-specific CD8+ T cells to expand and differentiate into effector cells (Badovinac et al., 2002; Bevan and Fink, 2001; Kaech and Ahmed, 2001; Mercado et al., 2000; van Stipdonk et al., 2001) that are capable of cytolysis and production of cytokines such as IFNγ and TNF (Harty et al., 2000). Recently, we showed that CD8+ T cell contraction to memory levels is also programmed and independent of the duration of infection or Ag display (Badovinac et al., 2002). As a consequence of their increased number and perhaps different threshold(s) of activation, memory CD8+ T cells are poised for rapid responses and early control of pathogens after reinfection. It is apparent that contraction of the secondary response is also programmed (Badovinac et al., 2002); however, contraction of the secondary CD8+ T cell response occurs over several weeks rather than several days as observed in contraction of the primary response to infection (Badovinac et al., 2002; Grayson et al., 2002).

In general, cytolysis and cytokine production by effector and memory CD8+ T cells are tightly regulated by the presence of Ag, likely as a mechanism to minimize immunopathology (Slifka and Whitton, 2000b). However, clear evidence from experimental models shows that the CD8+ T cell response to infection can be associated with lethal immunopathology. A classic example is intra-

cranial (IC) infection of adult mice with lymphocytic choriomeningitis virus (LCMV), which results in CD8+ T cell-mediated immunopathology in the brain and eventual death (Kagi et al., 1994; Walsh et al., 1994). Lethal immunopathology in this model depends on perforin-mediated CD8+ T cell cytolysis (Kagi et al., 1994; Walsh et al., 1994) and is also affected by the virulence and dosage of the LCMV strain (Oehen et al., 1992; Smelt et al., 2001).

Effective vaccination strategies generally evoke adaptive immune responses similar to those found after infection. However, vaccination with recombinant vaccinia virus expressing LCMV proteins resulted in CD8+ T cell-mediated fatal meningitis after IC infection with strains or doses of LCMV that did not evoke lethal immunopathology in naive hosts (Oehen et al., 1992). Thus, while vaccination generally enhances antimicrobial immunity, it has the potential to be harmful under certain circumstances. Evidence to support this notion is not limited to experimental models, as vaccination against respiratory syncytial virus and measles virus has been associated with exacerbation of disease in humans (Fulginiti et al., 1967; Kim et al., 1969).

Intraperitoneal infection of wild-type (WT) mice with LCMV strain Armstrong (Arm) results in vigorous CD8+ T cell expansion (Butz and Bevan, 1998; Homann et al., 2001; Murali-Krishna et al., 1998) and perforin-dependent clearance of the virus without overt clinical symptoms. Perforin-deficient mice fail to clear LCMV Arm and develop chronic infections with occasional mortality (Matloubian et al., 1999). In contrast, infection of WT mice with LCMV clone 13 results in exhaustion of LCMV-specific CD8+ T cells and chronic infection usually without mortality. Interestingly, perforin-deficient mice exhibit reduced CD8+ T cell exhaustion and increased mortality after infection with LCMV clone 13 and DOCILE (Gallimore et al., 1998; Matloubian et al., 1999), suggesting a role for perforin in the exhaustion process. Additionally, we found increased CD8+ T cell expansion in perforin-deficient compared to WT mice after infection with attenuated *Listeria monocytogenes* (LM) (Badovinac et al., 2000b). Together, the results suggest that, in addition to a major role in cytolysis of infected cells, perforin may also regulate important aspects of the CD8+ T cell response (Harty and Badovinac, 2002). Resolution of this issue has direct relevance for understanding the pathogenesis of familial hemophagocytic lymphohistiocytosis syndromes—uniformly fatal diseases associated with recurrent viral infections, as at least some afflicted individuals are deficient in perforin (de Saint Basile and Fischer, 2001; Stepp et al., 1999; Trapani and Smyth, 2002).

The requirement for perforin-mediated cytolysis in resistance to primary infection with LCMV is well documented (Kagi et al., 1996). Perforin-deficient mice also are impaired in clearance of primary infection with virulent LM (Jensen et al., 1998; Kagi et al., 1994). However, vaccination of perforin-deficient mice with attenuated LM generates CD8+ T cells that provide enhanced resistance to infection (White et al., 1999). In addition, adop-

*Correspondence: john-harty@uiowa.edu

tive transfer studies with perforin-deficient CD8$^+$ T cell lines provide clear evidence of perforin-independent pathways of protection against LM infection (White and Harty, 1998). Similarly, IFNγ- and TNF-deficient mice are extremely sensitive to virulent LM but can be vaccinated with attenuated LM to generate high level CD8$^+$ T cell-mediated resistance against secondary infection (Harty and Bevan, 1995; White et al., 2000). Together, these results demonstrate that vaccination can overcome the absence of effector molecules that are critical for resistance to primary infection, at least in the LM model. However, the generality of this observation for different pathogens, particularly viruses, is unknown. Since clearance of primary LCMV infection is absolutely dependent on perforin, it is the most stringent model to determine whether vaccination generates memory CD8$^+$ T cells that can overcome perforin deficiency and control viral infection. In addition, the impact of perforin deficiency on regulation of the secondary CD8$^+$ T cell response to infection remains to be determined. In this study, we analyzed the outcome of LCMV infection and the nature of the secondary CD8$^+$ T cell response in vaccinated perforin-deficient mice.

Results

NP$_{118}$-Specific CD8$^+$ T Cell Homeostasis after Viral and Bacterial Infections in the Absence of Perforin

Vaccination of BALB/c (H-2^d) mice with recombinant (r)LM-expressing the LCMV NP$_{118}$ epitope generated memory CD8$^+$ T cells that protected against LCMV challenge (Shen et al., 1998). We wished to use the same system to determine whether vaccination could protect against LCMV infection in the absence of perforin. However, all published studies on LCMV infection in the absence of perforin used B6 (H-2^b) perforin-deficient (PKO) mice (Gallimore et al., 1998; Kagi et al., 1994, 1999; Matloubian et al., 1999; Walsh et al., 1994). Thus, we first needed to determine the course of infection and CD8$^+$ T cell response in BALB/c-PKO mice after primary LCMV infection. We infected BALB/c and BALB/c-PKO mice with LCMV-Arm and measured the CD8$^+$ T cell response to the NP$_{118}$ epitope using tetrameric MHC class I-peptide complexes (L^d [NP$_{118}$]) (Altman et al., 1996) or intracellular cytokine staining (ICS) for IFNγ (Badovinac and Harty, 2000) (Figure 1A). As we recently reported, the expansion and early contraction of NP$_{118}$-specific CD8$^+$ T cells was similar in BALB/c and BALB/c-PKO mice, despite high virus titers in the spleen of PKO mice (>10^6 pfu/g at day 12 postinfection [Badovinac et al., 2002]). However, we detected no NP$_{118}$-specific CD8$^+$ T cells in BALB/c-PKO mice at 85 days postinfection, with either tetramer staining or ICS, whereas a large population of NP$_{118}$-specific memory CD8$^+$ T cells were detected at the same time in BALB/c mice (Figure 1A and Murali-Krishna et al., 1998). Furthermore, the significant mortality reported after LCMV infection of H-2^b PKO mice (Matloubian et al., 1999) was not observed after LCMV infection of BALB/c-PKO mice as 100% (40/40 from multiple experiments) of these mice survived >100 days p.i. Finally, BALB/c-PKO mice still harbored virus at day 100+ p.i. (~1 × 10^5 pfu/g spleen).
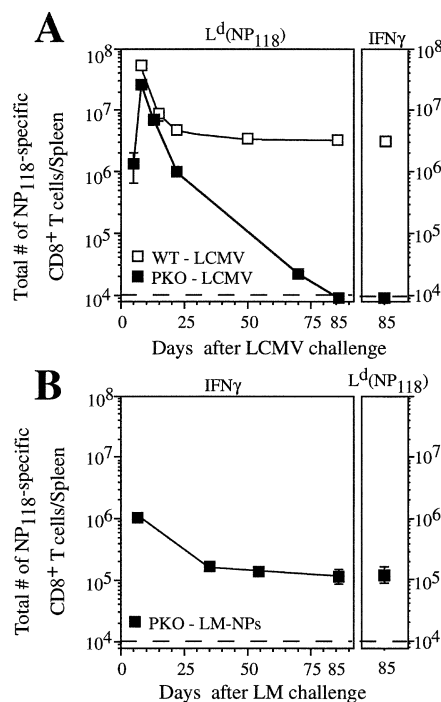

Figure 1. Disappearance of NP$_{118}$-Specific CD8$^+$ T Cells after LCMV Infection in Perforin-Deficient Mice

BALB/c (WT, H-2^d) and perforin-deficient mice (BALB/c-PKO, H-2^d) were infected (A) i.p. with 2 × 10^5 pfu LCMV-Arm or (B) ~1 × 10^6 cfu of Att LM-NPs, and NP$_{118}$-specific CD8$^+$ T cells were measured by L^d(NP$_{118}$) tetramers and/or intracellular IFNγ staining at the indicated days after challenge. Dashed line represents the limit of detection. Survival was 100% in all groups of mice (two to four mice per time point).

