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Critical Role for Alpha/Beta and Gamma Interferons in Persistence of Lymphocytic Choriomeningitis Virus by Clonal Exhaustion of Cytotoxic T Cells

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Under conditions of high antigenic load during infection with invasive lymphocytic choriomeningitis virus (LCMV) strains, virus can persist by selective clonal exhaustion of antigen-specific CD8$^+$ T cells. In this work we studied the down-regulation of the virus-specific CD8$^+$-T-cell response during a persistent infection of adult mice, with particular emphasis on the contribution of the interferon response in promoting host defense. Studies were conducted by infecting mice deficient in receptors for type I (alpha/beta interferon [IFN-α/β]), type II (IFN-γ), and both type I and II IFNs with LCMV isolates that vary in their capacity to induce T-cell exhaustion. The main conclusions of this study are as follows. (i) IFNs play a critical role in LCMV infection by reducing viral loads in the initial stages of infection and thus modifying both the extent of CD8$^+$-T-cell exhaustion and the course of infection. The importance of IFNs in this context varies with the biological properties of the LCMV strain. (ii) An inverse correlation exists between antigen persistence and responsiveness of virus-specific CD8$^+$ T cells. This results in distinct programs of activation or tolerance (functional unresponsiveness and/or physical elimination of antigen-specific cells) during acute and chronic virus infections, respectively. (iii) A successful immune response associated with definitive viral clearance requires an appropriate balance between cellular and humoral components of the immune system. We discuss the role of IFNs in influencing virus-specific T cells that determine the outcome of persistent infections.

Viruses use a number of strategies, including escape from immune recognition or induction of immunosuppression, to avoid immunological surveillance and thereby persist in the host (reviewed in references 1, 14, 35, 45, and 59). The immune response to viruses involves activation of both effector arms of the adaptive immune system, i.e., virus-specific CD8$^+$ T cells and neutralizing antibody production, as well as components of the innate response, including type I (alpha/beta interferon [IFN-α/β]) and type II (IFN-γ) IFNs(27, 56, 69, 72). IFNs are an essential part of both the innate and adaptive cytokine responses to viral infection, having important functions in the regulation of the immune system (12, 24, 38, 49). In addition to inducing an antiviral state (24, 38), IFNs are noted for their function in many immunoregulatory processes, including up-regulation of major histocompatibility complex (MHC) class I and II molecules, activation of macrophages and natural killer cells (68), augmentation of dendritic cell responses, and promotion of proliferation and survival of activated lymphocytes (15, 36, 60).

Infection of mice with the relatively noncytopathic lymphocytic choriomeningitis virus (LCMV) results in an early and dramatic elevation of IFN-α/β, within day 2 to 3 of infection (18, 67). The adaptive T-cell immune response, characterized by profound CD8$^+$-T-cell expansion and IFN-γ production, is elicited by day 7 to 9 after infection (11, 23, 73). The central concept derived from studies with this viral system is that in previously unexposed individuals a race occurs between the development of cell-mediated immunity and the extent of viral replication. Virus clearance or persistence is determined by a critical balance between the virus-specific immune response and the rate of virus replication. Consistent with this model, virus control and functional T-cell memory, or viral persistence and exhaustion of virus-specific CD8$^+$ T cells, reflect the ends of the spectrum of the virus-host interaction. Thus, infection with invasive strains of LCMV that can rapidly replicate and produce a high viral load can drive the activation and vigorous expansion of antigen-specific CD8$^+$ T cells, followed by their functional inactivation resulting in irreversible anergy and/or deletion (43, 71). This phenomenon, called clonal exhaustion, results in viral persistence. In contrast, infection with less invasive, slowly replicating LCMV strains induces virus-specific T cells capable of efficiently clearing the infection. Typically, a fraction of these cells persist as long-term memory cells after virus elimination. These distinct outcomes of LCMV infection are critically controlled by host factors, which determine the magnitude of the virus-specific cytotoxic-T-lymphocyte (CTL) response, and by the rapidity of spread of the virus, determined by the virus strain and the route and dose of infection (2, 40, 42). Thus, susceptibility to persistent infection by clonal exhaustion correlates with a quantitatively lower virus-specific CTL response from the host and with rapidly replicating LCMV strains.

We previously observed that the ability of individual virus strains to induce extensive spread of infection correlates with their relative resistance to IFN-α/β and IFN-γ (39); hence, fast-growing IFN-resistant isolates, such as Docile and CL 13 Armstrong, readily induce persistent infection, whereas slow-growing IFN-sensitive strains, such as WE, Aggressive, and Armstrong, do not. Thus, it is of interest to further understand

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the roles played by IFNs during the establishment of persistent infections, and LCMV serves as a valuable model for such studies. It is particularly important to determine the impact of IFNs on viral dissemination during the onset of infection and to elucidate their role in initiation and regulation of the T-cell response and therefore the outcome of infection. Clonal expansion and differentiation of LCMV-specific $CD8^+$ T cells reportedly proceed normally in the absence of IFN-γ, and the importance of this cytokine during acute LCMV infection is influenced by the invasiveness of the virus strains used (34, 39, 48, 58). However, complete inhibition of virus clearance following treatment with antibody specific to IFN-γ has been reported (61, 70). This correlated with a greatly reduced CTL response and suggested an essential role for IFN-γ in generation of virus-specific $CD8^+$ T cells. Similarly, studies with mice deficient in the IFN-α/β pathway revealed that LCMV-WE is able to initiate a persistent infection due to the absence of virus-specific $CD8^+$ T cells, while clearance of LCMV-Armstrong proceeds but with slower kinetics (19, 46, 63). Together, these studies imply an essential role for IFN-α/β or IFN-γ in LCMV infection, which can determine the outcome of acute infection. However, the mechanisms by which IFNs are operational in this respect (direct suppression of virus replication and/or regulation of adaptive antiviral responses) are not well understood.

In this work the kinetics of virus replication and the development of the virus-specific CTL response were studied in mice deficient in receptors for IFN-α/β, IFN-γ, or both IFN-α/β and IFN-γ during infection with LCMV strains with different potentials for causing persistent infection. The specific $CD8^+$-CTL response was examined by direct visualization with MHC class I tetramers complexed to LCMV epitopes and with stimulation of IFN-γ expression by viral peptides. The results reveal a critical role for both IFN-α/β and IFN-γ in restricting LCMV spread at the onset of infection and thus preventing extinction of the antiviral T-cell response. Our study shows that production of IFN-α/β and/or IFN-γ critically regulates the virus-host balance during the acute phase of infection, such that a high viral burden drives responding cells into different programs of exhaustion. This dampening of virus-specific $CD8^+$-T-cell responses in the early phase of infection results in a protracted or permanent persistence of infection.

MATERIALS AND METHODS

Mice. Mice deficient in IFN-α/β receptor (IFN-α/β$R^{-/-}$), IFN-γ receptor (IFN-γ$R^{-/-}$), or both IFN-α/β and IFN-γ receptors (IFN-α/β-γ$R^{-/-}$) on the 129/SvEv background (29, 46, 63), originally obtained from B&K Universal Limited (Hull, United Kingdom), were bred and maintained under specific-pathogen-free conditions. Age-matched 129/SvEv control mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All mice used in this study had the H-2^b MHC, and animals were kept and experiments were performed in accordance with institutional animal welfare guidelines.

