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Critical Role for Perforin-, Fas/FasL-, and TNFR1-Mediated Cytotoxic Pathways in Down-Regulation of Antigen-Specific T Cells during Persistent Viral Infection

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Viral persistence following infection with invasive strains of lymphocytic choriomeningitis virus (LCMV) can be achieved by selective down-regulation of virus-specific T lymphocytes. High viral burden in the onset of infection drives responding cells into functional unresponsiveness (anergy) that can be followed by their physical elimination. In this report, we studied down-regulation of the virus-specific $CD8^+$-T-cell response during persistent infection of adult mice with LCMV, with emphasis on the role of perforin-, Fas/FasL-, or tumor necrosis factor receptor 1 (TNFR1)-mediated cytolysis in regulating T-cell homeostasis. The results reveal that the absence of perforin, Fas-ligand, or TNFR1 has no significant effect on the kinetics of proliferation and functional inactivation of virus-specific $CD8^+$ T cells in the onset of chronic LCMV infection. However, these molecules play a critical role in the homeostatic regulation of T cells, influencing the longevity of the virus-specific $CD8^+$-T-cell population once it has become anergic. Thus, $CD8^+$ T cells specific to the dominant LCMV $NP_{396–404}$ epitope persist in an anergic state for at least 70 days in perforin-, FasL-, or TNFR1-deficient mice, but they were eliminated by day 30 in C57BL/6 controls. These effects were additive as shown by a deficit of apoptotic death of $NP_{396–404}$ peptide-specific $CD8^+$ T cells in mice lacking both perforin and TNFR1. This suggests a role for perforin-, FasL-, and TNFR1-mediated pathways in down-regulation of the antiviral T cell response during persistent viral infection by determining the fate of antigen-specific T cells. Moreover, virus-specific anergic $CD8^+$ T cells in persistently infected C57BL/6 mice contain higher levels of Bcl-2 and Bcl-XL than functionally intact T cells generated during acute LCMV infection. In the case of proapoptotic factors, Bax expression did not differ between T-cell populations and Bad was below the limit of detection in all samples. As expression of the Bcl-2 family members controls susceptibility to apoptosis, this finding may provide a molecular basis for the survival of anergic cells under conditions of prolonged antigen stimulation.

The outcome of any viral infection is the net result of a balance between virus replication in host tissues and the capacity of the host to mount and maintain an effective antiviral response. The murine lymphocytic choriomeningitis virus (LCMV) system has been used extensively to study the dynamics of virus-host interactions, and two extreme features in the interaction between the virus and the host immune system have been clearly demonstrated with this viral system (1, 31, 53, 55, 56). First, sustained $CD8^+$-T-cell responses result in viral clearance within 2 weeks postinfection, primarily mediated via perforin-dependent cytolysis. In this situation, antigen-specific $CD8^+$ T cells expand and differentiate into effector cells and after virus clearance, undergo a death phase in which the majority of the expanded T cells are eliminated. The remaining virus-specific memory $CD8^+$ T cells persist at relatively high levels throughout the life of the host. The magnitude of expansion varies depending on the infection, and reproducible hierarchies of immunodominance are observed in antigen-specific $CD8^+$ T cells. In contrast, a transient $CD8^+$-T-cell response, in which antigen-specific $CD8^+$ T cells are induced and proliferate, initially exhibiting antiviral function by progressively losing this ability (a phenomenon known as clonal exhaustion), results in viral persistence (31). Such functionally deficient T cells persist in the host for long periods but may eventually be eliminated (37, 54). Both outcomes (viral clearance or persistence) are of limited pathological consequence for the host and are determined by the strength and magnitude of the virus-specific immune response and the rate of virus replication (21, 28, 50). Thus, susceptibility to persistent infection by clonal exhaustion correlates with a quantitatively lower virus-specific $CD8^+$-T-cell response from the host and with rapidly replicating LCMV strains (2, 3, 28, 29). Hence, fast growing isolates, such as Docile and CL13 Armstrong, readily induce persistent infection, whereas more slowly growing strains such as WE, Aggressive, and Armstrong do not. A further distinction in the above scenario is provided under certain conditions of infection (selected virus strain and host combinations) where failure of the T-cell response to rapidly clear the infection due to functional exhaustion of virus-specific $CD8^+$ T cells may lead to a time-limited viral persistence. In this situation, $CD4^+$ helper T cells and neutralizing antibody produced by B cells can become the principle arm of antiviral defense, and viral clearance can be achieved at a later stage of infection.

The silencing of the immune response and the decline in the number and activation state of T cells during the course of an acute (usually limited to 10 to 14 days) or chronic viral infec-

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tion is a well-recognized but poorly understood phenomenon. Analyses of the complex regulatory networks leading to T-cell survival, death, and immunodeficiency in viral infections support the view that the strength of antigenic stimulation via the clonotypic T-cell receptor (TCR) after engagement with the major histocompatibility complex (MHC)-peptide complexes can dictate the fate of virus-specific T cells (20, 23, 51). Many different signaling molecules are engaged when T cells interact with virus-infected antigen-presenting cells (APC), and integration of these signals by the T cell ultimately decides whether it will be activated and remain functionally active, become unresponsive (anergic), or undergo apoptotic lysis. Factors such as the prior activation state of the T cell, the dose of antigen, affinity of the TCR for the MHC-peptide complex, and costimulation provided by professional APC play a role in this process. Among the immune regulatory mechanisms that have been identified so far, the secretory and membrane-lytic pathways involving perforin and granzyme and the nonsecretory receptor-mediated cytolytic pathway involving Fas/FasL or tumor necrosis factor (TNF) receptor 1 (TNFR1) interactions are major mechanisms implicated in antigen-induced downsizing of virus-specific T cells during acute or persistent viral infections (15, 16, 27). Initiation of apoptosis in target cells by granule exocytosis is the result of the action of two types of molecules, the pore-forming protein perforin and the lymphocyte granule-specific serine esterase granzyme B, which together can reproduce all the features of cytotoxic T lymphocyte (CTL)-induced apoptosis (17, 19, 26, 33, 46, 52). In addition to induction of granule exocytosis (perforin) pathways, activation of T cells by engagement of the TCR by the appropriate MHC-peptide complex leads to up-regulation of Fas and FasL, and their interaction causes the association of adapters such as Fas-associated death domain protein that ultimately result in caspase activation and cell death. Likewise, TNFR1 signaling is rapid and highly specific and occurs through two principal classes of cytoplasmic adapter proteins, TRAFs (TNFR-associated factors) and death domain molecules, causing caspase activation and cell death (49). A number of cytokines have been also proposed as factors either inducing apoptosis or conditioning cells to become susceptible to apoptosis (11, 22). Antigen-specific death or survival may be directed by the expression of such death factors, death receptors, and survival receptors by the T cells themselves. Alternatively, these mechanisms can influence antigen-specific T-cell regulation via termination of antigen processing through T cell-mediated killing of professional APC, occurring as a consequence of the infection of professional APC by the invading pathogen and rendering these critical accessory cells targets for destruction by the immune response (44). Thus, deficits in accessory cell cosignal delivery are likely to affect the initial expansion, differentiation, and death phase of homeostatic T-cell regulation.

The studies reported here were undertaken to explore the mechanisms and factors that determine the nature and kinetic patterns of functional inactivation and/or physical lysis of virus-specific $CD8^+$ T cells during persistent infection of a mature host with LCMV, with particular emphasis on the role of perforin, Fas/FasL, or TNFR1 pathways in regulating virus-specific T-cell responses. The experiments carried out during the infection of mice with the Docile or Aggressive strains of LCMV revealed comparable kinetics of expansion and functional inactivation of virus-specific $CD8^+$ T cells in the early phase of infection in mice deficient in TNFR1, FasL, or perforin and in C57BL/6 (B6) controls. However, the data underscore a critical role for these molecules in the persistence of the virus-specific $CD8^+$-T-cell population once it has become anergic.

MATERIALS AND METHODS

Mice. B6, B6-gld ($FasL^{-/-}$), and gamma interferon (IFN-γ)-deficient mice on the B6 background (IFN-$\gamma^{-/-}$) (10) were obtained from Jackson Laboratories, Bar Harbor, Maine. Perforin-deficient mice ($P^{-/-}$) were of a pure B6 genetic background, generated by homologous recombination in embryonic stem cells of B6 origin (17). TNFR1-deficient mice ($TNFR1^{-/-}$) on the B6 genetic background were kindly provided by Tak Mak (Toronto, Canada) (39). $P^{-/-}$ mice were crossed with $TNFR1^{-/-}$ or IFN-$\gamma^{-/-}$ mice to obtain mice deficient in either perforin and TNFR1 ($P^{-/-}$ $TNFR1^{-/-}$) or perforin and IFN-γ ($P^{-/-}$ IFN-$\gamma^{-/-}$). The genotypes of the mice were determined by PCR of tail DNA. Mice were bred and maintained under specific-pathogen-free conditions. Animals were kept and experiments were performed in accordance with institutional animal welfare guidelines.