Thus, perforin was essential for clearance of LCMV in mice expressing either H-2^b or H-2^d MHC molecules. However, perforin was not essential for exhaustion of NP$_{118}$-specific CD8$^+$ T cells in BALB/c-PKO mice. The reason(s) for the strain differences are unknown but may relate to differences in the complexity of the CD8$^+$ T cell response (four strong LCMV epitopes in B6 mice compared to one very dominant epitope in BALB/c) or to the influence of background genes. While resolution of this issue will be of interest, the major point from this experiment was that BALB/c-PKO failed to clear primary LCMV infection but exhibited 100% survival, which correlated with exhaustion of NP$_{118}$-specific CD8$^+$ T cells.

In contrast to LCMV infection, perforin was not required for resistance to primary infection with *actA*-deficient LM, which were cleared from BALB/c and BALB/c-PKO mice with similar kinetics (Badovinac et al., 2000b; Harty et al., 2000; White et al., 1999). Immunization of BALB/c-PKO mice with an a*ctA*-deficient strain of LM that expressed the LCMV-derived NP$_{118}$ epitope (Att LM-NPs) (Shen et al., 1998; Tvinnereim et al., 2002) generated vigorous expansion of NP$_{118}$-specific CD8$^+$ T cells, which exhibited normal contraction to a substantial number (>10^5/spleen) of functional (IFNγ-producing) NP$_{118}$-specific memory CD8$^+$ T cells 85 days after challenge (Figure 1B). We have previously reported similar

kinetics for CD8$^+$ T cell responses to endogenous LM epitopes (Badovinac et al., 2000b). The numbers of NP$_{118}$-specific CD8$^+$ T cells in BALB/c-PKO mice were virtually identical from day 35 to day 85 postinfection, demonstrating normal maintenance of Ag-specific memory CD8$^+$ T cells in BALB/c-PKO mice after Att LM-NPs infection (Figure 1B).

These data demonstrate that exhaustion of NP$_{118}$-specific CD8$^+$ T cells in BALB/c-PKO mice was a function of LCMV infection and not the specific epitope. In addition, the results showed that functional NP$_{118}$-specific memory CD8$^+$ T cells can be generated in BALB/c-PKO mice by immunization with Att LM-NPs.

CD8$^+$T Cell Vaccination of Perforin-Deficient Mice

We next asked whether vaccination altered the outcome of LCMV infection in the absence of perforin. BALB/c-PKO mice were immunized with either Att LM-NPs or a control strain Att LM, which did not express the NP$_{118}$ epitope (Figure 2A). The total numbers of Ag-specific CD8$^+$ T cells in the spleens were determined 37 days later, using ICS for IFNγ (Figures 2B and 2C). CD8$^+$ T cells specific for the LM-derived H-2K^d restricted LLO$_{91-99}$ epitope (Pamer et al., 1991) were detected after Att LM and Att LM-NPs immunization (Figures 2B and 2C). Att LM-NPs infection also evoked a substantial level of NP$_{118}$-specific memory CD8$^+$ T cells (2 × 10^5 ± 0.4 × 10^5/spleen), which was absent in Att LM immunized mice (Figures 2B and 2C).

Immunized BALB/c-PKO mice were challenged with LCMV-Arm at day 37 postbacterial infection. All Att LM immunized BALB/c-PKO mice survived infection and exhibited no overt signs of illness. In striking contrast, all Att LM-NPs vaccinated BALB/c-PKO mice died by day 8 post-LCMV challenge (Figure 2D). In other experiments, transfer of BALB/c-derived NP$_{118}$-specific CD8$^+$ T cells into BALB/c-PKO hosts resulted in survival after LCMV infection and generation of stable NP$_{118}$-specific memory (data not shown). Thus, perforin-deficient NP$_{118}$-specific memory CD8$^+$ T cells failed to protect BALB/c-PKO mice from LCMV, and their presence correlated with mortality rather than survival after infection.

Enormous Expansion of Ag-Specific CD8$^+$ T Cells after LCMV Challenge in Vaccinated BALB/c-PKO Mice

To address the mechanism(s) responsible for LCMV-induced mortality in Att LM-NPs vaccinated mice, we analyzed Ag-specific CD8$^+$ T cell expansion after LCMV challenge. Naive and Att LM immune BALB/c-PKO mice had substantial numbers (several million) of NP$_{118}$-specific CD8$^+$ T cells at day 5 post-LCMV infection as detected by tetrameric MHC class I-peptide complexes (L^d [NP$_{118}$]) (Figure 3). In sharp contrast, Att LM-NPs vaccinated BALB/c-PKO mice had an enormous number of NP$_{118}$-specific CD8$^+$ T cells (~10^8/spleen, Figure 3B) at day 5 after LCMV challenge. Almost 50% of all spleen cells in these mice were CD8$^+$- and NP$_{118}$-specific (Figure 3A). Strikingly, 85%–90% of all CD8$^+$ T cells in the spleen were NP$_{118}$-specific (Figure 3A). To the best of our knowledge this is the highest in vivo frequency and total number of single epitope-specific CD8$^+$ T cells detected in a nontransgenic system. The number of
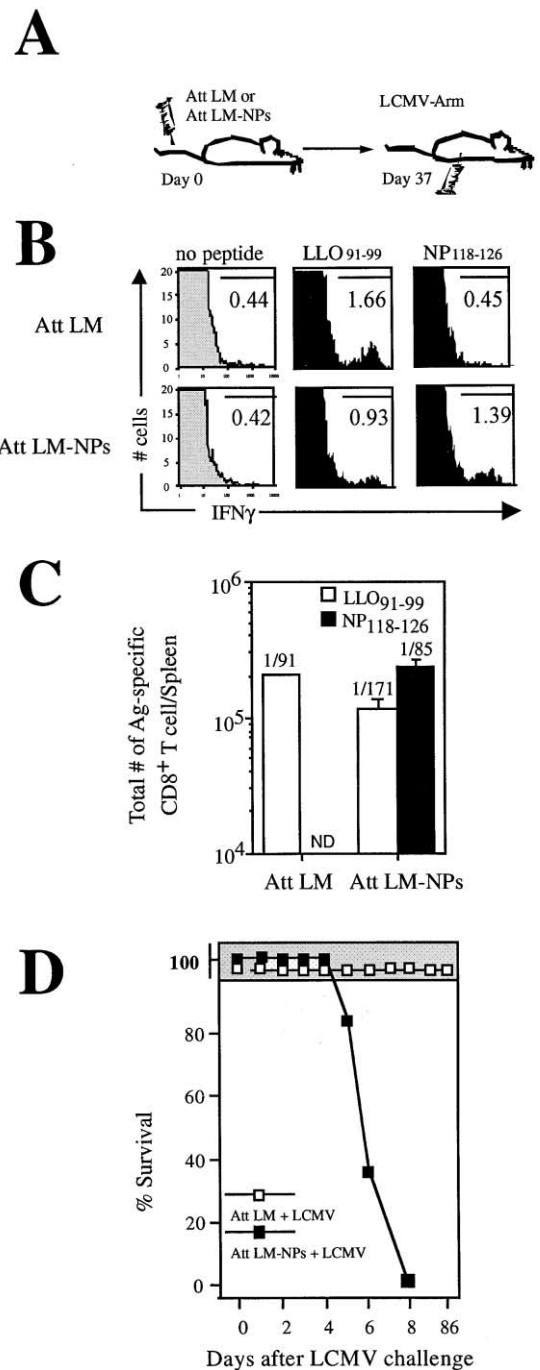

Figure 2. Vaccinated Perforin-Deficient Mice Succumb to LCMV Challenge

(A) Experimental design. BALB/c-PKO mice were infected i.v. with ~1 × 10^6 cfu of Att LM or Att LM-NPs. At day 37 postinfection mice were challenged i.p. with 2 × 10^5 pfu LCMV-Arm.

(B) Frequency of LLO$_{91-99}$ and NP$_{118}$-specific CD8$^+$ T cells from representative mice at day 37 post-LM challenge as determined by ICS. Numbers represent the percent of Ag-specific CD8$^+$ T cells that produce IFNγ in the absence (no peptide) or in the presence of the indicated peptides.

(C) Total number (mean ± SD) and frequencies of Ag-specific CD8$^+$ T cells per spleen from three mice. Total number of NP$_{118}$-specific CD8$^+$ T cells obtained after Att LM-NP infection was 2.3 ± 0.4 × 10^5 cells/spleen. ND, not detectable.

(D) Survival of BALB/c-PKO mice after LM and LCMV infections.