Viruses. LCMV-WE was originally obtained from F. Lehmann-Grube (Hamburg, Germany), and LCMV-Docile and LCMV-Aggressive (variants isolated from an LCMV-WE [UBC] carrier mouse) were obtained from C. J. Pfau (Troy, N.Y.) as plaque-purified second passage virus (52). LCMV-Armstrong and CL 13 Armstrong viruses were obtained originally from M. B. A. Oldstone (Scripps Clinic and Research Foundation, La Jolla, Calif.) (20) and Rafi Ahmed (Emory University Vaccine Center, Atlanta, Ga.) (2). CL 13 Armstrong is a variant isolated from the spleen cells of an adult BALB/WEHI mouse neonatally infected with LCMV-Armstrong.

Virus titers and neutralization assay. LCMV titers in the spleen and neutralizing antibody titers in serum were determined with an immunological focus assay (8). Neutralizing titers were defined as the dilution of serum causing half-maximal reduction of plaques of LCMV with the same amount of virus inoculated with control serum and measured as $-\log_2$, starting with a 1:10 dilution of serum.

Depletion of $CD8^+$- or $CD4^+$-T-cell subsets in vivo. Mice were depleted of $CD8^+$- or $CD4^+$-T-cell subsets by intraperitoneal injection of purified specific antibody (YTS169 anti-CD8 or YTS191 anti-CD4) as previously described (41). Antibody was administrated 1 day before and 2 days after infection as indicated. This treatment depleted >99% of the splenic $CD4^+$-T-cell population for a 3- to 4-week period, but within 2 months these mice had reconstituted their T-cell subsets and contained normal levels of $CD4^+$ T cells. However, although these mice recovered their T-cell populations, virus-specific $CD4^+$-T-cell activity was not detectable in the periphery (due to persisting virus antigen in the thymus resulting in negative selection), as determined by measuring virus-specific antibody activity by enzyme-linked immunosorbent assay (ELISA) and plaque neutralization assay (10, 44). Alternatively, $CD4^+$ T cells were eliminated on day 20 after infection by treatment with anti-CD4 antibody on day 20, on day 23, and continuing at monthly intervals throughout the experiment. Similarly, mice treated with antibody to CD8 depleted >90% of their $CD8^+$ T cells, and while the $CD8^+$-T-cell population eventually returned to normal levels, functional virus-specific $CD8^+$ T cells were undetectable.

Viral peptides. Peptides were synthesized at the Medical College of Georgia Molecular Biology Core Facility (Augusta, Ga.), 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 peptides $GP1_{33–41}$ (KAVYNFATC), $GP2_{276–286}$ (SGVENPGGYCL), $NP_{396–404}$ (FQPQNGQFI), and $GP1_{92–101}$ (CSANNSHHYI) and the H-$2K^b$-binding peptides $GP1_{34–43}$ (AVYNFATCGI) and $NP_{205–212}$ (YTVKYPNL). Except for LCMV-Docile, which contains an amino acid change in the peptide $GP2_{276–286}$ ($380_{N→S}$), all virus strains used in this study were conserved in epitopes recognized by virus-specific T cells. Note that the mutation ($380_{N→S}$) in LCMV-Docile substantially reduces the ability of the $GP2_{276–286}$ peptide to bind H-$2D^b$.

CTL response. CTL precursor activity was determined in a bulk culture system as described previously (45). 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 cells/well) or virus-infected peritoneal macrophages (5×10^5 cells/well) in 2 ml of Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum and 10 U of recombinant murine interleukin-2 (IL-2) per 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 using MC57G (H-2^b) cells infected with virus or pulsed with 10 μg of the indicated peptide per ml as target cells.

Quantitative analysis of virus-specific $CD8^+$ T cells in spleen. MHC-peptide tetramers for staining of epitope-specific T cells were prepared as previously described (3, 4, 21, 47). Soluble MHC class I (H-$2D^b$) with a specific biotinylation site and human β2-microglobulin were produced in large amounts as recombinant proteins by transforming *Escherichia coli* strain BL21(DE3) with the plasmid pET23-D^b-BSP, pET23-K^b-BSP, or pHN1-β2m (kindly provided by J. D. Altman, Emory University, Atlanta, Ga.). Expression of the proteins was induced with isopropyl-β-thiogalactopyranoside as described previously (4). Folding, purification, and biotinylation of H2-D^b peptide complexes were performed as described previously (3). Finally, biotinylated MHC-peptide complexes were tetramerized by addition of phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, Oreg.). Experiments utilized H-$2D^b$ tetramers complexed with LCMV $GP1_{33–41}$, $GP2_{276–286}$, or $NP_{396–404}$ peptide. Single-cell suspensions prepared from spleen were stained with H-$2D^b$ tetramer along with anti-CD8 fluorescein isothiocyanate-conjugated rat monoclonal antibody (clone 53-6.7) (Caltag, Burlingame, Calif.) 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 analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, Calif.).

Intracellular staining for IFN-γ following peptide stimulation. Splenocytes were cultured in 96-well U-bottom plates at 4×10^6 cells/well in 200 μl of RPMI 1640 (Gibco) supplemented with 10% fetal calf serum, 10 U of murine IL-2 per well, and 1 μg of Brefeldin A (Pharmingen, San Diego, Calif.) per well in the presence or absence of CTL epitope peptide at a concentration of 1 μg/ml (21, 47). The peptides used were the H-$2D^b$-binding $GP1_{33–41}$, $GP2_{276–286}$, $NP_{396–404}$, or $GP1_{92–101}$ and H-$2K^b$-binding $GP1_{34–43}$ or $NP_{205–212}$. After 6 h of culture, cells were harvested, washed once in FACS buffer, and surface stained with phycoerythrin-conjugated monoclonal rat antibody specific to mouse CD8α (clone