Viruses. LCMV Docile and LCMV Aggressive (variants isolated from an LCMV-UBC carrier mouse) were obtained from C. J. Pfau (Troy, N.Y.) as a plaque-purified second passage virus (38). Virus titers were determined with an immunological focus assay (8).

Viral peptides. Peptides were synthesized at the Medical College of Georgia Molecular Biology Core Facility (Augusta, Ga.) by using a Perkin-Elmer Applied Biosystems (Berkeley, Calif.) 433A peptide synthesizer. The LCMV-specific CTL epitope peptides used in this study were the H-$2D^b$-binding peptide $GP1_{33–41}$ (KAVYNFATC), $GP2_{276–286}$ (SGVENPGGYCL), $NP_{396–404}$ (FQPQNGQFI), and $GP1_{92–101}$ (CSANNSHHYI) and the H-$2K^b$-binding $GP1_{34–43}$ (AVYN FATCGI) and $NP_{205–212}$ (YTVKYPNL) peptides. Except for LCMV Docile, which contains an amino acid change in the peptide $GP2_{276–286}$ ($GP2_{280_N}$→S), all virus strains used in this study were conserved in epitopes recognized by virus-specific T cells. Note that the mutation ($GP2_{280_N}$→S) substantially reduces the ability of the $GP2_{276–286}$ peptide to bind H-$2D^b$.

Cytotoxic T-cell response. CTL precursor activity was determined in a bulk culture system as described previously (32). Briefly, splenocytes were prepared from LCMV-infected mice at the indicated time points. Cells were cultured for 5 days at densities of 4×10^6, 2×10^6, and 0.5×10^6 cells/well together with peptide-pulsed (0.1 μg/ml) irradiated (30 Gy) splenocytes (4×10^6) or virus-infected peritoneal macrophages (5×10^5) in 2 ml of Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum and 10 U of murine interleukin-2/ml. Restimulated cells were resuspended in 1 ml of medium per culture well and serial threefold dilutions of effector cells were tested in a ^{51}Cr release assay which used MC57G (H-2^b) cells infected with virus or pulsed with 10 μg of the indicated peptide/ml as target cells. The cytotoxic activity in the spleen of virus-infected mice was expressed in lytic units per spleen; a lytic unit was defined as the number of splenocytes required to lyse one-third of a standard number of target cells (10^4 cells per well).

Quantitative analysis of virus-specific $CD8^+$ T cells in spleen. MHC-peptide tetramers for the staining of epitope-specific T cells were prepared as previously described (4, 5, 37). Experiments utilized H-$2D^b$ tetramers complexed with the LCMV $GP1_{33–41}$, $GP2_{276–286}$, or $NP_{396–404}$ peptide. Single-cell suspensions prepared from the spleen were stained with H-$2D^b$ tetramer along with anti-CD8 fluorescein isothiocyanate (FITC)-conjugated rat monoclonal antibody (clone 53-6.7) in fluorescence-activated cell sorter (FACS) buffer (phosphate-buffered saline [PBS] with 1% bovine serum albumin and 0.2% sodium azide). After staining for 1 h at 4°C, cells were fixed in PBS containing 0.1% paraformaldehyde and acquired on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif.), and the data were analyzed using CellQuest software.

Intracellular staining for IFN-γ following peptide stimulation. Splenocytes were cultured in 96-well U-bottom plates at a density of 4×10^6 cells/well in 200 μl of RPMI 1640 (Gibco) supplemented with 10% fetal calf serum, 10 U of murine interleukin-2/well, and 1 μg of brefeldin A (Pharmingen, San Diego, Calif.)/well in the presence or absence of CTL epitope peptide at a concentration of 1 μg/ml (12, 34, 40). The peptides used were the H-$2D^b$-binding $GP1_{33–41}$, $GP2_{276–286}$, $NP_{396–404}$, or $GP1_{92–101}$ and the H-$2K^b$-binding $GP1_{34–43}$ or $NP_{205–212}$. After 6 h of culture, the cells were harvested, washed once in FACS buffer, and surface stained with phycoerythrin-conjugated monoclonal rat antibody specific to mouse CD8α (clone 53-6-72). After washing, the cells were

stained for intracellular cytokines by using the Cytofix/Cytoperm kit (Pharmingen) according to manufacturer's instructions. FITC-conjugated monoclonal rat antibodies specific to murine IFN-γ or TNF-α (clones XMG1.2 and MP6-XT22, respectively; Caltag, Burlingame, Calif.) and their isotype control antibodies (rat immunoglobulin G1 [IgG1] and IgG2a, respectively) were used to identify cytokine-positive cells. Stained cells were washed a further time and fixed in PBS containing 0.1% paraformaldehyde. Samples were acquired on a FACSCalibur flow cytometer (Becton Dickinson).

Bcl-2, Bcl-XL, and Bax expression by FACS. Splenocytes were surface stained with H-2D^b tetramer ($GP1_{33–41}$) along with anti-CD8 phycoerythrin-conjugated rat monoclonal antibody (clone 53-6.7), washed with PBS, and subjected to intracellular staining for Bcl-2, Bcl-XL, Bax, or Bad by using the CytoFix/Cytoperm kit according to the manufacturer's instructions. For intracellular Bcl-2 staining, FITC-conjugated hamster anti-mouse Bcl-2 antibody (clone 3F11) or its isotype control antibody (hamster IgG) (Pharmingen) was used. For intracellular staining for Bax, Bad, or Bcl-XL the cells were incubated with a hamster antibody specific to Bax or Bad (Pharmingen) or a rabbit antibody specific to Bcl-XL (B22630; Transduction Labs). The cells were washed twice, and FITC-conjugated antibody specific to hamster or rabbit IgG (Jackson, West Grove, Pa.) was added. Following incubation for 30 min, the cells were washed with PBS and analyzed by FACS. Isotype-matched control antibodies, purified hamster IgG, and normal rabbit serum were used as negative controls.

RESULTS

(i) Regulation of virus-specific CD8$^+$-T-cell responses by perforin and FasL during chronic infection of mice with LCMV Docile. Perforin and Fas/FasL pathways can control T-cell homeostasis by acting directly on virus-specific T cells to control cell proliferation or via T cell-mediated killing of virally infected APC. Although acute LCMV infection of mice deficient in perforin, Fas, or FasL has previously been characterized extensively (18, 24, 27, 36, 42), the potential role of these molecules in the exhaustion of virus-specific T-cell responses during chronic LCMV infection has not yet been explored. In particular, it was unclear how perforin or Fas/FasL pathways of cell death would impact the fate of virus-specific CD8$^+$-T-cell responses during a persistent viral infection. We addressed this issue by analyzing viral replication and the development of the virus-specific CD8$^+$-T-cell response in mice deficient in perforin or FasL in the context of infection with LCMV strains causing an acute (LCMV Aggressive) or persistent (LCMV Docile) infection in B6 mice. The specificity and function of the CD8$^+$-T-cell response were examined by direct visualization with binding of D^b tetramer molecules complexed to immunodominant LCMV peptides ($GP1_{33–41}$, $GP2_{276–286}$, or $NP_{396–404}$) and by IFN-γ production after peptide stimulation.