A

B

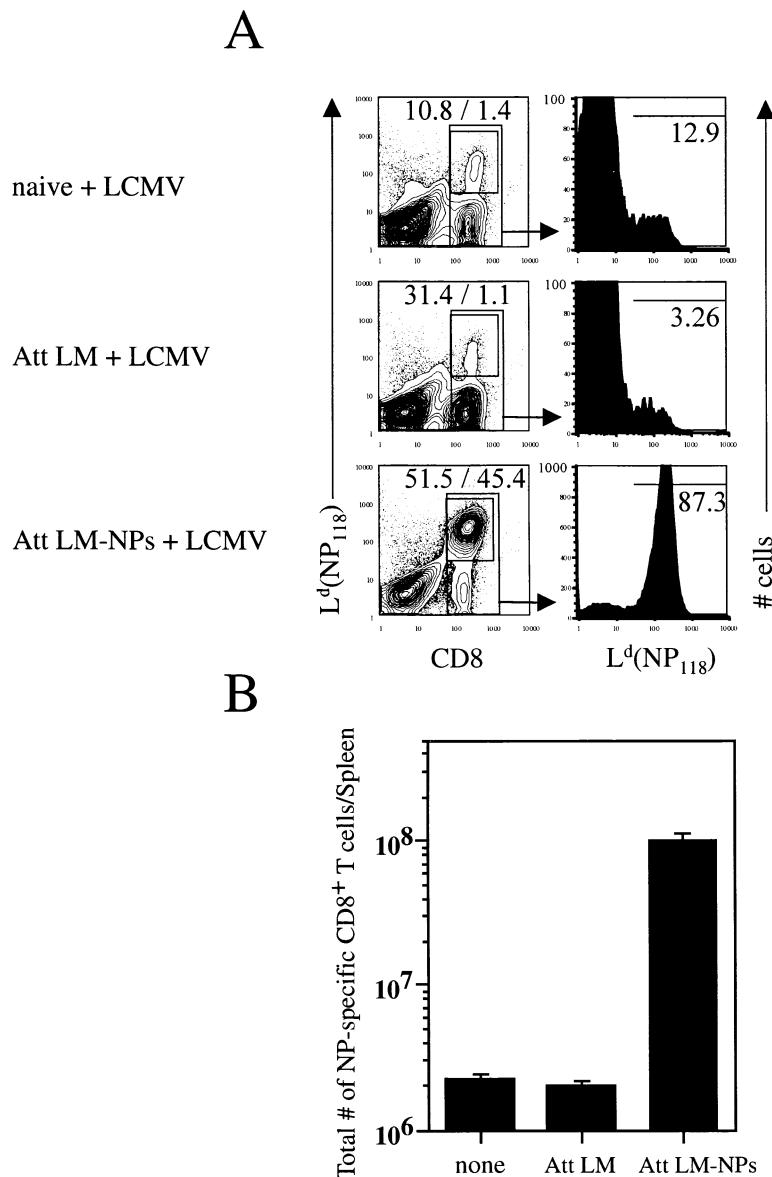
NP_{118}-specific $CD8^+$ T cells in LM-NPs vaccinated BALB/c-PKO mice far exceeded that reported for secondary responses to LCMV in immune BALB/c mice (Grayson et al., 2002). Thus, the absence of perforin resulted in an enormous secondary response of NP_{118}-specific $CD8^+$ T cells in the spleen after LCMV infection. Importantly, this was associated with mortality.

After infection, Ag-specific $CD8^+$ T cells are present in nonlymphoid as well as in lymphoid tissues (Marshall et al., 2001; Masopust et al., 2001). One possible explanation for the enormous NP_{118}-specific $CD8^+$ T cell numbers in the spleen was a failure of these cells to migrate into tissues. To address this issue, we isolated lymphocytes and measured NP_{118}-specific $CD8^+$ T cells in various tissues of Att LM-NPs vaccinated BALB/c and BALB/c-PKO mice after LCMV-Arm challenge (Figure 4). Robust NP_{118}-specific $CD8^+$ T cell responses, ranging from 1 out of 300 lymphocyte gated cells (bone marrow)

to 1 out of 3 lymphocyte gated cells (liver), were observed in tissues from vaccinated BALB/c mice at day 6 after LCMV-Arm infection (Figure 4). The frequency of NP_{118}-specific cells out of all $CD8^+$ T cells in various tissues of BALB/c mice ranged from 33%–80%. In contrast, ~90% of all $CD8^+$ T cells detected in various organs of vaccinated BALB/c-PKO mice were specific for the NP_{118} epitope at day 6 after LCMV infection (Figure 4B), resulting in 1.5 to 30 times more NP_{118}-specific $CD8^+$ T cells, depending on the tissue, than vaccinated BALB/c mice (Figure 4C). This estimation was based on frequency of NP_{118}-specific $CD8^+$ T cells rather than total numbers due to variability in recovery of lymphocytes from organs in different mice. However, in no case were fewer lymphocytes recovered from vaccinated BALB/c-PKO organs; thus, these animals contained substantially more nonlymphoid NP_{118}-specific $CD8^+$ T cells than vaccinated BALB/c mice. From these data we conclude
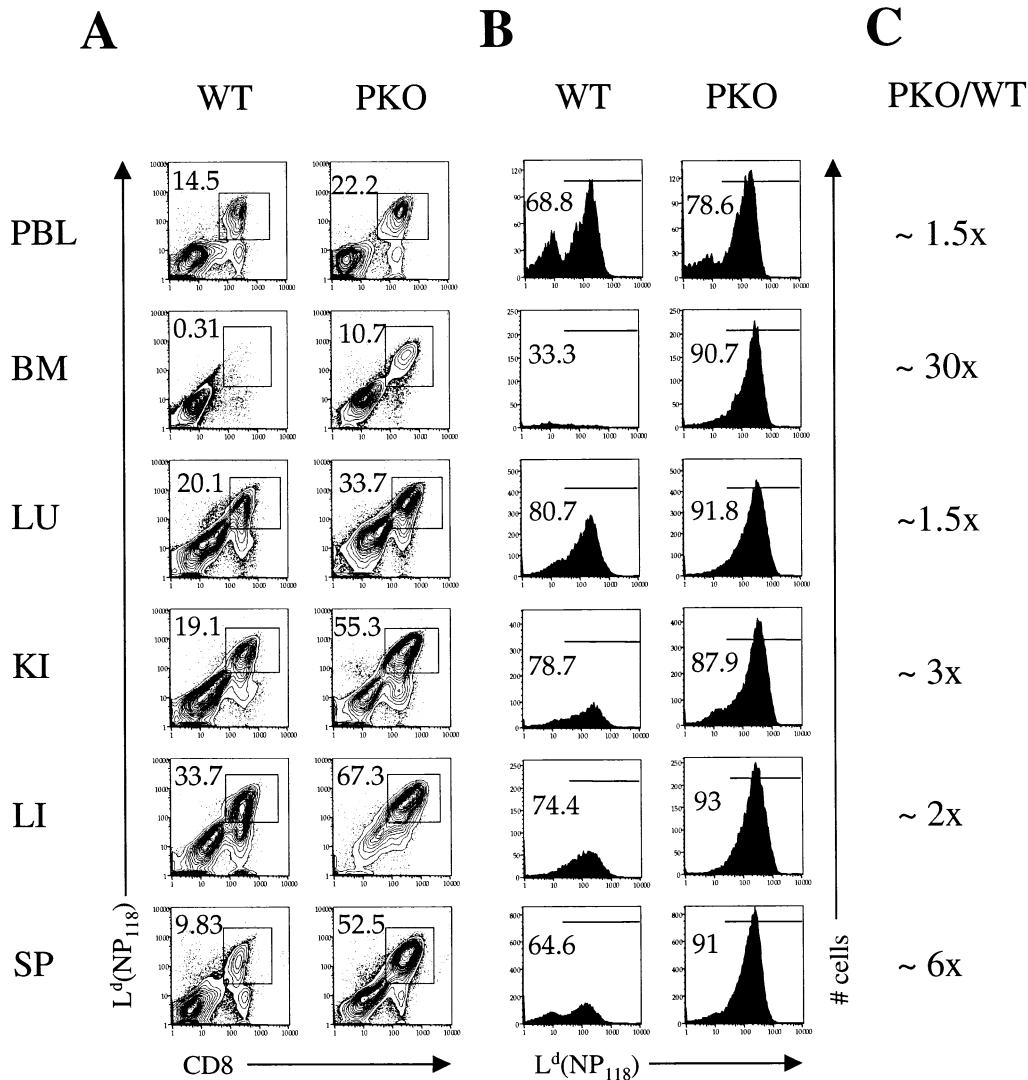
Figure 4. Expansion of Wild-Type and Perforin-Deficient NP_{118}-Specific $CD8^+$ T Cells in the Lymphoid and Nonlymphoid Tissues after LCMV Challenge

WT and BALB/c-PKO mice were infected with Att LM-NPs and at day 58 p.i. mice were challenged with LCMV-Arm. At day 6 post-LCMV infection lymphocytes from the indicated tissues were subjected to MHC class I tetramer ($L^d[NP_{118}]$) staining.

(A) The numbers inside the $L^d(NP_{118})$ versus CD8 contour plots represent the percentage of NP_{118}-specific $CD8^+$ T cells among all lymphocytes.

(B) The numbers inside the number of cells versus $L^d(NP_{118})$ histograms represent the percentage of $CD8^+$ T cells that stained with $L^d(NP_{118})$ tetramers. Data in (A) and (B) are representative of two mice in the experiment.

(C) Approximate fold increase in frequencies of $L^d(NP_{118})$-positive cells in BALB/c-PKO mice compared to WT mice. PBL, peripheral blood leukocytes; BM, bone marrow; LU, lung; KI, kidney; LI, liver; SP, spleen.

that the enormous expansion of NP_{118}-specific $CD8^+$ T cells in vaccinated BALB/c-PKO spleens was not a consequence of failure by these cells to migrate to tissues. In addition, a substantial number of NP_{118}-specific $CD8^+$ T cells were located in nonlymphoid tissues, and these cells may have contributed to mortality in vaccinated BALB/c-PKO mice.

**Phenotypic and Functional Comparison
of Ag-Specific $CD8^+$ T Cells after LCMV
Infection in BALB/c and BALB/c-PKO Mice**
The frequencies of $CD8^+$ T cells responding to NP_{118} were determined by phenotypic assessment using MHC

class I-peptide tetramers (Figures 3 and 4). To determine whether cells detected by tetramer staining were functional, splenocytes from vaccinated BALB/c and BALB/c-PKO mice were analyzed for the ability to produce IFNγ after 6 hr incubation in brefeldin A (BfA) in the presence or absence of NP_{118} peptide (Figure 5).