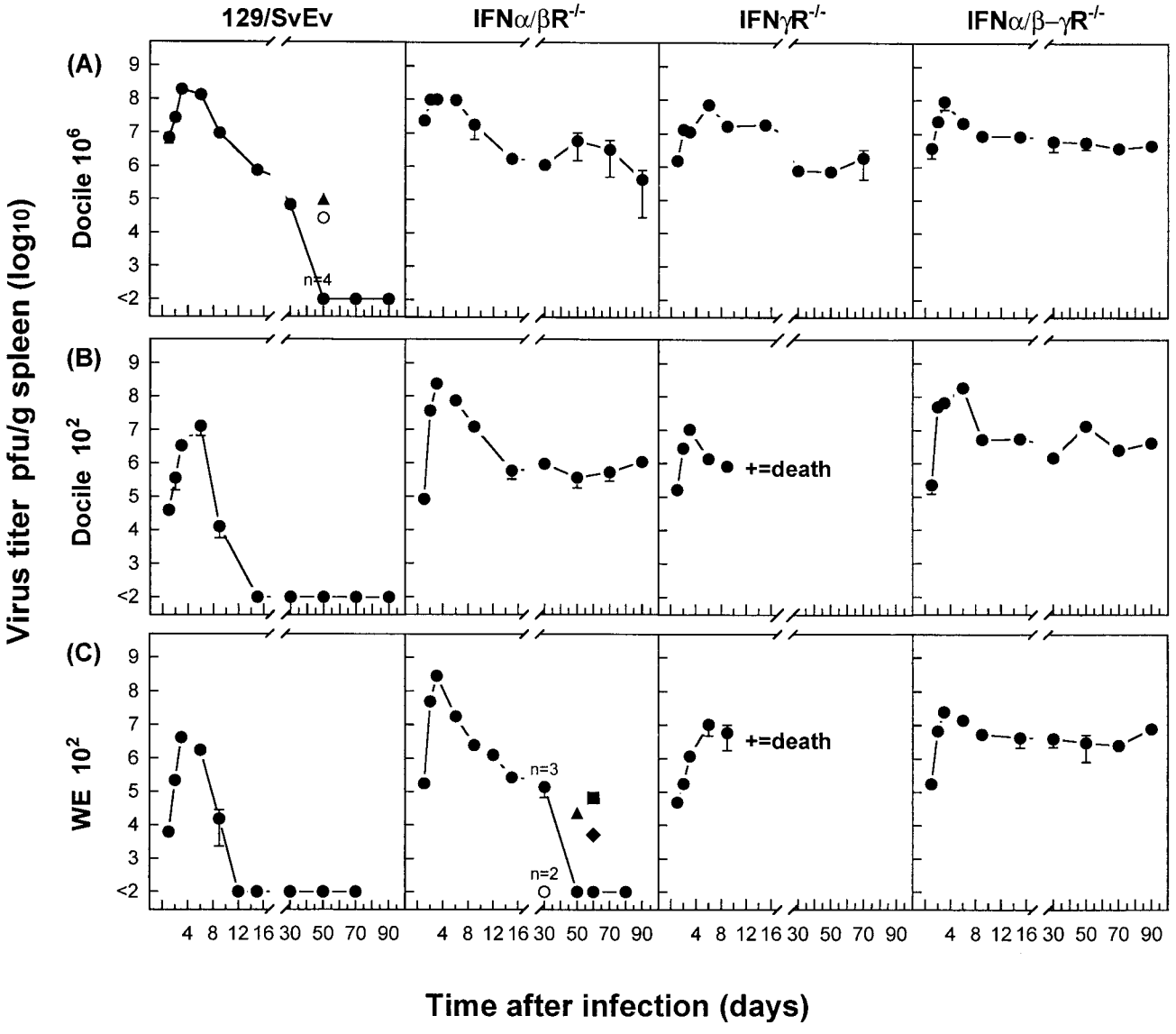

FIG. 1. Kinetics of LCMV-Docile and LCMV-WE replication in the spleens of mice deficient in type I, type II, or both type I and II IFN receptors compared to their 129/SvEv congenic controls. Virus titers in spleens were measured at the time points indicated following intravenous infection of IFN-α/βR$^{-/-}$, IFN-γR$^{-/-}$, IFN-α/β-γR$^{-/-}$, or 129/SvEv mice either with 10^2 PFU (A) or 10^6 PFU (B) of Docile or with 10^2 PFU of WE (C). Data are means and standard errors of the means for three to five mice. Different symbols used in the columns represent values obtained from individual mice.

53-6-72). After washing, cells were stained for intracellular IFN-γ using a Cytofix/Cytoperm kit (Pharmingen) according to the manufacturer's instructions. Fluorescein isothiocyanate-conjugated monoclonal rat antibody specific to murine IFN-γ (clone XMG1.2) and its isotype control antibody (rat immunoglobulin G1 [IgG1]) were used to identify cytokine-positive cells. Stained cells were washed a further time and fixed in PBS containing 0.1% paraformaldehyde. Samples were analyzed as described above.

RESULTS

Contributions of IFN-α/β and IFN-γ to control of acute infection with the WE or Docile strain of LCMV. Having previously demonstrated that the capacity of LCMV strains to replicate vigorously and generate high viral burdens in vivo correlates with their ability to initiate viral persistence (39), we questioned whether antiviral cytokines such as IFN-α/β and IFN-γ play a critical role in determining viral load and thus the outcome of infection in immune-competent adults. The purpose of these experiments was to define roles for IFN-α/β- and/or IFN-γ-mediated pathways in establishing an effective defense against LCMV strains differing in their capacities to induce persistent infection.

Adult mice infected with LCMV strain WE mount a vigorous CTL response that clears virus efficiently within 10 to 15 days. However, infection with the rapidly replicating LCMV-Docile leads to a different outcome. Kinetic studies of virus replication in the spleens of 129/SvEv mice infected with 10^2 PFU of LCMV-WE demonstrated that viral burdens peaked at around day 3 to 6 at levels of 7 $\log_{10}$ PFU/g of spleen and were below the limit of detection by day 12 (Fig. 1). Virus clearance

also proceeded efficiently in mice infected with 10^6 PFU of LCMV-WE, although viral titers peaked earlier (day 2) and declined by day 15 (data not shown). Similarly, 129/SvEv mice infected with 10^2 PFU of the Docile strain of LCMV cleared virus by day 15, but viral titers increased more rapidly and peaked at slightly higher levels (7.5 $\log_{10}$ PFU/g of spleen) compared to infection with 10^2 PFU of LCMV-WE (Fig. 1B and C). In contrast, 129/SvEv mice infected with a relatively high dose (10^6 PFU) of Docile initially failed to clear the virus and retained high virus levels in many tissues (spleen, thymus, and kidney) for several weeks (Fig. 1A and data not shown). Interestingly, virus levels declined over time to below the detection limit by day 50 after infection.

Extended studies of IFN-α/βR$^{-/-}$, IFN-γR$^{-/-}$, or IFN-α/β-γR$^{-/-}$ mice infected with LCMV-WE (10^2 PFU) or LCMV-Docile (10^2 or 10^6 PFU) revealed strikingly different outcomes for the viral infection compared to that in control (129/SvEv) mice with intact IFN responses (Fig. 1). Thus, kinetic studies of virus replication revealed that the absence of IFN-α/β responsiveness significantly increased sensitivity to LCMV infection, as determined by higher peak viral titers (up to 2 log units) at day 3 or 5 compared to the controls. This was most clearly seen for infection with 10^2 PFU of strain Docile or WE (Fig. 1B and C). As a consequence of this, IFN-α/βR$^{-/-}$ mice failed to clear infection with LCMV-Docile and these mice became virus carriers, retaining high levels of virus in several organs (>100 days). Infection with LCMV-WE resulted in time-limited persistence of virus infection, and virus levels declined over time to below the detection limit by day 80. Similarly, the absence of IFN-γ responsiveness also increased sensitivity to LCMV infection, such that IFN-γR$^{-/-}$ mice failed to control infection with 10^6 PFU of LCMV-Docile (Fig. 1). However, peak viral titers were not significantly different in IFN-γR$^{-/-}$ mice compared to the control (129/SvEv) population, which is consistent with our earlier observation of LCMV-Docile resistance to IFN-γ effects. In agreement with earlier reports (48), infection of IFN-γR$^{-/-}$ mice with 10^2 PFU of LCMV-Docile or -WE induced a severe fatal wasting disease, and the mice succumbed to infection by day 9 to 14 (Fig. 1). Inactivation of both IFN-α/β and IFN-γ responses drastically increased susceptibility to virus infection, resulting in persistence of the virus at high levels in IFN-α/β-γR$^{-/-}$ mice infected with either LCMV-WE or -Docile (Fig. 1). Thus, major contributions to virus replication and hence the course and outcome of LCMV infection are determined by both IFN-α/β- and IFN-γ-mediated antiviral mechanisms. Hence, susceptibility of LCMV strains to the antiviral effects of IFNs, IFN-α/β in particular, is a critical factor for the restriction of virus spread in vivo and thus protection from virus persistence.