As predicted, B6 mice infected with a relatively high dose (2×10^6 PFU) of Docile failed to clear the infection, and the virus persisted indefinitely (Fig. 1A, left panel). Kinetic studies of virus-specific CD8$^+$ T cells in the spleen of these mice initially showed a significant increase in D^b $GP1_{33–41}$ and D^b $NP_{396–404}$ binding of CD8$^+$ T cells (Fig. 1B and C, left panels). The numbers of $NP_{396–404}$ peptide-specific CD8$^+$ T cells declined over time and were below detectable levels by day 30, whereas $GP1_{33–41}$-specific CD8$^+$ T cells persisted at high levels over a 3-month observation period (>90 days). The number of $GP1_{33–41}$-specific T cells increased to a steady-state level of around 10% of total splenic CD8$^+$ T cells by day 9 postinfection, whereas the number of $NP_{396–404}$-specific T cells dropped from a maximum of 3 to 5% at day 6 or 9 to undetectable levels (less than 0.1%) by day 30. Both tetramer binding T-cell populations in these mice initially exhibited antiviral functions (day 6), producing IFN-γ after stimulation with the appropriate peptide, but they rapidly became unresponsive by day 15 postinfection. Data obtained from FasL$^{-/-}$ mice infected with 2×10^6 PFU of LCMV Docile revealed a general pattern of virus replication and persistence in the spleen similar to that seen in B6 mice (Fig. 1A, middle panel). However, in FasL$^{-/-}$ mice, antigen-specific CD8$^+$ T cells were induced at slightly higher levels than in the controls and they persisted, retaining the nonfunctional phenotype, either for >90 days ($GP1_{33–41}$-specific T cells) or around 70 days ($NP_{396–404}$ peptide specific). The death phase of the $NP_{396–404}$-specific T cells, which was essentially completed by day 30 in the control mice, occurred by day 90 in FasL$^{-/-}$ mice, indicating a significant role for the Fas/FasL pathway in the lysis of virus-specific T cells. Note that the kinetics of functional inactivation by day 15 postinfection of antigen-specific CD8$^+$ T cells (based on IFN-γ production) were identical for both FasL$^{-/-}$ and control mice. Finally, as expected, perforin-deficient mice infected with 2×10^6 PFU of Docile did not clear the infection (Fig. 1A, right panel). While a vigorous virus-specific CD8$^+$-T-cell response was detected by tetramers and IFN-γ staining, T-cell function was lost by day 15 as observed with control mice. Functionally unresponsive cells persisted with comparable kinetics to FasL$^{-/-}$ mice (Fig. 1B and C, right panels). It is worthy to note that in agreement with earlier reports, perforin-deficient mice were not free of disease signs, and we have observed mortality (around 40%) in these experiments. Taken together, these data indicate a role for perforin or Fas/FasL effector cytolytic pathways in the clonal exhaustion of virus-specific CD8$^+$ T cells. Deficiency in either the perforin or the FasL pathway increases the levels and life span of anergic T cells, perhaps by preventing apoptotic cell death during chronic LCMV infection.

(ii) Regulation of virus-specific CD8$^+$-T-cell responses through perforin or FasL during acute infection of mice with LCMV Docile or LCMV Aggressive. We next examined virus-specific CD8$^+$-T-cell homeostasis under conditions of an acute LCMV infection, which induced in wild-type mice a vigorous CD8$^+$-T-cell response with rapid control of the virus. Infection of B6 mice with 10^2 PFU of LCMV Docile resulted in the expansion of $GP1_{33–41}$ or $NP_{396–404}$ CD8$^+$ T cells that cleared the infection within 2 weeks (Fig. 2A, left panel) and persisted at high frequencies, retaining a functional phenotype (Fig. 2B and C, left panels). After virus clearance, a death phase ensued in which a substantial fraction of the expanded antigen-specific T cells were eliminated. Similar results were obtained with FasL$^{-/-}$ mice under these infection conditions (Fig. 2, middle panels), except the peak number of virus-specific CD8$^+$ T cells was approximately fivefold greater than in the controls. However, the kinetics of the death phase after virus clearance did not differ significantly, although the level of antigen-specific memory CD8$^+$ T cells was somewhat higher in FasL$^{-/-}$ mice than in the controls. As expected, perforin-deficient mice infected with 10^2 PFU of LCMV Docile did not clear the infection (Fig. 2A, right panel). Virus-specific CD8$^+$ T cells were initially induced and progressively lost effector function by day 30 (2 weeks later than mice infected with 2×10^6 PFU of LCMV Docile, as shown in Fig. 1). When functionally deficient $GP1_{33–41}$ peptide-specific T cells were maintained at high levels throughout the period of this study (90 days), the level of $NP_{396–404}$ peptide-specific T cells declined below detectable levels by day 50 only in a fraction of the infected mice (around

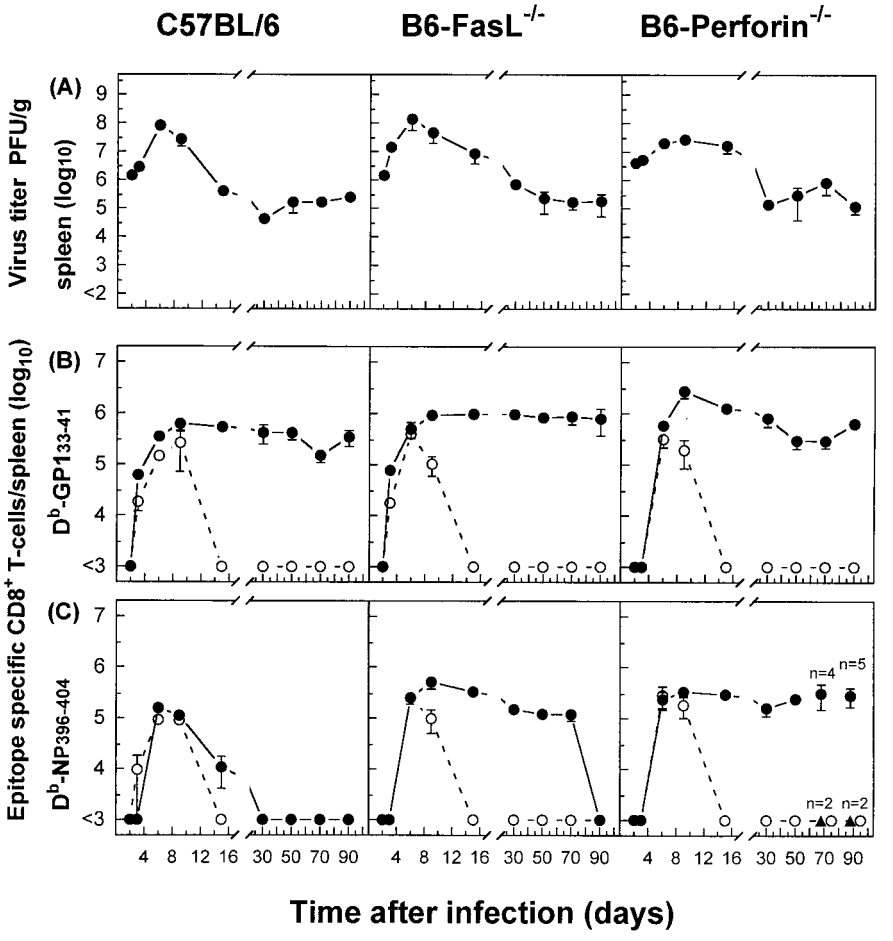

FIG. 1. Kinetics of virus replication and virus-specific $CD8^+$-T-cell response in spleens of perforin- or FasL (gld)-deficient mutant mice compared to those of B6 congenic control mice following infection with a relatively high dose of LCMV Docile. Analyses were performed to correlate the kinetics of virus replication (A) with the kinetics of virus-specific $CD8^+$-T-cell response (B and C). Mice were infected with 2×10^6 PFU of LCMV Docile, and virus titers in the spleens were measured at the time points indicated. The data shown are means ± standard errors of the means for 3 to 5 mice (in $\log_{10}$ PFU/gram of tissue). In addition, total numbers of $GP1_{33-41}$ or $NP_{396-404}$ peptide-specific $CD8^+$ T cells were measured by staining for H-$2D^b$ tetramer (filled circles) or intracellular IFN-γ (open circles) production following stimulation of cells with the appropriate peptide to determine the functional responsiveness of these cells. The data shown are means ± standard errors of the means for 3 to 5 mice (in $\log_{10}$ virus-specific T cells per spleen). In the case of perforin-deficient mice, some variation in the number of $NP_{396-404}$ tetramer-positive cells was observed at late time points. The symbols (▲,●) used in the right column of panel C represent values obtained from the number of mice indicated.

60%). Extended analyses of infection with 10^2 PFU of LCMV Aggressive essentially reproduced the picture obtained from infection with 10^2 PFU of LCMV Docile (Fig. 3A to C). The latter analyses include the profile of the $GP2_{276-286}$ epitope-specific $CD8^+$-T-cell response. Note that the delayed kinetics of functional inactivation of virus-specific $CD8^+$ T cells in perforin-deficient mice infected with 10^2 PFU of either Docile or Aggressive were reflected by higher mortality rates (around 80%) than that seen with mice infected with 2×10^6 PFU of Docile. However, sufficient numbers of surviving animals were obtained for these experiments, and such survivors were generally free of severe disease signs, and no abnormalities in lymphatic tissues were observed (data not shown).