Similar frequencies of Ag-specific $CD8^+$ T cells were detected by tetramer and ICS for IFNγ at day 5 after LCMV infection of vaccinated BALB/c mice (Figures 5A and 5B). Importantly, NP_{118}-specific $CD8^+$ T cells from BALB/c mice did not produce IFNγ in the absence of peptide stimulation. Thus, regulation of direct ex vivo IFNγ production by NP_{118}-specific $CD8^+$ T cells in
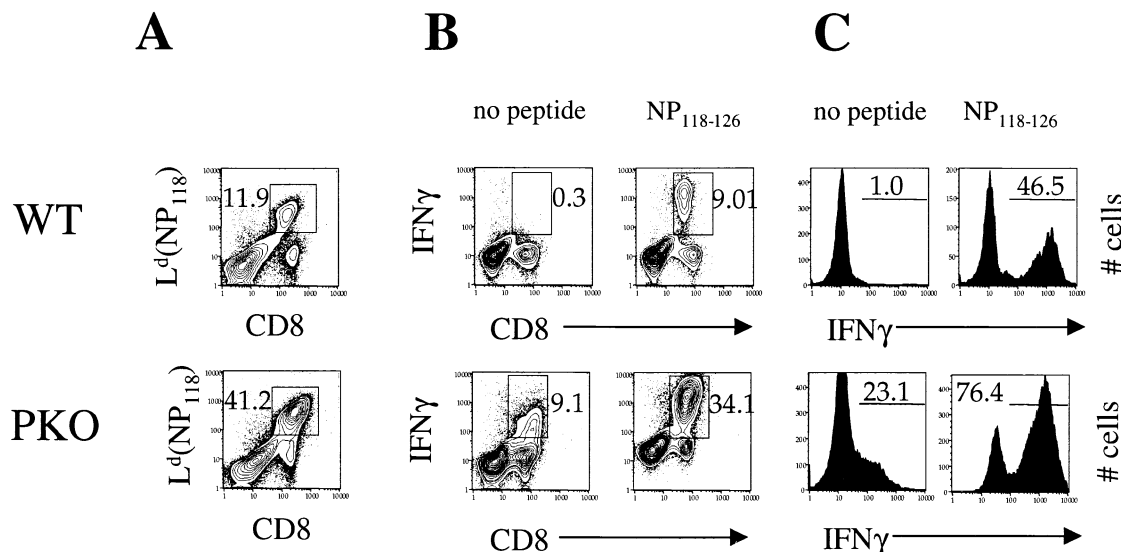
A   **B**   **C**

Figure 5. IFNγ Production by Wild-Type and Perforin-Deficient NP$_{118}$-Specific CD8$^+$ T Cells after LCMV Infection

WT and BALB/c-PKO mice were infected with Att LM-NPs and at day 58 p.i. mice were challenged with LCMV-Arm.

(A) At day 5 post-LCMV challenge, the frequencies of NP$_{118}$-specific CD8$^+$ T cells in the spleen were determined by MHC class I tetramer (L^d[NP$_{118}$]) staining. The numbers inside the L^d(NP$_{118}$) versus CD8 dot plots represent the percentage of NP$_{118}$-specific CD8$^+$ T cells among all splenocytes.

(B) The same mice as in (A) were analyzed for Ag-specific IFNγ production in the absence (no peptide) or in the presence of NP$_{118}$ peptide after 6 hr incubation in the presence of BfA. The numbers inside the IFNγ versus CD8 contour plots represent the percentage of NP$_{118}$-specific CD8$^+$ T cells among all splenocytes.

(C) The numbers inside the number of cells versus L^d(NP$_{118}$) histograms represent the percentage of CD8$^+$ T cells that stained with IFNγ in the presence or absence of NP$_{118}$ peptide stimulation.

BALB/c mice was Ag dependent (Badovinac et al., 2000a; Slifka et al., 1999). In contrast, cytokine production by perforin-deficient NP$_{118}$-specific CD8$^+$ T cells was substantially different. In the absence of peptide stimulation one out of three antigen-specific CD8$^+$ T cells (23% compared to 76% in peptide-stimulated cells, Figure 5C) produced high levels of IFNγ after 6 hr of in vitro incubation. These data demonstrate aberrant regulation of direct ex vivo cytokine production by NP$_{118}$-specific CD8$^+$ T cells from LCMV-infected BALB/c-PKO mice.

Kinetics of Ag-Specific CD8$^+$ T Cell Expansion in Vaccinated Perforin-Deficient Mice after LCMV Infection

We next addressed the kinetics of the CD8$^+$ T cell response after LCMV infection of vaccinated BALB/c-PKO mice. More than 10 weeks after vaccination with Att LM-NPs, BALB/c-PKO mice contained $1 \times 10^5 \pm 0.4 \times 10^5$ NP$_{118}$-specific CD8$^+$ T cells in the spleens. Two days after LCMV infection, we were unable to find NP$_{118}$-specific CD8$^+$ T cells (detection limit $\sim 10^4$ cells/spleen), either by tetramer staining or functional assays for cytokine production (Figures 6A–6C). The reason for this decline is not known; however, similar results were reported after high-dose secondary challenge with LM (Busch et al., 2000). At day 4 after LCMV infection 20% of splenic CD8$^+$ T cells stained with L^d(NP$_{118}$) tetramers. One day later, more than 80% of CD8$^+$ T cells were specific for NP$_{118}$ (Figure 4A), representing $\sim 10^8$ cells/ spleen (Figure 6B). The elevated frequency of the NP$_{118}$-

specific CD8$^+$ T cell response was maintained at later time points and all of the mice died by day 8 post-LCMV challenge (Figure 6B). If we assume $\sim 10^4$ NP$_{118}$-specific CD8$^+$ T cells at day 2 postinfection (an upper limit, Figure 6B) and that none of the NP$_{118}$-specific CD8$^+$ T cells would die or leave the spleen, then these cells divided 13 times over the ensuing 72 hr to reach 10^8 NP$_{118}$-specific CD8$^+$ T cells/spleen at day 5 post-LCMV infection. This represents a doubling rate of ~ 5.5 hr (death and emigration of Ag-specific CD8$^+$ T cells from the spleen would decrease the doubling time), a rate that is even faster than that suggested for the expansion of NP$_{118}$-specific CD8$^+$ T cells after primary infection of BALB/c mice (Goldrath and Bevan, 1999; Murali-Krishna et al., 1998). These data revealed dramatic expansion NP$_{118}$-specific CD8$^+$ T cells over the short period of time immediately preceding the onset of fatal disease.

Cytokine Production by Perforin-Deficient NP$_{118}$-Specific CD8$^+$ T Cells

After direct ex vivo peptide stimulation most WT Ag-specific CD8$^+$ T cells that made IFNγ also make TNF (Badovinac and Harty, 2000; Slifka and Whitton, 2000a). Direct ex vivo IFNγ production continued while Ag was present but rapidly ceased upon Ag removal. In contrast, TNF production by the same Ag-specific cells ceased after 3–4 hr of stimulation, whether or not the Ag was removed (Badovinac et al., 2000a). Consistent with the results in Figure 5, a substantial fraction of NP$_{118}$-specific CD8$^+$ T cells ($\sim 50\%$ of all CD8$^+$ T cells in these animals) produced IFNγ even without in vitro peptide stimulation,
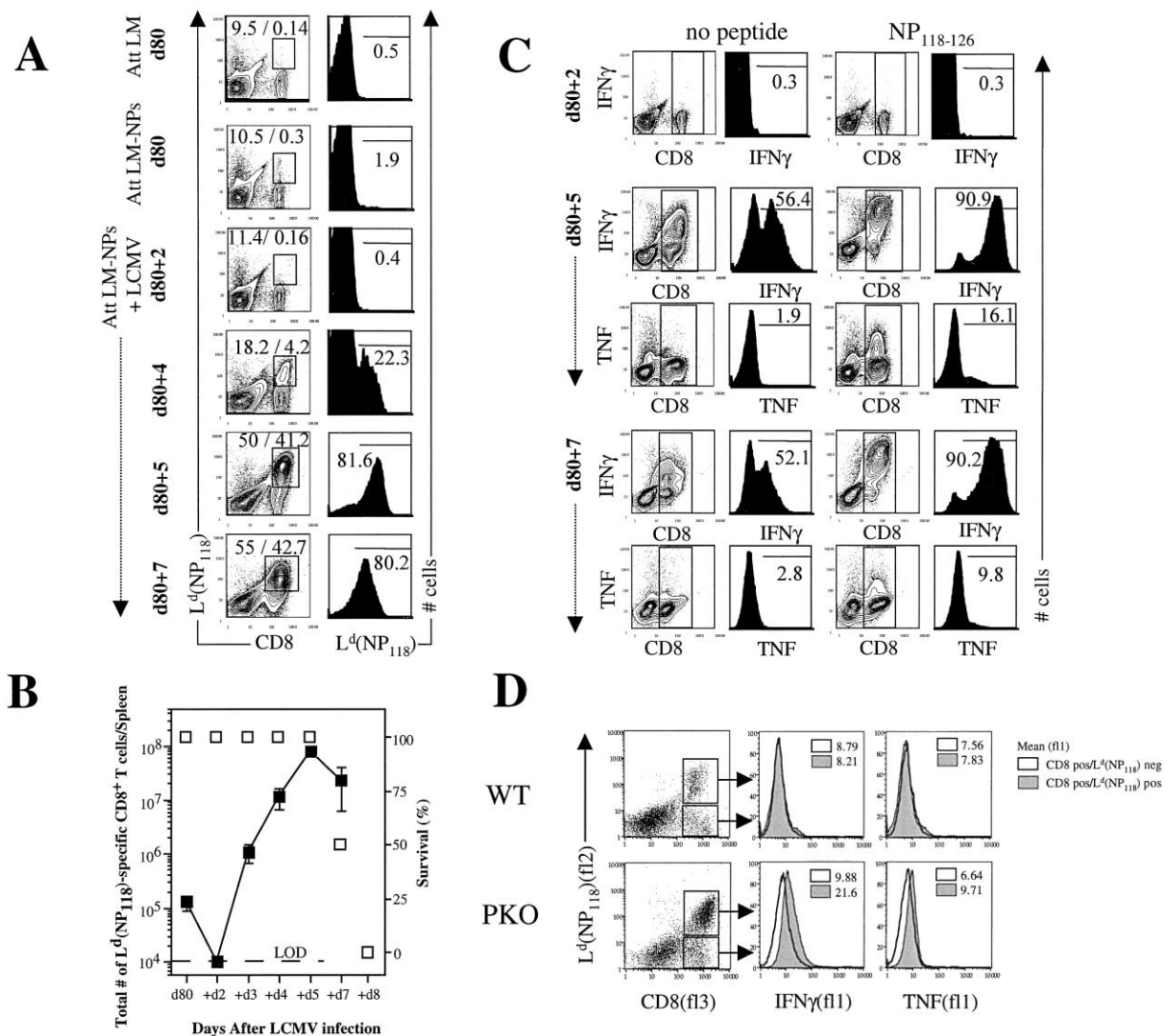
Figure 6. Kinetic Analysis of Expansion and Cytokine Regulation of Perforin-Deficient NP$_{118}$-Specific CD8$^+$ T Cells after LCMV Infection