Contribution of IFN-α/β and IFN-γ to control of acute infection with the CL 13 Armstrong or Armstrong strain of LCMV. Next, to assess whether the significant contribution of the IFN response towards viral clearance noted for Docile and WE infection can be generalized to other LCMV strains, kinetic studies of virus clearance were carried out with LCMV strain Armstrong and its variant CL 13 Armstrong. As expected, virus clearance proceeded efficiently in 129/SvEv mice infected with LCMV-Armstrong. Thus, studies of infection with 10^2 PFU (not shown) or 10^5 PFU of Armstrong demonstrated an acute infection with viral burdens below the limit of detection by day 10 or 15 after infection, respectively (Fig. 2 and data not shown). Consistent with the experiments shown in Fig. 1, 10^2 PFU of CL 13 Armstrong was efficiently eliminated from the spleen by day 15; however, a relatively high dose of 10^6 PFU persisted for several weeks. Interestingly, virus burdens declined over time and were below limits of detection by day 70 after infection.

The absence of IFN-α/β responsiveness significantly increased sensitivity to infection with Armstrong (10^5 PFU) or CL 13 Armstrong (10^2 or 10^6 PFU). Relative to 129/SvEv control mice, IFN-α/βR$^{-/-}$ mice showed accelerated kinetics of virus replication and higher peak viral titers, conditions which favor protraction of infection for an extended period varying between 2 and 8 weeks (Fig. 2). In the absence of IFN-γ responsiveness, long-term persistence of virus infection was observed only in mice infected with 10^6 PFU of CL 13 Armstrong (except for one mouse of seven that cleared the infection at day 90). Note that these mice were not free of disease signs, and we have, albeit infrequently, observed mortality in our experiments. In contrast, 10^2 PFU of CL 13 Armstrong was cleared efficiently, and no sign of disease development was observed. Likewise, clearance of infection with 10^2 PFU of Armstrong was achieved within 7 to 10 days (data not shown), while mice infected with 10^5 PFU developed a lethal wasting disease and died by days 9 to 14 after infection (Fig. 2). Finally, elimination of both IFN-α/β and IFN-γ pathways renders mice susceptible to infection with either virus strain, and permanent virus persistence was observed irrespective of the dose of infection (Fig. 2). Taken together with the kinetic studies of virus clearance, this shows that the absence of either IFN-α/β or IFN-γ pathways increases sensitivity to LCMV infection, as observed by a delay in or abolition of virus clearance compared to that in animals with intact IFN responsiveness. The development of wasting syndrome and the subsequent death of mice in the absence of IFN-γ responsiveness are dependent on the conditions of infection. Thus, under conditions where either an initial high viral load caused persistent infection or initial low viral burdens were cleared rapidly by the host immune system, mice developed few symptoms of disease and recovered from infection. In contrast, where an imbalance between virus replication and the host immune system occurred, animals succumbed to a fatal wasting disease, which has previously been recognized to be mediated by virus-specific T cells (28, 48).

IFN-α/β- and IFN-γ-mediated regulation of the virus-specific CD8$^+$-CTL response during infection of mice with the WE or Docile strain of LCMV. As host CD8$^+$ T cells are critical for efficient clearance of LCMV infection and their inactivation (via deletion and/or anergy) during the acute phase of infection results in persistence of infection, the development of the virus-specific CD8$^+$-T-cell response was examined in IFN-α/βR$^{-/-}$, IFN-γR$^{-/-}$, IFN-α/β-γR$^{-/-}$, or 129/SvEv control mice infected with LCMV. The specificity and function of the CD8$^+$-T-cell response were examined by direct visualization with binding of D^b MHC class I tetramer molecules complexed to LCMV peptides immunodominant for CD8$^+$-T-cell responses ($GP1_{33–41}$, $GP2_{276–286}$, or $NP_{396–404}$) and by stimulation of expression of IFN-γ with viral peptides. The results revealed a distinct pattern of virus-specific CD8$^+$-T-cell responses associated with an acute or chronic (protracted or