(iii) $CD8^+$-T-cell effector functions are lost in mice with persistent LCMV infection. The MHC-tetramer peptide complex and IFN-γ secretion staining assay techniques are limited by their ability to resolve a signal over background staining by FACS (around 0.1% of $CD8^+$ T cells). Hence, as an independent test of functional activity, we measured the ability of splenocytes from $FasL^{-/-}$ or B6 mice infected with 2×10^6 or 10^2 PFU of Docile to develop cytotoxic activity to the virus or individual peptide epitopes following stimulation in vitro. As shown in Fig. 4, $CD8^+$-T-cell responses to virus or peptides of dominant ($GP1_{33-41}$, $NP_{396-406}$, or $GP1_{34-43}$) or subdominant ($GP1_{92-101}$ or $NP_{205-212}$) epitopes were detectable in mice with chronic permanent infection during the initial phase of infection but not in the later phase. Consistent with the results of IFN-γ staining, splenocytes from B6 mice infected with Docile (2×10^6 PFU) exhibited substantial cytotoxic activity in the initial phase of infection but lost their cytotoxic activity by day 15 (Fig. 4A). Similar results were obtained with $FasL^{-/-}$ mice under these infection conditions (Fig. 4C). To further address the functional behavior of virus-specific T cells, parallel studies were conducted of mice infected with 10^2 PFU of Docile (Fig.

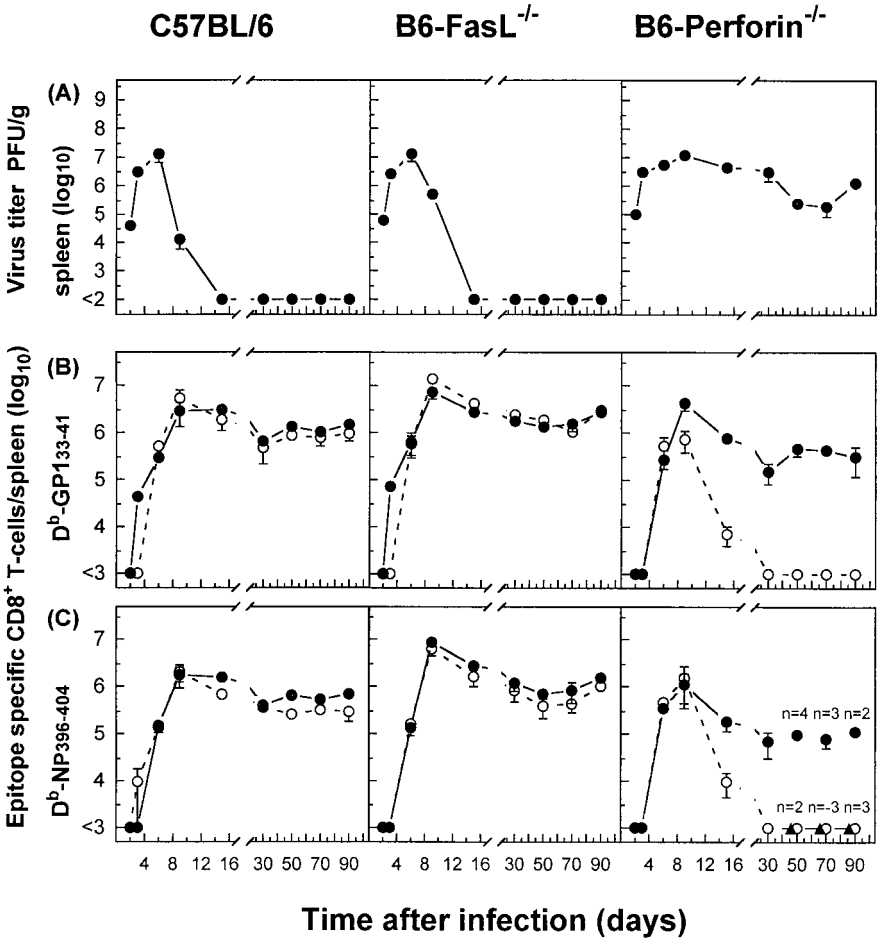

FIG. 2. Kinetics of virus replication and virus-specific $CD8^+$-T-cell response in spleens of perforin- or FasL (gld)-deficient mutant mice compared to those of B6 congenic control mice following infection with a low dose of LCMV Docile. Mice were infected with 10^2 PFU of LCMV Docile, and virus titers in the spleens were measured at the time points indicated (A). The data shown are means ± standard errors of the means for 3 to 5 mice (in $\log_{10}$ PFU/gram of tissue). In addition, total numbers of $GP1_{33–41}$ or $NP_{396–404}$ peptide-specific $CD8^+$ T cells were measured by staining for H-$2D^b$ tetramer (filled circles) or intracellular IFN-γ (open circles) production following stimulation of cells with the appropriate peptide to determine the functional responsiveness of these cells (B and C). The data shown are means ± standard errors of the means for 3 to 5 mice (in $\log_{10}$ virus-specific T cells per spleen). In the case of perforin-deficient mice, some variation in the number of $NP_{396–404}$ tetramer-positive cells was observed at late time points. The symbols (▲,●) used in the right column of panel C represent values obtained from the number of mice indicated.

4B and D). In general, the magnitude of CTL activity obtained following in vitro restimulation with virus-infected macrophages as APC correlates with the IFN-γ staining data. Thus, cytotoxic T-cell responses were elicited in B6 or $FasL^{-/-}$ mice in the initial phase of infection and were sustained at high levels after virus elimination over the period of 3 months. Taken together, the above findings indicate that during chronic infection, virus-specific $CD8^+$ T cells become incapable of eliciting their normal array of effector functions, including cytotoxicity and cytokine production. In addition, the rapid functional inactivation of $CD8^+$ T cells specific to subdominant epitopes suggest that such T cells are susceptible to clonal exhaustion.

(iv) Regulation of virus-specific $CD8^+$-T-cell responses in mice deficient in TNFR1, perforin and TNFR1, or perforin and IFN-γ during infection with LCMV Docile. In addition to perforin and Fas/FasL effector pathways, TNFR1 has also been implicated in homeostatic apoptotic regulation of T lymphocytes. Thus, we studied the $CD8^+$-T-cell responses in $TNFR1^{-/-}$ mice infected with LCMV Docile (Fig. 5, left panels). $TNFR1^{-/-}$ mice were unable to clear infection with 2×10^6 PFU of Docile, and the levels of $GP1_{33–41}$ and $NP_{396–404}$ peptide-specific $CD8^+$ T cells increased initially and either persisted ($GP1_{33–41}$) over the period of this experiment (to day 70) or progressively declined ($NP_{396–404}$) below detectable levels by day 70. In these mice, $CD8^+$ T cells for either epitope initially exhibited antiviral functions, producing IFN-γ after stimulation with the appropriate peptide, but progressively became unresponsive by day 50 ($GP1_{33–41}$) or 30 ($NP_{396–404}$) postinfection. Thus, in contrast to control mice, in which antigen-specific cells have essentially lost function by day 15 and the elimination of $NP_{396–404}$ was completed by day 30, TNFR1 deficiency resulted in prolonged persistence of antigen-specific T cells and in a delay in the kinetics of functional inactivation. Extended studies on $P^{-/-}$ $TNFR1^{-/-}$ mice confirmed the normal course of viral persistence observed in control mice (Fig. 5,