BALB/c-PKO mice were infected with Att LM-NPs, and at day 80 p.i. mice were challenged with LCMV-Arm. The kinetics of NP$_{118}$-specific CD8$^+$ T cell response was determined by phenotypic and functional assays.

(A) At the indicated days prior and post-LCMV challenge, the frequencies of NP$_{118}$-specific CD8$^+$ T cells in the spleen were determined by MHC class I tetramer (L^d[NP$_{118}$]) staining. The numbers inside the L^d(NP$_{118}$) versus CD8 dot plots represent the percentage of all CD8$^+$ T cells/ percentage of NP$_{118}$-specific CD8$^+$ T cells among all splenocytes. The numbers inside the numbers of cells versus L^d(NP$_{118}$) histograms represent the percentage of CD8$^+$ T cells that stained with L^d(NP$_{118}$) tetramers.

(B) The total number of NP$_{118}$-specific CD8$^+$ T cells per spleen determined by tetramer staining (filled squares). Data are presented as mean ± SD for two to three mice per time point. LOD represents the limit of detection. Survival of vaccinated BALB/c-PKO mice after LCMV infection (open squares).

(C) At the indicated days post-LCMV challenge, the same mice as in (A) were analyzed for Ag-specific IFNγ or TNF production in the absence (no peptide) or in the presence of NP$_{118}$ peptide.

(D) Direct ex vivo cytokine production by wild-type (WT) and perforin-deficient NP$_{118}$-specific CD8$^+$ T cells after LCMV infection. WT and BALB/ c-PKO mice were infected with Att LM-NPs and at day 58 p.i. mice were challenged with LCMV-Arm. At day 5 post-LCMV infection, splenocytes from WT and BALB/c-PKO mice were stained with αCD8 and tetramers (L^d[NP$_{118}$]), fixed, permeabilized, and immediately stained for intracellular IFNγ or TNF in the absence of peptide stimulation and BfA. Mean fluorescence indexes of CD8pos/L^d(NP$_{118}$)neg and CD8pos/L^d(NP$_{118}$)pos populations are presented for both cytokines. Representative data from one mouse of two in one of three similar experiments are shown.

with an increase to ∼90% upon addition of the NP$_{118}$ peptide (Figure 6C). In sharp contrast, TNF production by the same IFNγ-producing cells was not detected without peptide stimulation and was minimal after addition of peptide. These data were consistent with the notion that NP$_{118}$-specific CD8$^+$ T cells in BALB/c-PKO mice were chronically stimulated to produce IFNγ but

extinguished TNF due to continued Ag presentation. However, given the relatively long incubation in vitro, we cannot rule out the possibility that generation of a single-cell suspension alters access of CD8$^+$ T cells to LCMV antigens or permits Ag display in vitro that was absent in vivo.

In order to determine whether cytokine dysregulation

Table 1. Depletion of CD8$^+$ Cells and Treatment with Anti-IFNγ Neutralizing Antibodies Prevent Mortality of Vaccinated Perforin-Deficient Mice after LCMV Challenge

Infection(s) Prior to LCMV Challenge	Treatment	Mortality
None	None	0% (0/40)
Att LM	None	0% (0/7)
Att LM-NPs	+/− Rat IgG	88% (21/24)
Att LM-NPs	αCD8	0% (0/5)
Att LM-NPs	αIFNγ	0% (0/7)

BALB/c-PKO mice were infected with Att LM-NPs or Att LM strains of LM and challenged with LCMV-Arm at least 50 days after initial LM challenge. All antibodies were i.p. administered at 1 mg/mouse on the day before LCMV challenge (αCD8) or at day 5 post-LCMV challenge (αIFNγ). Survival was followed at least 30 days after LCMV challenge. Data from multiple experiments are shown.

could be observed without extensive incubation in brefeldin A, splenocytes from vaccinated BALB/c and BALB/c-PKO mice were obtained at 5 days post-LCMV-Arm infection and were immediately stained with L^d(NP$_{118}$) (tetramers) and then fixed and permeabilized before intracellular staining for IFNγ or TNF. Cytokine expression was assessed by comparing the mean fluorescence between tetramer-positive and -negative CD8$^+$ T cells. As predicted, no increase in cytokine levels was detected in BALB/c-derived NP$_{118}$-specific CD8$^+$ T cells analyzed directly ex vivo (Figure 6D). In contrast, direct ex vivo IFNγ expression was observed in BALB/c-PKO-derived NP$_{118}$-specific CD8$^+$ T cells (tetramer-positive) when compared to the tetramer-negative CD8$^+$ T cells from the same mice (Figure 6D). Interestingly, TNF was also detected in BALB/c-PKO-, but not BALB/c-derived, NP$_{118}$-specific CD8$^+$ T cells (Figure 6D). The reason(s) why TNF production was detected by direct ex vivo analysis and not after in vitro incubation remains to be determined; however, these data suggested that chronic cytokine production by NP$_{118}$-specific CD8$^+$ T cells occurred in vivo and contributed to mortality in vaccinated BALB/c-PKO mice after LCMV infection.

CD8$^+$ T Cells and Cytokines Contributed to Mortality in Vaccinated Perforin-Deficient Mice after LCMV-Arm Infection

In multiple experiments ~90% of Att LM-NPs vaccinated BALB/c-PKO mice died by day 9 post-LCMV infection (Table 1). The enormous expansion of NP$_{118}$-specific CD8$^+$ T cells and dysregulated cytokine production correlated with mortality, but it was formally possible that these elements of the immune system did not account for mortality. In order to show that NP$_{118}$-specific CD8$^+$ T cells were responsible for the observed mortality, vaccinated BALB/c-PKO mice were depleted of CD8$^+$ T cells by antibody treatment prior to LCMV-Arm challenge. Depletion of CD8$^+$ T cells prevented LCMV triggered mortality. Thus, CD8$^+$ T cells were responsible for vaccination-induced mortality in perforin-deficient mice (Table 1).

To address the contribution of cytokines to mortality, we administrated anti-IFNγ mAb on day 5 post-LCMV challenge of Att LM-NPs vaccinated BALB/c-PKO mice. Seven out of seven (anti-IFNγ treatment) vaccinated

BALB/c-PKO mice survived LCMV infection (Table 1). In addition, two out of three Att LM-NPs vaccinated BALB/c-PKO mice treated with anti-TNF neutralizing mAb on day 5 after LCMV infection survived (data not shown). Further experiments are needed in order to show whether and under what instances anti-TNF treatment might be beneficial to the host.

Taken together, these data suggested that Ag-specific CD8$^+$ T cells were responsible for vaccination-induced mortality in the absence of perforin and that cytokine production (IFNγ) by these cells was a major factor that contributed to mortality after LCMV infection.

Discussion

One hundred percent of naive BALB/c-PKO (H-2^d MHC) mice survived infection with LCMV-Arm. These mice exhibited a vigorous NP$_{118}$-specific CD8$^+$ T cell response, similar in magnitude to that observed in WT BALB/c, although LCMV was not cleared from BALB/c-PKO hosts. As previously reported, NP$_{118}$-specific CD8$^+$ T cells undergo programmed contraction in BALB/c-PKO hosts despite the high virus load in the spleen (Badovinac et al., 2002). However, NP$_{118}$-specific CD8$^+$ T cells were eventually deleted (exhausted) by 85 days postinfection in the BALB/c-PKO hosts. Thus, perforin was not essential for CD8$^+$ T cell exhaustion after LCMV infection.