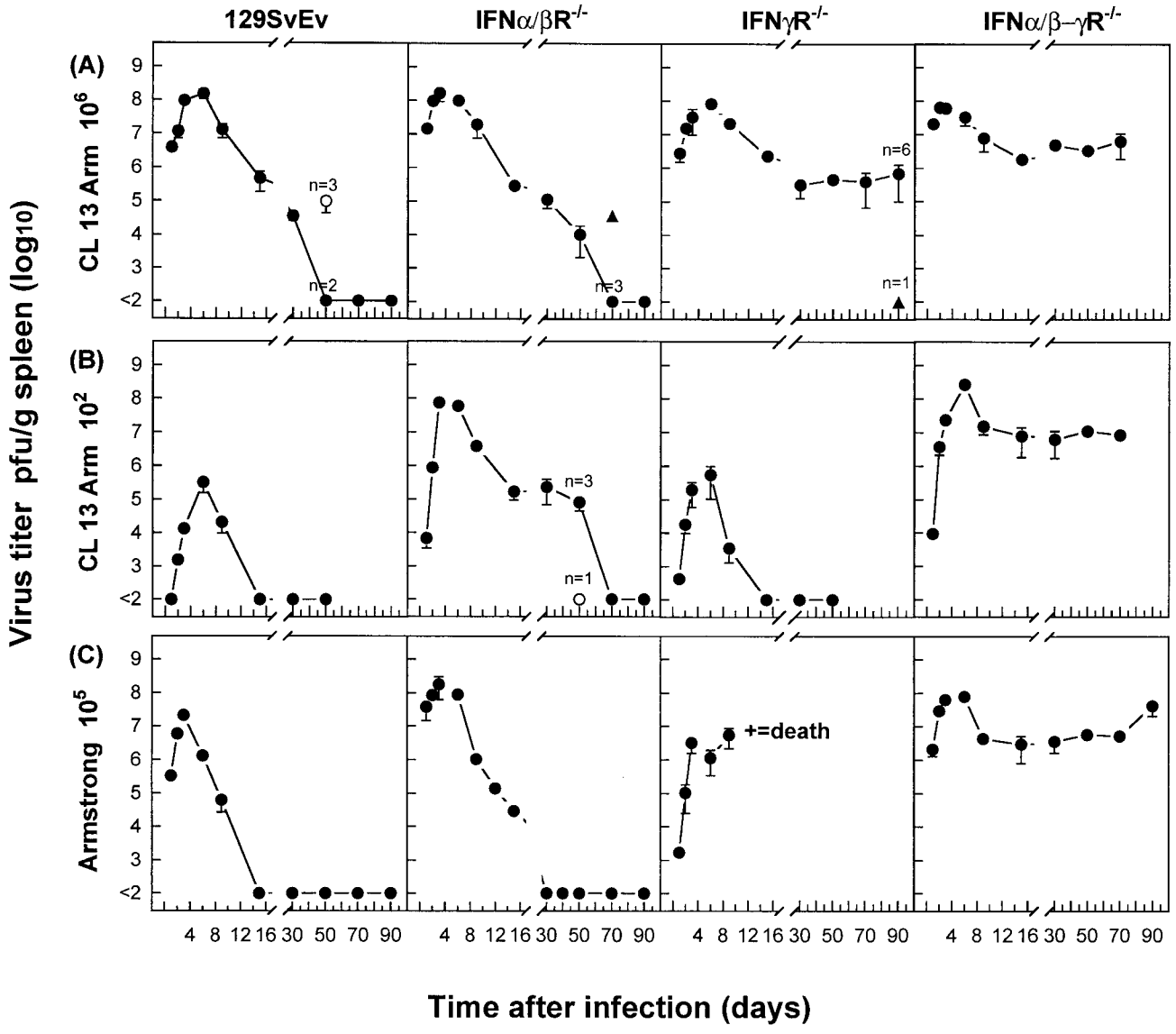

FIG. 2. Kinetics of LCMV CL 13 Armstrong and Armstrong replication in the spleens of mice deficient in type I, type II, or both type I and II IFN receptors compared to their 129/SvEv congenic controls. Virus titers in spleens were measured at the time points indicated following intravenous infection of IFN-$\alpha/\beta R^{-/-}$, IFN-$\gamma R^{-/-}$, IFN-α/β-$\gamma R^{-/-}$, or 129/SvEv mice either with 10^2 PFU (A) or 10^6 PFU (B) of CL 13 Armstrong or with 10^5 PFU of Armstrong (C). Data are means and standard errors of the means for three to five mice. The different symbols used in the columns represent values obtained from individual mice.

permanent) course of infection based on the experimental findings in Fig. 1 and 2.

Kinetic studies of virus-specific $CD8^+$ T cells in the spleens of 129/SvEv mice infected with LCMV-Docile (10^6 PFU) initially showed a significant increase in D^b-$GP1_{33–41}$- and D^b-$NP_{396–404}$-binding $CD8^+$ T cells (Fig. 3A and B). Interestingly, the numbers of $NP_{396–404}$ peptide-specific $CD8^+$ T cells declined over time and were below detectable levels by day 30. In striking contrast, $GP1_{33–41}$-specific $CD8^+$ T cells persisted at high levels over a 3-month observation period (>100 days). Both tetramer-binding T-cell populations in these mice exhibited antiviral functions initially (day 6), producing IFN-γ after stimulation with the appropriate peptide, but rapidly became unresponsive by day 9 after infection. However, even when the infection had been controlled (around day 50), $GP1_{33–41}$-specific T cells did not rapidly regain their capacity to elicit antiviral functions. Infection of 129/SvEv with 10^2 PFU of LCMV-Docile resulted in a vigorous expansion of $GP1_{33–41}$ and $NP_{396–404}$ $CD8^+$ T cells that cleared the infection within 2 weeks and persisted at high frequencies, retaining a functional phenotype (Fig. 3C and D). Likewise, studies on mice infected with LCMV-WE (10^2 PFU), including the profile of the $GP2_{276–286}$ epitope-specific $CD8^+$-T-cell response, yielded comparable results (Fig. 3E to G).

Infection of IFN-$\alpha/\beta R^{-/-}$ mice with LCMV-Docile (10^2 or 10^6 PFU), causing permanent persistence of infection, revealed another interesting phenotype (Fig. 3 A to D). In these mice $GP1_{33–41}$-specific $CD8^+$ T cells became completely unre-