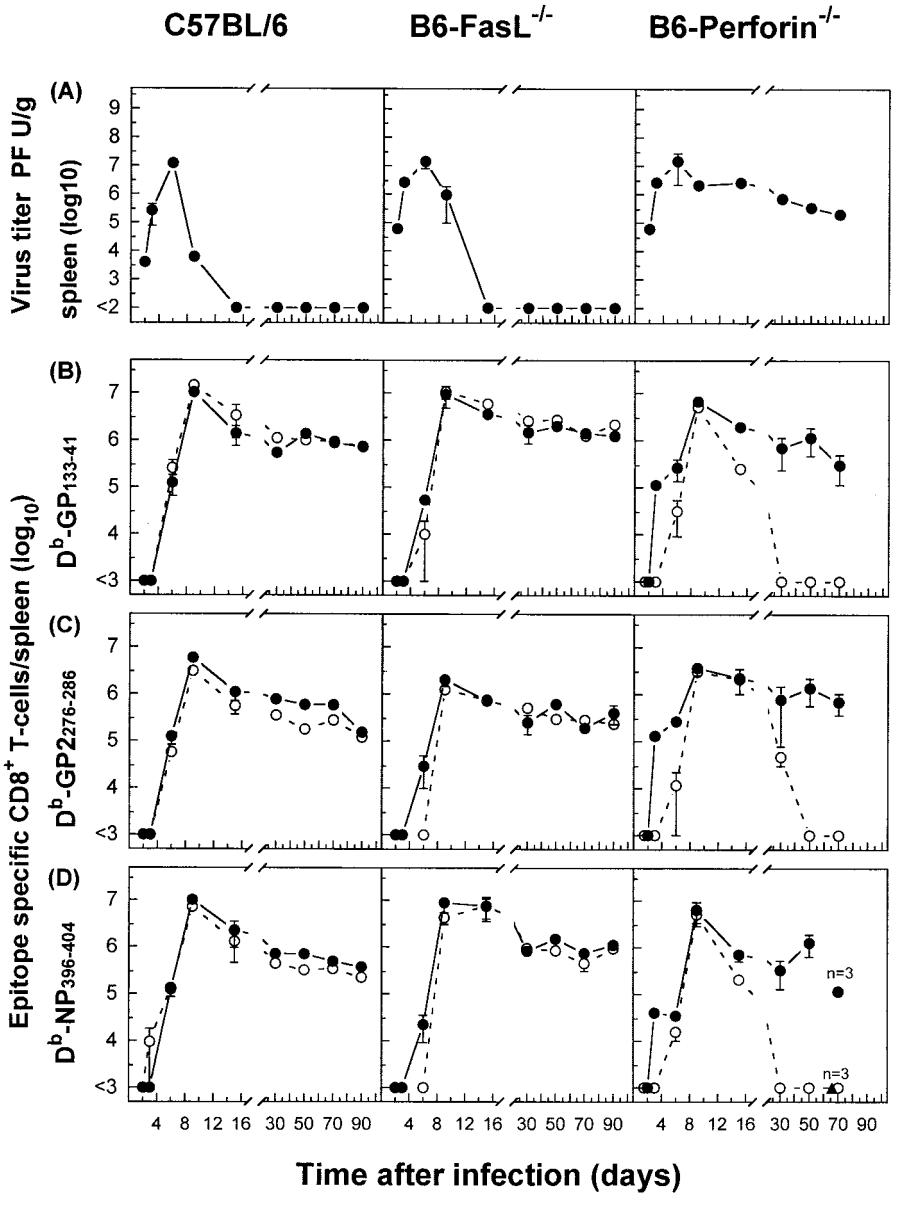

FIG. 3. Kinetics of virus replication and virus-specific $CD8^+$-T-cell response in spleens of perforin- or FasL (gld)-deficient mutant mice compared to those of B6 congenic control mice following infection with LCMV Aggressive. Mice were infected with 10^2 PFU of LCMV Aggressive, and virus titers in the spleens were measured at the time points indicated (A). The data shown are means ± standard errors of the means for 3 to 5 mice (in $\log_{10}$ PFU/gram of tissue). In addition, total numbers of $GP1_{33–41}$, $GP2_{276–286}$, or $NP_{396–404}$ peptide-specific $CD8^+$ T cells were measured by staining for H-$2D^b$ tetramer (filled circles) or intracellular IFN-γ (open circles) production following stimulation of cells with viral epitope peptide to determine the functional responsiveness of these cells (B, C, and D). The data shown are means ± standard errors of the means for 3 to 5 mice (in $\log_{10}$ virus-specific T cells per spleen).

middle panels). However, the number of virus-specific $CD8^+$ T cells specific for the $NP_{396–404}$ epitope did not decrease and remained elevated at day 90 postinfection. Strikingly, a major fraction of $NP_{396–404}$ epitope-specific T cells retained a functional phenotype until day 50. Finally, as a role for IFN-γ in the homeostasis of T cells through pathways independent of their cytotoxic effector function has been proposed (15), we analyzed antigen-specific induction, proliferation, and death in perforin- and IFN-γ-deficient mice (Fig. 5, right panels). As expected, infection of these mice with 2×10^6 PFU of Docile resulted in viral persistence, and in contrast to the control mice, neither peptide-specific $CD8^+$-T-cell population declined but persisted at relatively high levels. To test their functional states, we studied TNF-α production after peptide stimulation in vitro, but we were unable to detect any activity when splenocytes from perforin- and IFN-γ-deficient mice harvested at the early or late stage of infection were analyzed. This outcome was not unexpected in light of recent reports that, in contrast to IFN-γ, the production of TNF-α by $CD8^+$ T cells following antigen stimulation ceases after a short period even when antigen contact is sustained (6, 45). With regard to the mortality described for LCMV-infected mice with disruption

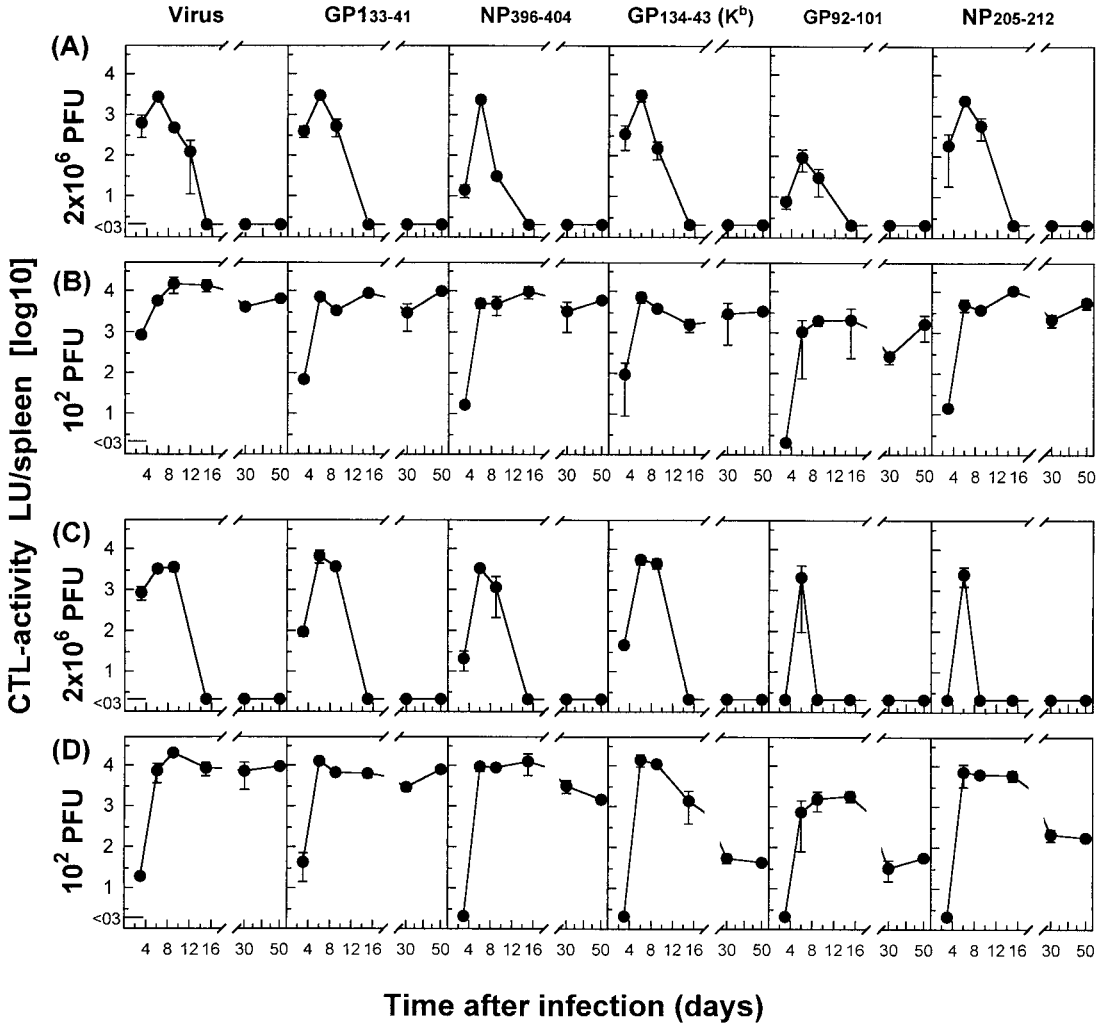

FIG. 4. Virus-specific cytotoxic activities in spleens of control B6 mice in comparison to those of FasL (gld) mice. B6 control mice (A and B) or FasL (gld) mutant mice (C and D) were infected with 2×10^6 or 10^2 PFU of LCMV Docile as indicated. Splenocytes isolated on days 3, 6, 9, 15, 30, and 50 postinfection were stimulated in vitro with $GP1_{33-41}$ or $NP_{396-404}$ peptide-pulsed splenocytes as described in Materials and Methods. The cytolytic activity of restimulated splenocytes cultured at a density of 4×10^6 cells/well was measured in a ^{51}Cr release assay as described in Materials and Methods. Virus-specific cytotoxic activity was expressed in lytic units (LU) per spleen. The data shown are means ± standard errors of the means for 3 to 5 mice (in $\log_{10}$ virus-specific T cells per spleen).

of the perforin or IFN-γ gene (17, 27, 35), perforin- and TNFR1- or perforin- and IFN-γ-deficient mice were generally free of disease signs, and we have only occasionally observed lethality in the above experiments.