In previous studies, we used gene knockout mice as models for vaccination of immunocompromised hosts against infection with LM. Our results clearly showed that vaccination can overcome the absence of molecules, including perforin, that were essential for resistance of the naive host to bacterial infection (Harty and Badovinac, 2002). Thus, we sought to determine whether vaccination could protect perforin-deficient hosts from persistent LCMV infection. However, rather than enhanced clearance, the majority (21/24) of vaccinated BALB/c-PKO mice succumbed to LCMV by 8–9 days postinfection. These mice, which contained ~10^5 LCMV-specific memory CD8$^+$ T cells/spleen (0.5%–1% of CD8$^+$ T cells) at the time of infection, exhibited a massive expansion of NP$_{118}$-specific cells to levels that represented 85%–90% of CD8$^+$ T cells in both lymphoid and nonlymphoid tissues by 5–6 days postinfection. By this time, ~10^8 NP$_{118}$-specific CD8$^+$ T cells could be found in the spleen. Since the spleens of these mice contained only ~2 × 10^8 total cells, the NP$_{118}$-specific CD8$^+$ T cells represented ~50% of total spleen cells at day 5 p.i. The spleen is thought to contain ~25% of the total number of CD8$^+$ T cells in a mouse (Blattman et al., 2002); thus, a conservative estimate would suggest that each BALB/c-PKO animal contained >2 × 10^8 NP$_{118}$-specific CD8$^+$ T cells. Thus, LCMV infection of vaccinated BALB/c-PKO mice essentially replaced the normal diversity of the immune system with CD8$^+$ T cells specific for a single epitope.

In contrast to data obtained here with H-2^d perforin-deficient mice, naive H-2^b perforin-deficient mice (B6-PKO) exhibited substantial, although not 100%, mortality after primary LCMV infection (Gallimore et al., 1998; Kagi et al., 1994, 1999; Matloubian et al., 1999; Walsh et al., 1994). Although the reason(s) for the strain differences is currently unknown, differences in the complex-

ity of the CD8$^+$ T cell response or the influence of background genes may account for the results. To determine whether vaccine induced mortality in the absence of perforin was generalizable to H-2^b mice, we vaccinated B6-PKO mice with recombinant Att LM that expressed the LCMV GP$_{33-41}$ (H-2D^b restricted) epitope (van der Most et al., 1996) and challenged with LCMV. All vaccinated B6-PKO rapidly succumed to LCMV infection (data not shown). In addition, vaccinated B6-PKO mice showed massive and rapid GP$_{33}$-specific CD8$^+$ T cell expansion and dysregulated cytokine production after LCMV challenge (>80% of CD8$^+$ T cells, representing 40% to 50% of all cells in the spleen, were GP$_{33}$-specific at day 6 postchallenge of vaccinated mice [data not shown]). Therefore, vaccination also resulted in massive CD8$^+$ T cell expansion and accelerated LCMV induced mortality in H-2^b perforin-deficient mice.

The NP$_{118}$-specific CD8$^+$ T cells from vaccinated BALB/c-PKO mice exhibited abnormal regulation of IFNγ and TNF in direct ex vivo assays, suggesting that persistent infection led to overproduction of CD8$^+$ T cell-derived cytokines and mortality in the absence of perforin. This notion was supported by rescue of vaccinated mice by treatment with neutralizing mAb for IFNγ. Importantly, depletion of CD8$^+$ T cells prior to LCMV infection also prevented death; thus, these cells were responsible for vaccine-induced mortality.

Naive and vaccinated BALB/c-PKO mice both fail to clear LCMV infection. Why then do vaccinated mice succumb while naive mice survive? One major difference in the two situations was the number of NP$_{118}$-specific CD8$^+$ T cells present at the time of infection. It is thought that the naive precursor pool for most CD8$^+$ T cell epitopes consists of several hundred clones in the spleen (Blattman et al., 2002). In contrast, vaccinated BALB/c-PKO mice contained ~10^5 NP$_{118}$-specific memory CD8$^+$ T cells/spleen at the time of LCMV infection. Recent studies demonstrated that the magnitude of CD8$^+$ T cell expansion after primary infection depended on the number of precursors recruited into the response (Kaech and Ahmed, 2001). Thus, the difference in available NP$_{118}$-specific precursors in naive and vaccinated perforin-deficient mice may have dictated survival or death by controlling the eventual magnitude of the CD8$^+$ T cell response after infection.

Vaccinated BALB/c-PKO mice contained high levels of memory cells, and it remains to be determined whether the number of memory cells dictates mortality versus survival after LCMV infection. In addition, while LCMV-induced mortality required vaccination with LM expressing the NP$_{118}$ epitope, it is also possible that LM infection helps to prime vaccinated mice for mortality. The latter notion predicts that generation of NP$_{118}$-specific memory CD8$^+$ T cells using other vaccination strategies would fail to sensitize BALB/c-PKO mice to LCMV-induced mortality. In direct contrast to this notion, ~30% (two out of seven) BALB/c-PKO mice that contained NP$_{118}$-specific CD8$^+$ T cells generated after vaccination with peptide-coated dendritic cells (Hamilton and Harty, 2002) succumbed to LCMV infection. These mice, which had ~1 × 10^4 NP$_{118}$ memory CD8$^+$ T cells/spleen prior to infection (~10- to 20-fold fewer than the Att LM-NPs vaccinated mice), also exhibited massive expansion of LCMV-specific CD8$^+$ T cells (approximately one out of

three cells in the spleen at day 5 postinfection; data not shown). Although additional experiments will be required to determine whether the number of memory cells determines the outcome after infection, these preliminary data suggest that delivery of vaccine antigens by LM is not required to sensitize vaccinated mice to LCMV-induced mortality.

Recent in vitro studies revealed that cytokine production by CD8$^+$ T cells was tightly regulated by the presence of Ag (IFNγ [Slifka et al., 1999; Badovinac et al., 2000a]) or programmed to cease after a burst of production even in the continued presence of Ag (TNF [Badovinac et al., 2000a]). This tight regulation was suggested to limit immunopathology and focus the CD8$^+$ T cell response on cells expressing pathogen-derived antigens (Slifka and Whitton, 2000b). Consistent with this notion, CD8$^+$ T cells undergoing secondary expansion in WT mice exhibited no direct ex vivo expression of either IFNγ or TNF but rapidly produced both upon peptide stimulation. In contrast, we showed that NP$_{118}$-specific CD8$^+$ T cells obtained from vaccinated BALB/c-PKO mice at various times after LCMV infection produced both IFNγ and TNF when analyzed directly ex vivo and exhibited dysregulated cytokine production after in vitro culture in the absence of additional peptide stimulation. Dysregulated cytokine production was also observed after DC immunization as well as in vaccinated B6-PKO mice after LCMV infection (data not shown). Since the levels of cytokine detected in the absence of stimulation, brefeldin A, and prolonged incubation were modest, we could not tell whether only a subset of BALB/c-PKO-derived NP$_{118}$-specific CD8$^+$ T cells were producing cytokines in vivo. However, given the enormous numbers of NP$_{118}$-specific CD8$^+$ T cells that respond to LCMV in vaccinated BALB/c-PKO mice, continuous cytokine production from even a small fraction of cells could result in substantial immunopathology.

In addition to the role of perforin as an effector molecule, experiments in various models such as chronic LCMV infection (Binder et al., 1998; Gallimore et al., 1998; Matloubian et al., 1999), bone marrow transplantation/GVHD (Spaner et al., 1999), and autoimmunity (Spielman et al., 1998) suggested a possible immunoregulatory role for perforin, perhaps specific for deletion of T cells in situations of chronic antigen exposure (Harty and Badovinac, 2002). Although both vaccinated WT and BALB/c-PKO mice mount a significant secondary CD8$^+$ T cell response to LCMV, the response in PKO mice is extraordinary, and only the perforin-deficient mice succumb to infection. What specific role does perforin deficiency play in this process? We speculate that the adverse outcome of LCMV infection in vaccinated PKO mice results from a combination of perforin-regulated events. First, the inability to rapidly clear LCMV by perforin-dependent cytolysis will result in a substantial antigen load in PKO mice. In turn, this will ensure that most, if not all, NP$_{118}$-specific memory CD8$^+$ T cells are recruited to undergo a programmed degree of expansion (Kaech and Ahmed, 2001), generating massive numbers of CD8$^+$ T cells. In contrast, WT memory CD8$^+$ T cells rapidly control LCMV infection, limiting the amount of antigen and likely causing only a subset of memory cells to be recruited into the secondary response. This notion is consistent with data demonstra-

ting that primary and secondary responses are of similar magnitude in WT mice after LCMV infection (Grayson et al., 2002).

In addition to the massive expansion, CD8$^+$ T cells in vaccinated PKO mice would be subjected to chronic antigen exposure since the virus was not cleared. Although our results with naive BALB/c-PKO mice showed that perforin was not essential for exhaustive deletion of NP$_{118}$-specific CD8$^+$ T cells, complete elimination of these cells required several months as opposed to the relatively short period of time required to eliminate WT Ag-specific cells after chronic LCMV infection (Zajac et al., 1998). Extended survival of perforin-deficient CD8$^+$ T cells, based on increased resistance to AICD in the face of chronic antigen exposure, could also contribute to cytokine dysregulation and mortality in vaccinated PKO mice.