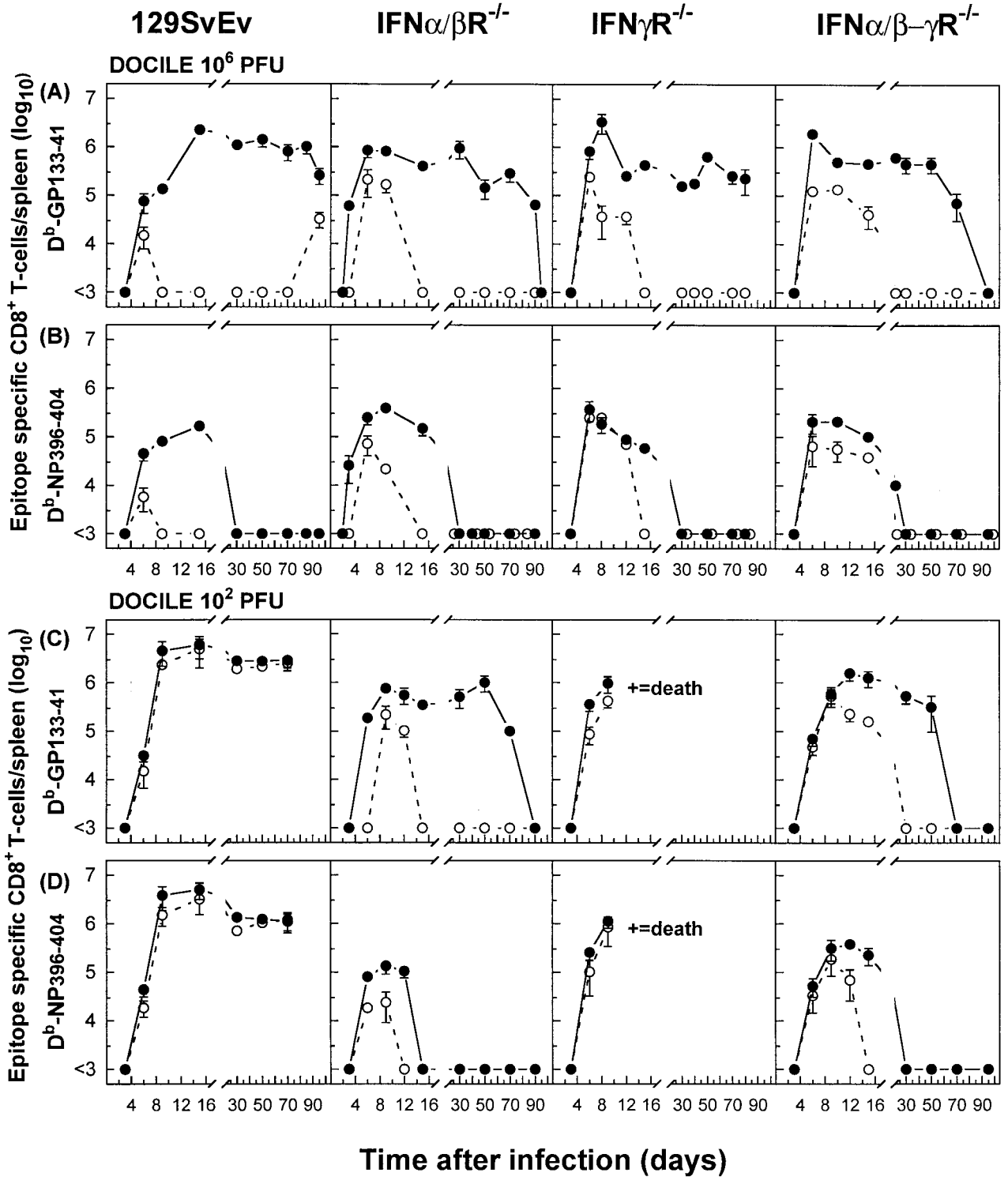

FIG. 3. Kinetics of the virus-specific $CD8^+$-T-cell response in the spleens of mice deficient in type I, type II, or both type I and II IFN receptors compared to their 129/SvEv congenic controls following infection with the Docile or WE LCMV strain. IFN-$\alpha/\beta R^{-/-}$, IFN-$\gamma R^{-/-}$, IFN-α/β-$\gamma R^{-/-}$, or 129/SvEv mice were infected with 10^6 PFU (A and B) or 10^2 PFU (C and D) of Docile or with 10^2 PFU of WE (E to G). Total numbers of $GP1_{33-41}$, $GP2_{276-286}$, or $NP_{396-404}$ peptide-specific $CD8^+$ T cells were measured by staining with H-$2D^b$ tetramers (filled circles) or intracellular IFN-γ production (open circles) following stimulation of cells with viral epitope peptide to determine the functional responsiveness of these cells. Data are means and standard errors of the means for three to five mice. The limit of detection was 10^3 antigen-specific cells per spleen. These analyses were performed to correlate the kinetics of virus replication (Fig. 1) with the kinetics of virus-specific $CD8^+$-T-cell responses (panels A, B, and C in Fig. 1 correspond to panels A and B, C and D, and E to G here, respectively).

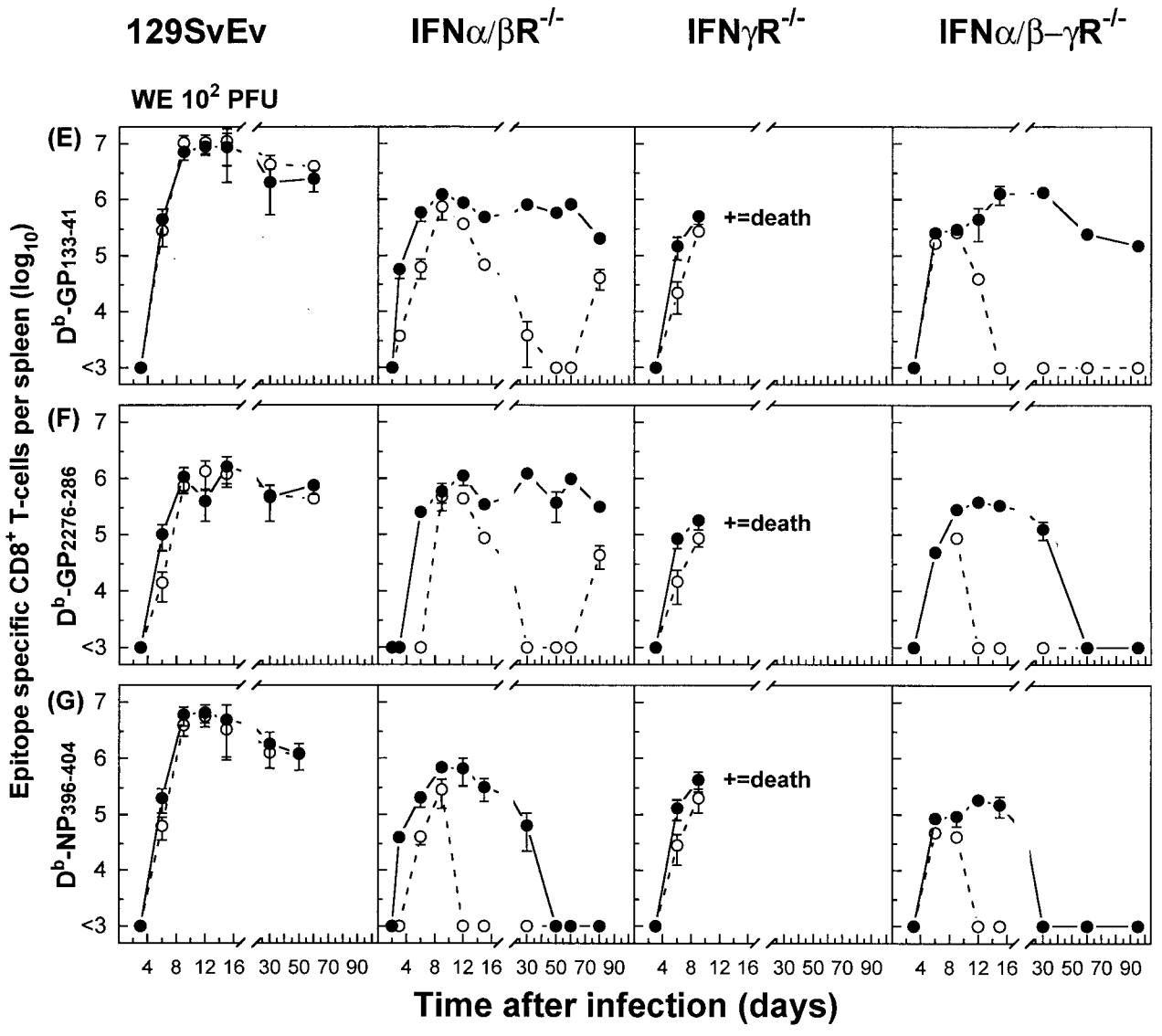

FIG. 3—*Continued.*

sponsive (based on IFN-γ production) by day 15 after infection and persisted at high levels, retaining the nonfunctional phenotype over a 3-month period; however, their levels declined to below detectable levels between days 80 and 100 after infection. $NP_{396–404}$-specific $CD8^+$ T cells were eliminated by day 40 after infection. Extended studies with IFN-$\alpha/\beta R^{-/-}$ mice with a protracted course of LCMV-WE infection (10^2 PFU) revealed a phenotype different from that seen in 129/SvEv mice (Fig. 3E to G). Thus, $GP1_{33–41}$- or $GP2_{276–286}$-specific $CD8^+$ T cells are functional during the initial phase of infection and subsequently persist at high levels. However, the fraction of epitope-specific $CD8^+$ T cells showing a functional deficit increased progressively until no functional T cells were detectable between days 50 and 60. However, this unresponsiveness was transient, with IFN-γ production on restimulation resuming at around day 70. In contrast, $NP_{396–404}$-specific $CD8^+$ T cells were functionally unresponsive by day 12 and were subsequently physically deleted by day 50 after infection.