We next examined antigen-specific $CD8^+$-T-cell responses after infection with 10^2 PFU of LCMV Docile. $TNFR1^{-/-}$ mice elicited vigorous $CD8^+$-T-cell priming against the dominant $GP1_{33-41}$ and $NP_{396-404}$ epitopes (Fig. 6, left panels). After clearance of infection by day 15, antigen-specific T cells persist, retaining a functional phenotype. Consistent with the data presented in Fig. 5, in $P^{-/-}$ $TNFR1^{-/-}$ and $P^{-/-}$ IFN-$\gamma^{-/-}$ mice infected with 10^2 PFU of Docile, the infection was not cleared and antigen-specific $CD8^+$ T cells persisted after losing their antiviral function by day 30 (Fig. 6, middle and right panels). Together, these data show that antigen-specific $CD8^+$-T-cell functional inactivation and lysis are disrupted in TNFR1-, perforin- and TNFR1-, or perforin- and IFN-γ-deficient mice. The fact that perforin-, FasL-, or TNFR1-deficient mice showed comparable viral titers in different tissues at the onset of infection with 2×10^6 PFU of LCMV Docile, suggests that it is unlikely that different viral loads could account for our results (data not shown).

(v) Relative expression of Bcl-2 family members by virus-specific $CD8^+$ T cells during chronic viral infection. Long-term survival of anergic $GP1_{33-41}$-specific $CD8^+$ T cells in the chronic infected host is observed, despite persistent antigen stimulation that can lead to apoptotic cell lysis as shown in different experimental settings. This suggests that survival pathways are activated to prevent physical lysis of these virus-specific T-cell populations by apoptosis. In a classical immune response, the initial phase of expansion of T cells is followed by a second phase of elimination of activated cells by apoptosis. In contrast to activated cells, memory T cells and naive T cells are relatively resistant to apoptosis. These distinct phases of susceptibility to apoptosis are likely to reflect different expression profiles for members of the Bcl-2 family with proapoptotic

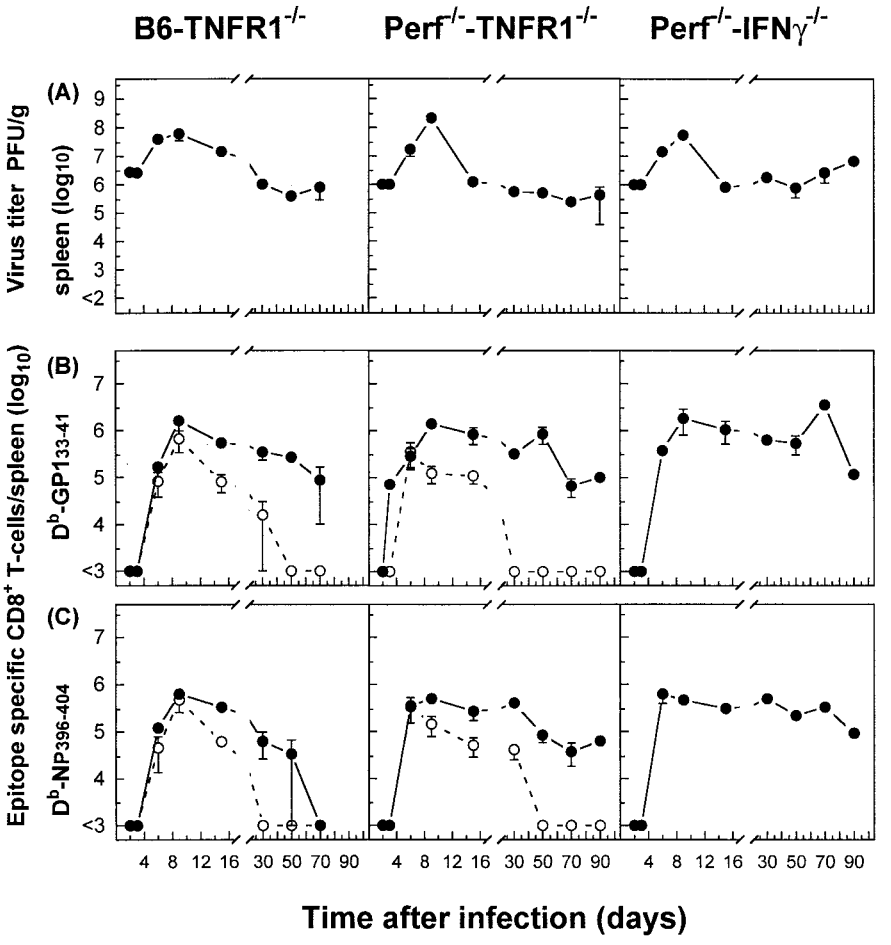

FIG. 5. Kinetics of virus replication and virus-specific $CD8^+$-T-cell response in spleens of mice deficient in TNFR1, perforin and TNFR1, or perforin and IFN-γ following infection with a relatively high dose of LCMV Docile. Mice were infected with 10^6 PFU of LCMV Docile, and virus titers in the spleens were measured at the time points indicated (A). The data shown are means ± standard errors of the means for 3 to 5 mice (in $\log_{10}$ PFU/gram of tissue). In addition, total numbers of $GP1_{33–41}$ or $NP_{396–404}$ peptide-specific $CD8^+$ T cells were measured by staining for H-$2D^b$ tetramer (filled circles) or intracellular IFN-γ (open circles) production following stimulation of cells with the appropriate peptide to determine the functional responsiveness of these cells (B and C). The data shown are means ± standard errors of the means for 3 to 5 mice (in $\log_{10}$ virus-specific T cells per spleen).

(Bad, Bax) or antiapoptotic (Bcl-2, Bcl-XL) properties. We therefore examined expression of these molecules by $GP1_{33–41}$-specific $CD8^+$ T cells derived from B6 mice infected 1, 9, 15, 30, or 70 days previously with 2×10^6 or 10^2 PFU of LCMV Docile (Fig. 7). The levels of Bcl-2 found on $GP1_{33–41}$-specific $CD8^+$ T cells isolated from acutely infected mice decreased around the time of the peak T-cell response (day 9 and 15) to levels lower than in naive ($CD8^+$-$CD44^{low}$) T cells. After completion of the death phase, the expression of Bcl-2 in this T-cell population increased to a level similar to that found in naive T cells. In contrast, these changes were not observed in functionally unresponsive $GP1_{33–41}$-specific $CD8^+$ T cells from persistently infected mice (2×10^6 PFU) which had Bcl-2 levels similar to those found in naive $CD8^+$ T cells isolated early in the infection (day 9 or 15) and slightly higher than those found later in the course of chronic infection (day 30 or 70). Functionally deficient $GP1_{33–41}$-specific $CD8^+$ T cells showed levels of Bcl-XL which were slightly higher than those of naive $CD8^+$ T cells, whereas levels of Bcl-XL were significantly decreased in active memory T cells generated during acute infection. Levels of Bax were at the limit of detection in all cell populations analyzed in this study, whereas levels of Bad remained below the limit of detection (data not shown).