Differential survival after LCMV infection of naive and vaccinated BALB/c-PKO mice could also result from unique properties of CD8$^+$ T cells undergoing primary and secondary responses to infection. It has long been known that activation of memory CD8$^+$ T cells is less dependent on costimulatory signals than activation of naive CD8$^+$ T cells. In addition, recent evidence showed that WT CD8$^+$ T cells underwent programmed contraction after primary infection that was rapid, with the majority of CD8$^+$ T cell death occurring within 3–5 days after the peak of expansion and independently of pathogen clearance or Ag display (Badovinac et al., 2002). In contrast, while the program of contraction in the secondary CD8$^+$ T cell response was also set by early events after infection, these cells exhibited a substantially delayed contraction phase, which took several weeks to reach secondary memory levels (Badovinac et al., 2002; Grayson et al., 2002). It remains to be determined whether the secondary response of perforin-deficient CD8$^+$ T cells also undergoes prolonged contraction and whether this feature of memory CD8$^+$ T cells facilitates immunopathology during chronic infection. In addition, CD8$^+$ T cell exhaustion in the LCMV model has only been shown after primary infection, using strains or doses of LCMV that cause chronic infections in naive WT mice. In contrast, memory CD8$^+$ T cells in vaccinated WT mice rapidly clear even these LCMV strains. Perhaps memory CD8$^+$ T cells were more resistant to exhaustion, and this property determined mortality in vaccinated BALB/c-PKO mice.

The concept of "vaccination for disease" was based on studies showing perforin-mediated meningitis in vaccinated but not control mice after IC infection with certain LCMV strains (Oehen et al., 1992). Our results showed that vaccination of perforin-deficient hosts with a single LCMV epitope converted a nonlethal peripheral infection into devastating disease mediated by CD8$^+$ T cell-derived cytokines. Thus, at least in some cases, vaccination of immunocompromised individuals could be counterproductive, even if these individuals tolerated the initial vaccine. Rescue of vaccinated mice from LCMV-induced mortality with neutralizing mAb specific for IFNγ not only identified an important effector molecule for mortality but also suggested that transient inhibition of cytokine bioactivity could rescue mice from cytokine-mediated immunopathology. This finding could have implications for treatment of perforin-defi-

cient individuals with familial hemophagocytic lympho-histiocytosis (de Saint Basile and Fischer, 2001; Stepp et al., 1999; Trapani and Smyth, 2002).

Experimental Procedures

Mice
BALB/c (H-2^d MHC) mice were obtained from the National Cancer Institute (Frederick, MD). BALB/c-PKO mice (H-2^d MHC) were previously described (Badovinac et al., 2000b; White et al., 1999). BALB/c-PKO 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 the experiments with LCMV and/or LM, at which point the mice were transferred to standard housing at the appropriate biosafety level. All mice were used at 8–16 weeks of age.

Bacteria, Virus, and Immunization
Virulent LM strain XFL303 (NP$_{118}$-expressing strain; Vir LM-NPs [Shen et al., 1998] and attenuated [actA-deficient] LM strains DP-L1942 [Att LM] [Brundage et al., 1993]) and XFL303actA- (Att LM-NPs) (Tvinnereim et al., 2002) are resistant to streptomycin and were used as previously described (Harty and Bevan, 1995). In brief, frozen stocks of bacteria were diluted in a tryptic soy broth and grown in a bacterial shaker at 37°C 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. The Armstrong strain of LCMV (2 × 10^5 pfu/mouse; intraperitoneally) was used as previously described (Badovinac et al., 2000b). Viral titers in homogenates of spleen and lungs were determined by plaque assay on VERO cells as described (Shen et al., 1998).

Monoclonal Antibodies and Peptides
The following mAbs were used: αIFNγ-PE or FITC (clone XMG 1.2, eBioscience, San Diego, CA), αTNF-PE or FITC (clone MP6-XT22, eBioscience), αCD8-FITC or CyChrome (clone 53-6.7, PharMingen, San Jose, CA). Synthetic peptides representing the defined LCMV (NP$_{118-126}$ H-2L^d restricted; van der Most et al., 1996) or LM (LLO$_{91-99}$ and p60$_{217-225}$ H-2K^d restricted; Harty and Pamer, 1995; Pamer et al., 1991) epitopes were synthesized at the University of Iowa Protein Structure Facility. In vivo CD8 T cell depletion and IFNγ or TNF neutralization were performed at the indicated days before or after LCMV-Arm infection by injection of anti-CD8 mAb 2.43, anti-IFNγ mAb XMG1.2 (Harty and Bevan, 1995), and anti-TNF mAb XT22 (White and Harty, 1998). All antibodies, including rat IgG (Sigma), were administred intraperitoneally at 1 mg/mouse.

Quantification of Ag-Specific CD8$^+$ T Cells in the Spleen
The magnitude of the epitope-specific CD8$^+$ T cell response was determined by intracellular cytokine staining (ICS) for TNF and/or IFNγ after 6 hr of incubation in brefeldin A (BfA; PharMingen), in the presence or absence of peptide, or by MHC class I tetramer staining as previously described (Badovinac et al., 2000a; Badovinac and Harty, 2000). For ICS, the percent of TNF$^+$ or IFNγ^+ CD8$^+$ T cells in the unstimulated sample for each mouse was subtracted from the peptide-stimulated value to determine the frequency of Ag-specific CD8$^+$ T cells. Total number of epitope-specific CD8$^+$ T cells per spleen was calculated from this frequency, the percent of CD8$^+$ T cells in each sample, and total number of cells per spleen. In experiments where ICS was performed directly ex vivo, splenocytes from LCMV-Arm-infected mice were stained immediately with MHC class I tetramers and αCD8 mAb for 1 hr on ice, fixed, and permeabilized with Cytofix/Cytoperm solution (PharMingen) before the addition of αIFNγ or αTNF mAbs.

Isolation of Lymphocytes from Tissues
Prior to tissue removal, samples of blood were obtained by retroorbital puncture. Anesthetized mice were then perfused in the left ventricle with phosphate-buffered saline containing 70 U/ml heparin (Sigma). Tissues (liver, lung, kidney) were cut into small pieces and incubated at 37°C for 90 min in collagenase (150 U/ml) (Gibco-

BRL, Rockville, MD) in RPMI with 5% fetal calf serum. The resulting mixture was made into a single-cell suspension, filtered, and washed. Liver and kidney cell suspensions were underlaid with a 35% Percoll (Sigma) solution and lung cell suspensions were underlaid with both a 40% and 75% Percoll solution. Samples were centrifuged at 2500 rpm for 20 min at room temperature. The single interface of lymphocytes from liver and kidney preparations and the lower interface of lung lymphocytes were collected and treated with Tris-ammonium chloride to remove red blood cells and then washed extensively before staining with MHC class I tetramers and αCD8 mAb. Bone marrow cells were obtained from femurs as previously described (Hamilton and Harty, 2002). Single-cell suspensions from spleen and bone marrow were treated with Tris-ammonium chloride and washed prior to tetramer staining.

References

Altman, J.D., Moss, P., Goulder, P., Barouch, D.H., McHeyzer, W.M., Bell, J.I., McMichael, A.J., and Davis, M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science *274*, 94–96.

Badovinac, V.P., and Harty, J.T. (2000). Intracellular staining for TNF and IFN-gamma detects different frequencies of antigen-specific CD8(+) T cells. J. Immunol. Methods *238*, 107–117.

Badovinac, V.P., Corbin, G.A., and Harty, J.T. (2000a). Cutting edge: OFF cycling of TNF production by antigen-specific CD8(+) T cells is antigen independent. J. Immunol. *165*, 5387–5391.

Badovinac, V.P., Tvinnereim, A.R., and Harty, J.T. (2000b). Regulation of antigen-specific CD8(+) T cell homeostasis by perforin and interferon-gamma. Science *290*, 1354–1357.

Badovinac, V.P., Porter, B.B., and Harty, J.T. (2002). Programmed contraction of CD8(+) T cells after infection. Nat. Immunol. *3*, 619–626.

Bevan, M.J., and Fink, P.J. (2001). The CD8 response on autopilot. Nat. Immunol. *2*, 381–382.

Binder, D., van den Broek, M.F., Kagi, D., Bluethmann, H., Fehr, J., Hengartner, H., and Zinkernagel, R.M. (1998). Aplastic anemia rescued by exhaustion of cytokine-secreting CD8+ T cells in persistent infection with lymphocytic choriomeningitis virus. J. Exp. Med. *187*, 1903–1920.

Blattman, J.N., Antia, R., Sourdive, D.J., Wang, X., Kaech, S.M., Murali-Krishna, K., Altman, J.D., and Ahmed, R. (2002). Estimating the precursor frequency of naive antigen-specific CD8 T cells. J. Exp. Med. *195*, 657–664.

Brundage, R.A., Smith, G.A., Camilli, A., Theriot, J.A., and Portnoy, D.A. (1993). Expression and phosphorylation of the *Listeria monocytogenes* ActA protein in mammalian cells. Proc. Natl. Acad. Sci. USA *90*, 11890–11894.

Busch, D.H., Kerksiek, K.M., and Pamer, E.G. (2000). Differing roles of inflammation and antigen in T cell proliferation and memory generation. J. Immunol. *164*, 4063–4070.