Studies with IFN-$\gamma R^{-/-}$ mice infected with LCMV-Docile (10^6 PFU) revealed that $GP1_{33–41}$-specific $CD8^+$ T cells persist for at least 80 days, having lost antiviral function at around day 30 after infection, whereas $NP_{396–404}$-specific cells were deleted by day 30 (Fig. 3A and B). IFN-$\gamma R^{-/-}$ mice infected with a low dose (10^2 PFU) of LCMV-Docile (Fig. 3C and D) or -WE (Fig. 3E to G) died within 2 weeks after infection but developed a rigorous virus-specific $CD8^+$-T-cell response, characterized by a rapid and extensive burst of tetramer-binding cells that were fully functional as assessed by IFN-γ production. Previous studies have shown that the lack of IFN-γ receptor does not impair the ability of $CD8^+$ T cells to produce IFN-γ on peptide stimulation (5, 54). These findings are compatible with and provide further experimental evidence for the

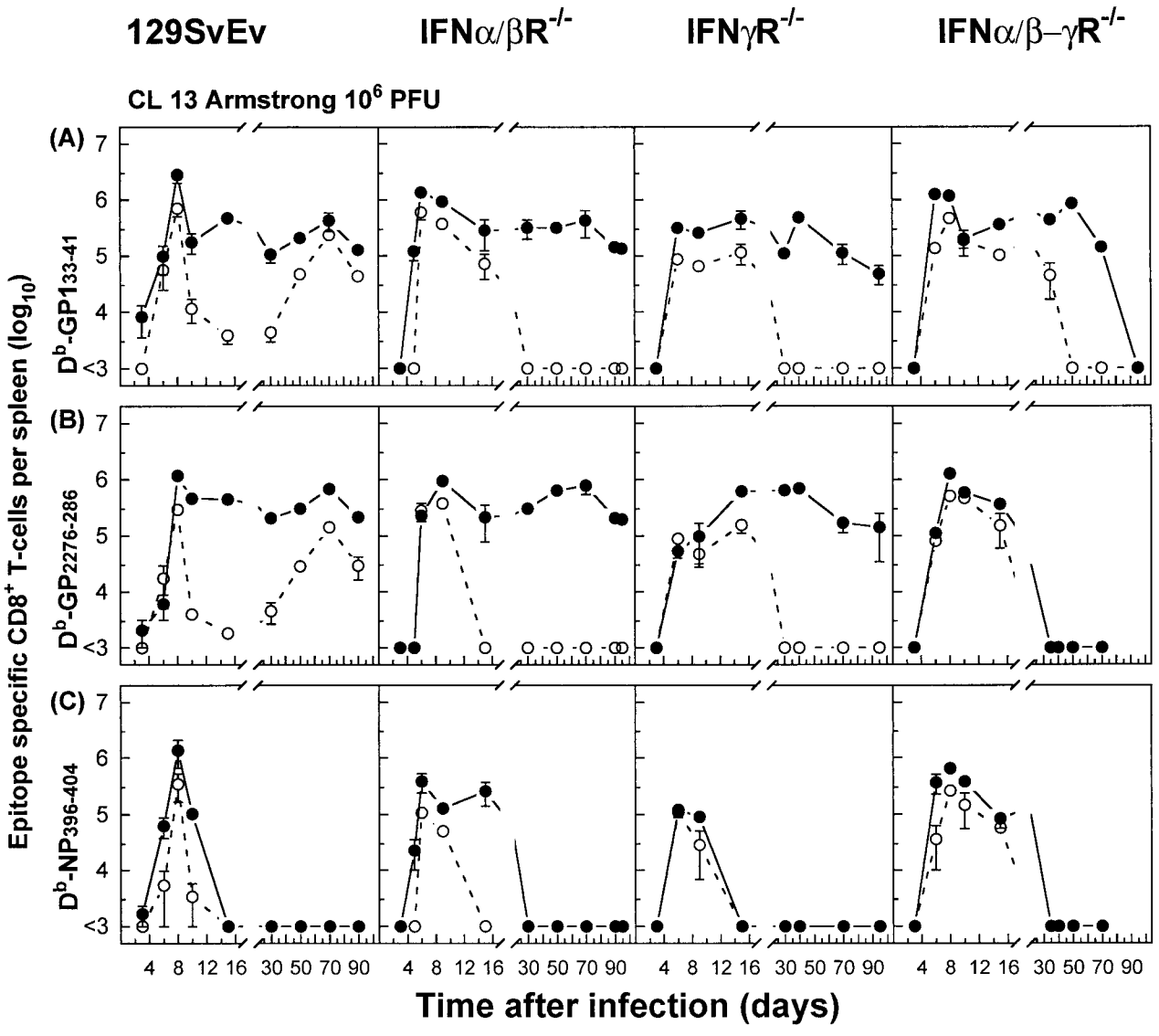

FIG. 4. Kinetics of the virus-specific $CD8^+$-T-cell response in the spleens of mice deficient in type I, type II, or both type I and II IFN receptors compared to their 129/SvEv congenic controls following infection with the CL 13 Armstrong or Armstrong LCMV strain. IFN-$\alpha/\beta R^{-/-}$, IFN-$\gamma R^{-/-}$, IFN-α/β-$\gamma R^{-/-}$, or 129/SvEv mice were infected with 10^6 PFU (A to C) or 10^2 PFU (D to F) of CL 13 Armstrong or with 10^5 PFU of Armstrong (G to I). Total numbers of $GP1_{33-41}$, $GP2_{276-286}$, or $NP_{396-404}$ peptide-specific $CD8^+$ T cells were measured by staining with H-$2D^b$ tetramers (filled circles) or intracellular IFN-γ production (open circles) following stimulation of cells with viral epitope peptide to determine the functional responsiveness of these cells. Data are means and standard errors of the means for three to five mice. The limit of detection was 10^3 antigen-specific cells per spleen. These analyses were performed to correlate the kinetics of virus replication (Fig. 2) with the kinetics of virus-specific $CD8^+$-T-cell responses (panels A, B, and C in Fig. 2 correspond to panels A to C, D to F, and G to I here, respectively).

hypothesis that an imbalance between virus spread and the host immune response results in the immune-mediated pathology that is instrumental in death of the animal. Finally, the virus-specific $CD8^+$-T-cell response in IFN-α/β-$\gamma R^{-/-}$ mice infected with LCMV-Docile (10^6 or 10^2 PFU) was comparable that seen in IFN-$\alpha/\beta R^{-/-}$ mice under similar conditions of infection, although functional unresponsiveness proceeded over a more extended period in these mice. Similarly, infection of IFN-α/β-$\gamma R^{-/-}$ mice with LCMV-WE (10^2 PFU) resulted in a drastic expansion of $GP1_{33-41}$-specific $CD8^+$ T cells, which persisted as long as day 100 after infection, having lost the capacity to elicit antiviral function around day 15, but $NP_{396-404}$- or $GP2_{276-286}$-specific cells were deleted by day 30 or 50, respectively (Fig. 3E to G). Combined, the tetramer binding and IFN-γ staining results indicate that acute infection is characterized by expansion and maintenance of functional T cells, whereas persistent infection is characterized by expansion and loss of T-cell function with eventual physical deletion.