DISCUSSION

As the net balance between induction and inactivation of antiviral T cells can determine the final outcome of a viral infection, we have explored here the contribution of perforin, Fas/FasL, or TNFR1 pathways in the clonal exhaustion of virus-specific $CD8^+$ T cells. The main observation is that deficiencies in perforin, FasL, or TNFR1 prolong the life span of unresponsive T cells, probably by preventing cell death, for several weeks (up to 70 days). This was most clearly seen for $CD8^+$ T cells specific to $NP_{396–404}$, which are lysed rapidly in wild-type mice (within 30 days). The effects of perforin and TNFR1 on the kinetics of antigen-specific T-cell lysis were additive, because deficiency in perforin and TNFR1 promotes survival of virus-specific $CD8^+$ T cells for at least for 90 days. Similar kinetic profiles for antigen-specific T-cell responses

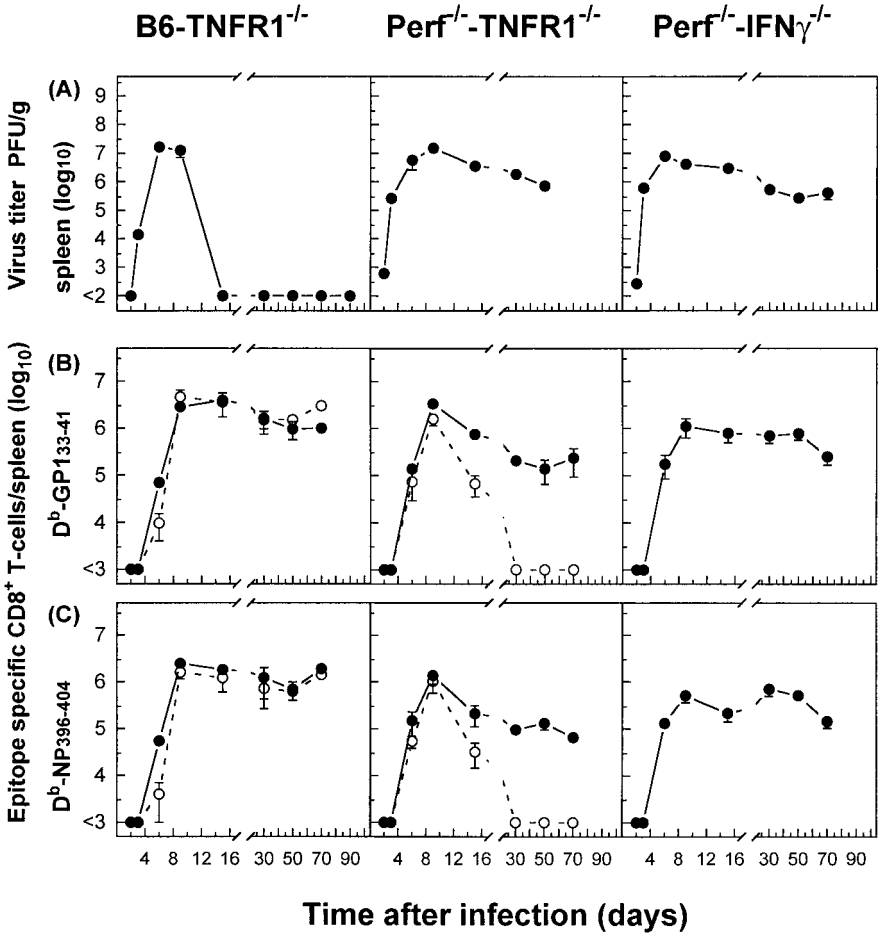

FIG. 6. Kinetics of virus replication and virus-specific $CD8^+$-T-cell response in spleens of mice deficient in TNFR1, perforin and TNFR1, or perforin and IFN-γ following infection with a low dose of LCMV Docile. Mice were infected with 10^2 PFU of LCMV Docile, and virus titers in the spleens were measured at the time points indicated (A). The data shown are means ± standard errors of the means for 3 to 5 mice (in $\log_{10}$ PFU/gram of tissue). In addition, total numbers of $GP1_{33–41}$ or $NP_{396–404}$ peptide-specific $CD8^+$ T cells were measured by staining for H-$2D^b$ tetramer (filled circles) or intracellular IFN-γ (open circles) production following stimulation of cells with the appropriate peptide to determine the functional responsiveness of these cells (B and C). The data shown are means ± standard errors of the means for 3 to 5 mice (in $\log_{10}$ virus-specific T cells per spleen).

were obtained for perforin- and IFN-γ-deficient mice. Moreover, the data presented in Fig. 1, 2, and 3 reveal two additional important features in the regulation of the virus-specific $CD8^+$-T-cell response. (i) The level of peak virus-specific $CD8^+$-T-cell expansion is of a considerably smaller magnitude in mice with viral persistence than in animals which rapidly cleared the infection. Thus, for example, the peak levels of $GP1_{33–41}$ peptide-specific $CD8^+$ T cells in B6 mice infected with 2×10^6 or 10^2 PFU of LCMV Docile or 10^2 PFU of LCMV Aggressive were 5.5 $\log_{10}$, 6.5 $\log_{10}$, or 7 $\log_{10}$, respectively, by day 9 postinfection. (ii) The death phase of virus-specific $CD8^+$ T cells was abnormal in mice infected with 2×10^6 PFU of LCMV Docile. No significant decrease in antigen-specific $CD8^+$ T cells was observed by day 15 or 30, a time at which the death phase is essentially completed in mice which clear the infection. Remarkably, the levels of antigen-specific memory $CD8^+$ T cells were comparable between the different groups of mice. Together these data provide direct evidence that under conditions of overwhelming infection death of antigen-specific T cells does not occur during the acute phase. It is worthy of note that previous studies documented the elimination of adoptively transferred $GP1_{33–41}$-specific transgenic T cells over an extended period of infection (deletion occurred by day 50 postinfection) (30). However, subsequent reports by us and others (37, 54), including the data in this study, show that anergic $GP1_{33–41}$-specific cells are maintained during persistent infection in B6 mice. It is likely that this apparent disparity is a result of the clonality of the TCR expression by $GP1_{33–41}$-specific T cells. The adoptive transfer approach utilized high-affinity transgenic T cells of a single clonotype, in contrast to the de novo-generated $GP1_{33–41}$-specific polyclonal T-cell response in which a broad spectrum of TCR clonotypes emerge, conferring a range of affinities to the MHC-$GP1_{33–41}$ peptide complex. This different spectrum of TCR clonality likely correlates to a spectrum of sensitivity to apoptotic death relative to the affinity of individual TCRs to the MHC-peptide complex.

The mechanisms and factors which determine down-regulation of the antiviral immune response during persistent infection with LCMV are poorly understood. However, functional