Butz, E.A., and Bevan, M.J. (1998). Massive expansion of antigen-specific CD8$^+$ T cells during an acute virus infection. Immunity *8*, 167–175.

de Saint Basile, G., and Fischer, A. (2001). The role of cytotoxicity in lymphocyte homeostasis. Curr. Opin. Immunol. *13*, 549–554.

Fulginiti, V.A., Eller, J.J., Downie, A.W., and Kempe, C.H. (1967). Altered reactivity to measles virus. Atypical measles in children previously immunized with inactivated measles virus vaccines. JAMA *202*, 1075–1080.

Gallimore, A., Glithero, A., Godkin, A., Tissot, A.C., Pluckthun, A., Elliott, T., Hengartner, H., and Zinkernagel, R. (1998). Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I peptide complexes. J. Exp. Med. *187*, 1383–1393.

Goldrath, A.W., and Bevan, M.J. (1999). Selecting and maintaining a diverse T-cell repertoire. Nature *402*, 255–262.

Grayson, J.M., Harrington, L.E., Lanier, J.G., Wherry, E.J., and Ahmed, R. (2002). Differential sensitivity of naive and memory CD8(+) T cells to apoptosis in vivo. J. Immunol. *169*, 3760–3770.

Hamilton, S.E., and Harty, J.T. (2002). Quantitation of CD8(+) T cell expansion, memory, and protective immunity after immunization with peptide-coated dendritic cells. J. Immunol. *169*, 4936–4944.

Harty, J.T., and Bevan, M.J. (1995). Specific immunity to *Listeria monocytogenes* in the absence of IFN γ. Immunity *3*, 109–117.

Harty, J.T., and Pamer, E.G. (1995). CD8 T lymphocytes specific for the secreted p60 antigen protect against Listeria monocytogenes infection. J. Immunol. *154*, 4642–4650.

Harty, J.T., and Badovinac, V.P. (2002). Influence of effector molecules on the CD8(+) T cell response to infection. Curr. Opin. Immunol. *14*, 360–365.

Harty, J.T., Tvinnereim, A.R., and White, D.W. (2000). CD8(+) T cell effector mechanisms in resistance to infection. Annu. Rev. Immunol. *18*, 275–308.

Homann, D., Teyton, L., and Oldstone, M.B. (2001). Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. Nat. Med. *7*, 913–919.

Jensen, E.R., Glass, A.A., Clark, W.R., Wing, E.J., Miller, J.F., and Gregory, S.H. (1998). Fas (CD95)-dependent cell-mediated immunity to Listeria monocytogenes. Infect. Immun. *66*, 4143–4150.

Kaech, S.M., and Ahmed, R. (2001). Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. Nat. Immunol. *2*, 415–422.

Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M., and Hensgartner, H. (1994). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature *369*, 31–37.

Kagi, D., Ledermann, B., Burki, K., Zinkernagel, R.M., and Hengartner, H. (1996). Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis. Annu. Rev. Immunol. *14*, 207–232.

Kagi, D., Odermatt, B., and Mak, T.W. (1999). Homeostatic regulation of CD8+ T cells by perforin. Eur. J. Immunol. *29*, 3262–3272.

Kim, H.W., Canchola, J.G., Brandt C.D., Pyles G., Chanock R.M., Jensen K., and Parrott R.H. (1969). Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. *89*, 422–434.

Marshall, D.R., Turner, S.J., Belz, G.T., Wingo, S., Andreansky, S., Sangster, M.Y., Riberdy, J.M., Liu, T., Tan, M., and Doherty, P.C. (2001). Measuring the diaspora for virus-specific CD8+ T cells. Proc. Natl. Acad. Sci. USA *98*, 6313–6318.

Masopust, D., Vezys, V., Marzo, A.L., and Lefrancois, L. (2001). Preferential localization of effector memory cells in nonlymphoid tissue. Science *291*, 2413–2417.

Matloubian, M., Suresh, M., Glass, A., Galvan, M., Chow, K., Whitmire, J.K., Walsh, C.M., Clark, W.R., and Ahmed, R. (1999). A role for perforin in downregulating T-cell responses during chronic viral infection. J. Virol. *73*, 2527–2536.

Mercado, R., Vijh, S., Allen, S.E., Kerksiek, D., Pilip, I.M., and Pamer, E.G. (2000). Early programming of T cell populations responding to bacterial infection. J. Immunol. *165*, 6833–6839.

Murali-Krishna, K., Altman, J.D., Suresh, M., Sourdive, D.J., Zajac,

A.J., Miller, J.D., Slansky, J., and Ahmed, R. (1998). Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity *8*, 177–187.

Oehen, S., Ohashi, P.S., Aichele, P., Burki, K., Hengartner, H., and Zinkernagel, R.M. (1992). Vaccination or tolerance to prevent diabetes. Eur. J. Immunol. *22*, 3149–3153.

Pamer, E.G., Harty, J.T., and Bevan, M.J. (1991). Precise prediction of a dominant class I MHC-restricted epitope of *Listeria monocytogenes*. Nature *353*, 852–855.

Shen, H., Miller, J.F., Fan, X., Kolwyck, D., Ahmed, R., and Harty, J.T. (1998). Compartmentalization of bacterial antigens: differential effects on priming of CD8 T cells and protective immunity. Cell *92*, 535–545.

Slifka, M.K., and Whitton, J.L. (2000a). Activated and memory CD8+ T cells can be distinguished by their cytokine profiles and phenotypic markers. J. Immunol. *164*, 208–216.

Slifka, M.K., and Whitton, J.L. (2000b). Antigen-specific regulation of T cell-mediated cytokine production. Immunity *12*, 451–457.

Slifka, M.K., Rodriguez, F., and Whitton, J.L. (1999). Rapid on/off cycling of cytokine production by virus-specific CD8+ T cells. Nature *401*, 76–79.

Smelt, S.C., Borrow, P., Kunz, S., Cao, W., Tishon, A., Lewicki, H., Campbell, K.P., and Oldstone, M.B. (2001). Differences in affinity of binding of lymphocytic choriomeningitis virus strains to the cellular receptor alpha-dystroglycan correlate with viral tropism and disease kinetics. J. Virol. *75*, 448–457.

Spaner, D., Kaliannan, R., Rabinovich, B., and Miller, R.G. (1999). A role for perforin in activation-induced T cell death in vivo: increased expansion of allogeneic perforin-deficient T cell in SCID mice. J. Immunol. *162*, 1192–1199.

Spielman, J., Lee, R.K., and Podack, E.R. (1998). Perforin/Fas-ligand double deficiency is associated with macrophage expansion and severe pancreatitis. J. Immunol. *161*, 7063–7070.

Stepp, S.E., Dufourcq, L.R., Le, D.F., Bhawan, S., Certain, S., Mathew, P.A., Henter, J.I., Bennett, M., Fischer, A., Saint, B.G., and Kumar, V. (1999). Perforin gene defects in familiar hemophagocytic lymphohistiocytosis. Science *286*, 1957–1959.

Trapani, J.A., and Smyth, M.J. (2002). Functional significance of perforin/granzyme cell death pathway. Nat. Rev. Immunol. *2*, 735–747.

Tvinnereim, A.R., Hamilton, S.E., and Harty, J.T. (2002). CD8(+)-T-cell response to secreted and nonsecreted antigens delivered by recombinant *Listeria monocytogenes* during secondary infection. Infect. Immun. *70*, 153–162.

van der Most, R.G., Sette, A., Oseroff, C., Alexander, J., Murali-Krishna, K., Lau, L.L., Southwood, S., Sidney, J., Chesnut, R.W., Matloubian, M., and Ahmed, R. (1996). Analysis of cytotoxic T cell responses to dominant and subdominant epitopes during acute and chronic lymphocytic choriomeningitis virus infection. J. Immunol. *157*, 5543–5554.

van Stipdonk, M.J., Lemmens, E.E., and Schoenberger, S.P. (2001). Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. Nat. Immunol. *2*, 423–429.

Walsh, C.M., Matloubian, M., Liu, C.C., Ueda, R., Kurahara, C.G., Christensen, J.L., Huang, M.T., Young, J.D., Ahmed, R., and Clark, W.R. (1994). Immune function in mice lacking the perforin gene. Proc. Natl. Acad. Sci. USA *91*, 10854–10858.

White, D.W., and Harty, J.T. (1998). Perforin-deficient CD8+ T cells provide immunity to *Listeria monocytogenes* by a mechanism that is independent of CD95 and IFN-gamma but requires TNF-alpha. J. Immunol. *160*, 898–905.

White, D.W., MacNeil, A., Busch, D.H., Pilip, I.M., Pamer, E.G., and Harty, J.T. (1999). Perforin-deficient CD8+ T cells: in vivo priming and antigen-specific immunity against *Listeria monocytogenes*. J. Immunol. *162*, 980–988.

White, D.W., Badovinac, V.P., Kollias, G., and Harty, J.T. (2000). Cutting edge: antilisterial activity of CD8+ T cells derived from TNF-deficient and TNF/perforin double-deficient mice. J. Immunol. *165*, 5–9.

Zajac, A.J., Blattman, J.N., Murali-Krishna, K., Sourdive, D.J., Suresh, M., Altman, J.D., and Ahmed, R. (1998). Viral immune evasion due to persistence of activated T cells without effector function. J. Exp. Med. *188*, 2205–2213.