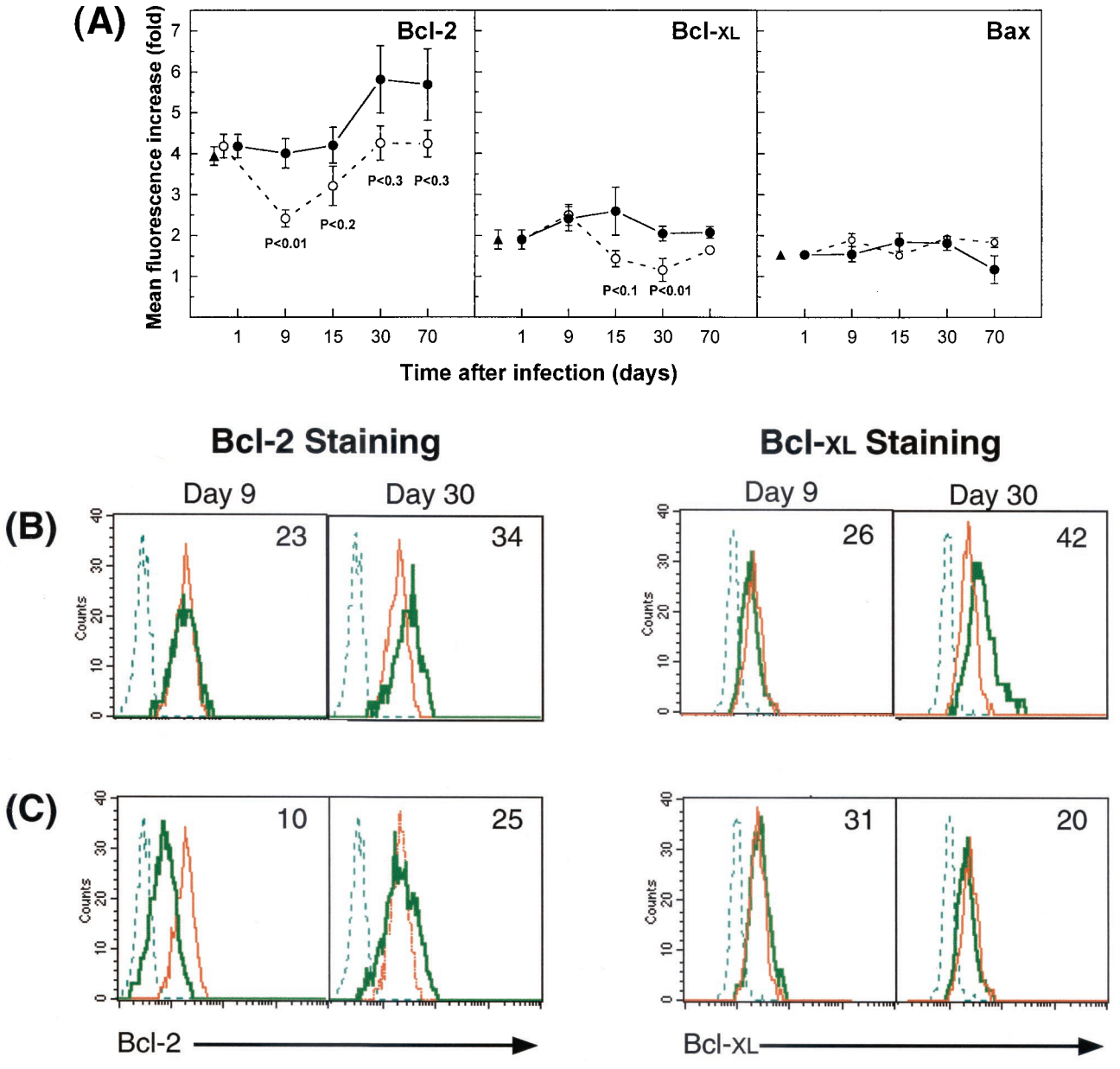

FIG. 7. Expression of Bcl-2 family members in virus-specific $CD8^+$ T cells during chronic LCMV infection of B6 mice. (A) Spleen cells from naive mice (▲) or mice infected with 2×10^6 PFU (●) or 10^2 PFU (○) of LCMV Docile analyzed at the indicated time points after infection were triple stained with anti-CD8α; antibody specific to Bcl-2, Bcl-XL, or Bax; and either anti-CD44 (naive mice) or $GP1_{33–41}$ tetramer (infected mice). The mean fluorescence of Bcl-2 family expression on gated $CD8^+$-$CD44^{low}$ or $GP1_{33–41}$ tetramer-positive T cells was measured by FACS. Bcl-2, Bcl-XL, or Bax expression was calculated as the mean (± standard error of the mean) fluorescence increase in relation to isotype antibody control expression for 3 to 6 mice. Statistical analysis (Student's *t* test) comparing $GP1_{33–41}$-specific T cells between mice infected with a high or low dose of LCMV Docile was conducted and revealed significant differences ($P < 0.01$) between Bcl-2 levels at day 9 and Bcl-XL levels at day 30. *P* values are indicated in the panels where appropriate. (B and C) Bcl-2 and Bcl-XL levels in $GP1_{33–41}$-specific T cells are indicated for mice infected with high (B) and low (C) doses of LCMV Docile on days 9 and 30. In these panels, the green line represents $GP1_{33–41}$ tetramer-positive T cells, the red line represents $CD8^+$-$CD44^{low}$ T cells in uninfected mice, and the broken blue line represents isotype antibody control staining. Mean fluorescence intensity values for $GP1_{33–41}$ tetramer-positive cells from infected mice are indicated in the upper right hand corner of each panel. The mean fluorescence intensities were 20 and 25 for $CD8^+$-$CD44^{low}$ cells and 5 and 16 for antibody isotype controls for Bcl-2 and Bcl-XL staining, respectively.

inactivation is the first step in the down-regulation of the $CD8^+$-T-cell response, and physical deletion proceeds subsequent to this, providing direct support for a linear differentiation model of T-cell regulation during chronic LCMV infection. According to such models, the life span of functionally deficient T cells will depend on the expression of various T-cell survival factors. It is worthy of note that functionally deficient T cells express a variety of markers (CD44, CD69, CD25) associated with a memory T-cell phenotype (data not shown). Eventually, after a period of persistence, functionally inactive

$CD8^+$ T cells will be lysed via several possible (but not mutually exclusive) mechanisms described below.

The first death mechanism results from excessive TCR stimulation (above a threshold level) causing antigen-specific cell lysis by controlled expression of death factors or by increasing the sensitivity of cells to death signals. It has been shown that prolonged viral antigen stimulation of T cells triggers feedback mechanisms that limit their proliferation and differentiation, resulting in activation-induced cell death (AICD) and termination of the T-cell immune response (41, 43, 47–49). Our findings illustrate that this can occur as a consequence of Fas/FasL- or TNFR1-mediated AICD. Intracellular factors, such as the Bcl-2/Bcl-XL antiapoptotic proteins and the Bad/Bax proapoptotic proteins, have also been implicated in the regulation of AICD in the acutely infected host (13, 14). To understand the role of such factors, it is critical to note that such pro- and antiapoptotic proteins can heterodimerize and effectively titrate each other's function, suggesting that the balance between competing activities of these factors determines the fate of individual T cells. The lack of down-regulation of Bcl-2 expression by functionally inactive T cells in chronically infected hosts, compared to that seen in functional T cells in acutely infected hosts during virus resolution, implies that the persistence of functionally inactive antigen-specific T cells is (at least in part) controlled through mechanisms that regulate these proteins. A second death mechanism may be senescence of antigen-specific T cells, a process occurring when T cells exceed a certain number of divisions. This is an attractive hypothesis which will be addressed in ongoing studies.

A final potential death mechanism is via the selective elimination of virus-infected professional APC by activated T cells. In this scenario, destruction of APC via cytotoxic pathways, especially perforin-mediated cytolysis, would eventually result in complete absence of cosignal delivery by accessory cells and this would facilitate T-cell death (9, 44). How does perforin regulate the T-cell response? Following the above arguments, several possibilities are apparent. First, perforin deficiency could enhance $CD8^+$-T-cell expansion through decreased killing of APC, resulting in prolonged antigen display and the stimulation of additional naive precursor cells. However, induction of virus-specific $CD8^+$ T cells proceeds normally, and some of these cells persist for many weeks while others are eliminated, which may argue for a TCR-specific regulated process rather than a deficit in stimulation of T cells by accessory cells. Clearly, the possibility that the infection of dendritic cells in chronically infected mice may disrupt the survival and activation of virus-specific T cells requires further analysis, and this is currently under investigation in our laboratory. Alternatively, perforin may act directly on antigen-specific $CD8^+$ T cells by controlling the balance between division and death during the expansion phase (18). The contribution of this mechanism is less clear. In our study we observed that perforin-deficient mice contain more antigen-specific $CD8^+$ T cells than do wild-type mice on day 9 postinfection, which supports such a view. However, this enhanced expansion is transient because by the time memory is established, perforin-deficient and wild-type mice contain comparable numbers of functionally inactive $CD8^+$ T cells.

The roles of FasL and TNFR1 in the regulation of the antiviral response are less clear. Although there is not evidence in the LCMV literature for a contribution of Fas/FasL or TNFR1 death pathways in virus clearance, a direct effect of these pathways on T-cell regulation is more likely and is supported by this study. Several studies of the T-cell activation and expansion, contraction, and memory phases in acute LCMV infection revealed normal death of virus-specific $CD8^+$ T cells following viral clearance in the absence of Fas or TNFR1 or of both Fas and TNFR1 molecules (24, 42). Similarly, studies with IFN-γ- or IFN-γ-receptor-deficient mice demonstrate a role for IFN-γ in the homeostatic regulation of T cells by preventing AICD, but the levels of virus-specific memory $CD8^+$ T cells in these mice were comparable to those in wild-type control animals (7, 25, 37). It has also been reported that viral persistence can be established, and $CD8^+$ CTL precursor frequency greatly declined during persistent infection of IFN-γ-receptor- and Fas (*lpr*)-deficient mice with LCMV strain CL13 Armstrong, suggesting that clonal exhaustion of T cells does not require IFN-γ and Fas, even though both molecules influence AICD (25). In many respects our results confirm the above studies with an important difference. In this study, we have systematically analyzed the contribution of perforin, FasL, TNFR1, and IFN-γ with regards to the long-term course of virus-specific $CD8^+$-T-cell responses by performing parallel analyses of visualization (by tetramers) of antigen-specific T cells and measuring antiviral functions (IFN-γ staining, cytotoxicity). Our study reveals a not yet defined contribution of the above pathways of cytolysis and/or T-cell regulation in the maintenance and persistence of functionally deficient virus-specific $CD8^+$ T cells. On the basis of our results, we speculate that down-regulation of virus-specific $CD8^+$ T cells during chronic viral infection is a complex multifactorial process partially controlled by interactions between pro-survival and -cell death factors. Because identification of methods to restore function to unresponsive antigen-specific T cells is of critical importance for immunotherapeutic strategies to curtail persistent infections, the findings in this report may help to define measures to prevent physical elimination of virus-specific T cells during persistent viral infection.

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