IFN-α/β- and IFN-γ-mediated regulation of virus-specific $CD8^+$ T cells during infection of mice with the Armstrong or CL 13 Armstrong strain of LCMV. To further correlate the link between susceptibility of the host to chronic infection and the extent of virus-specific $CD8^+$-T-cell exhaustion, the kinetics of the LCMV-specific T-cell response were observed in

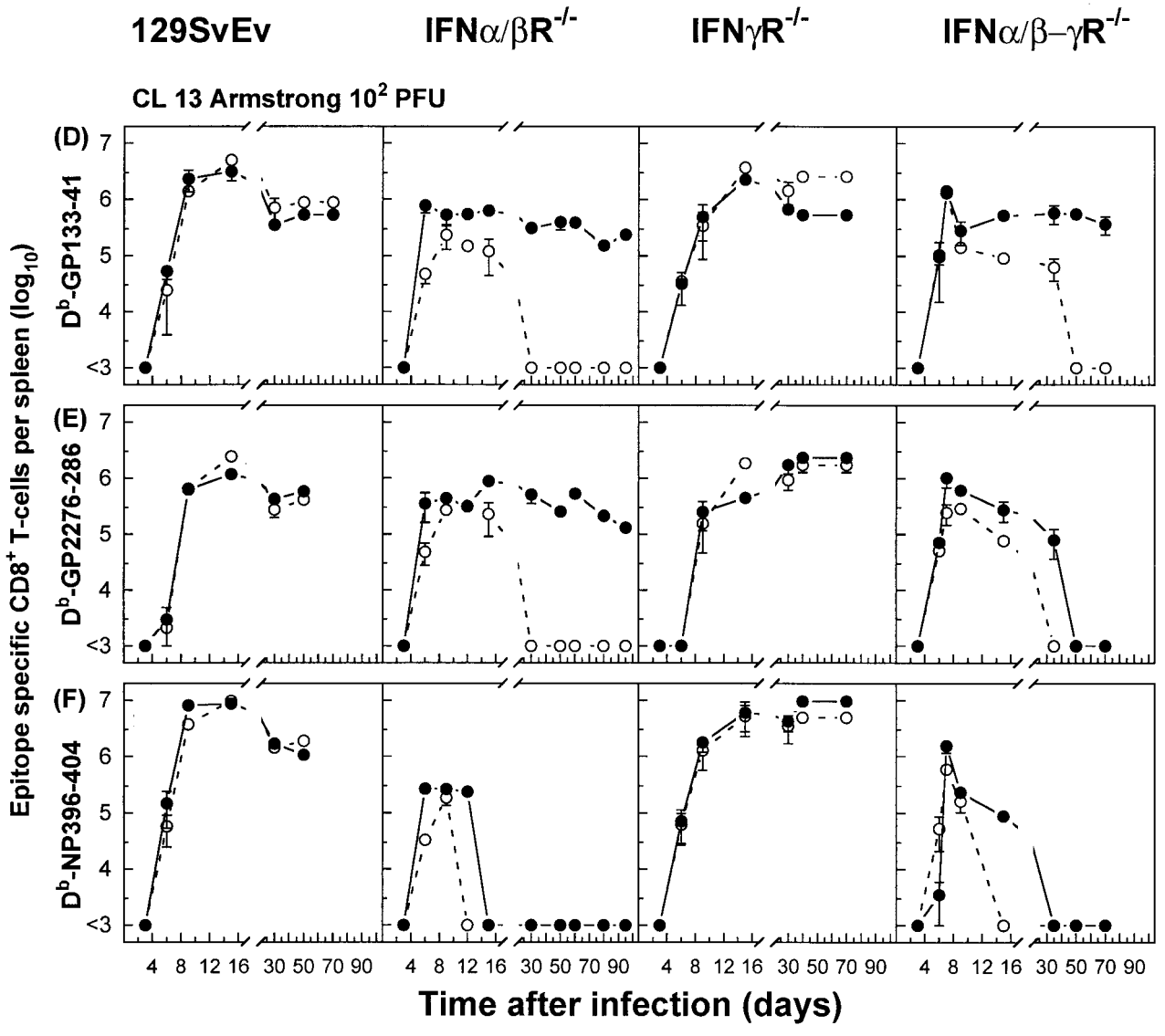

FIG. 4—*Continued.*

Armstrong- and CL 13 Armstrong-infected mice deficient in IFN pathways.

As evident from Fig. 4A to C, data obtained from 129/SvEv mice infected with CL 13 Armstrong (10^6 PFU) revealed an interesting new pattern for virus-specific $CD8^+$-T-cell regulation. Thus, $GP1_{33–41}$ or $GP2_{276–286}$ peptide-specific $CD8^+$ T cells were elicited efficiently during the acute phase of infection and persisted at high levels in the host. These cells exhibited a fully functional phenotype in the initial phase of infection and progressively lost function (around 99%) by day 15, but later (by day 70), after virus clearance was completed, they regained a fully functional phenotype. $NP_{396–404}$ $CD8^+$ T cells were rapidly deleted within the first 2 weeks after infection This finding demonstrated that a threshold of functional virus-specific $CD8^+$-T-cell activity must be maintained for resolution of a chronic infection. During the course of acute infection of 129/SvEv mice with a relatively low dose of either CL 13 Armstrong (10^2 PFU) or Armstrong (10^5 PFU), virus-specific $CD8^+$ T cells directed against the major epitopes expanded to high frequencies and exhibited antiviral effector functions (Fig. 4D to I).

Next, we evaluated the fate of virus-specific $CD8^+$ T cells in IFN-$\alpha/\beta R^{-/-}$ mice. Similar patterns of T-cell regulation were obtained during protracted infection with CL 13 Armstrong (10^6 or 10^2 PFU). Thus, $GP1_{33–41}$ or $GP2_{276–286}$ virus-specific $CD8^+$ T cells persisted in a nonresponsive state for as long as 100 days (beyond the time of virus clearance by around day 70). Deletion of $NP_{396–404}$ $CD8^+$ T cells proceeded rapidly within 15 to 30 days after infection (Fig. 4A to F). Infection of IFN-$\alpha/\beta R^{-/-}$ mice with Armstrong (10^5 PFU) elicited a vigorous response, with high levels of $GP1_{33–41}$- and $GP2_{276–286}$-specific $CD8^+$ T cells (Fig. 4G to I). In these mice a fraction (around 10%) of these cells remained functional, and the LCMV infection was resolved by day 30, approximately 2

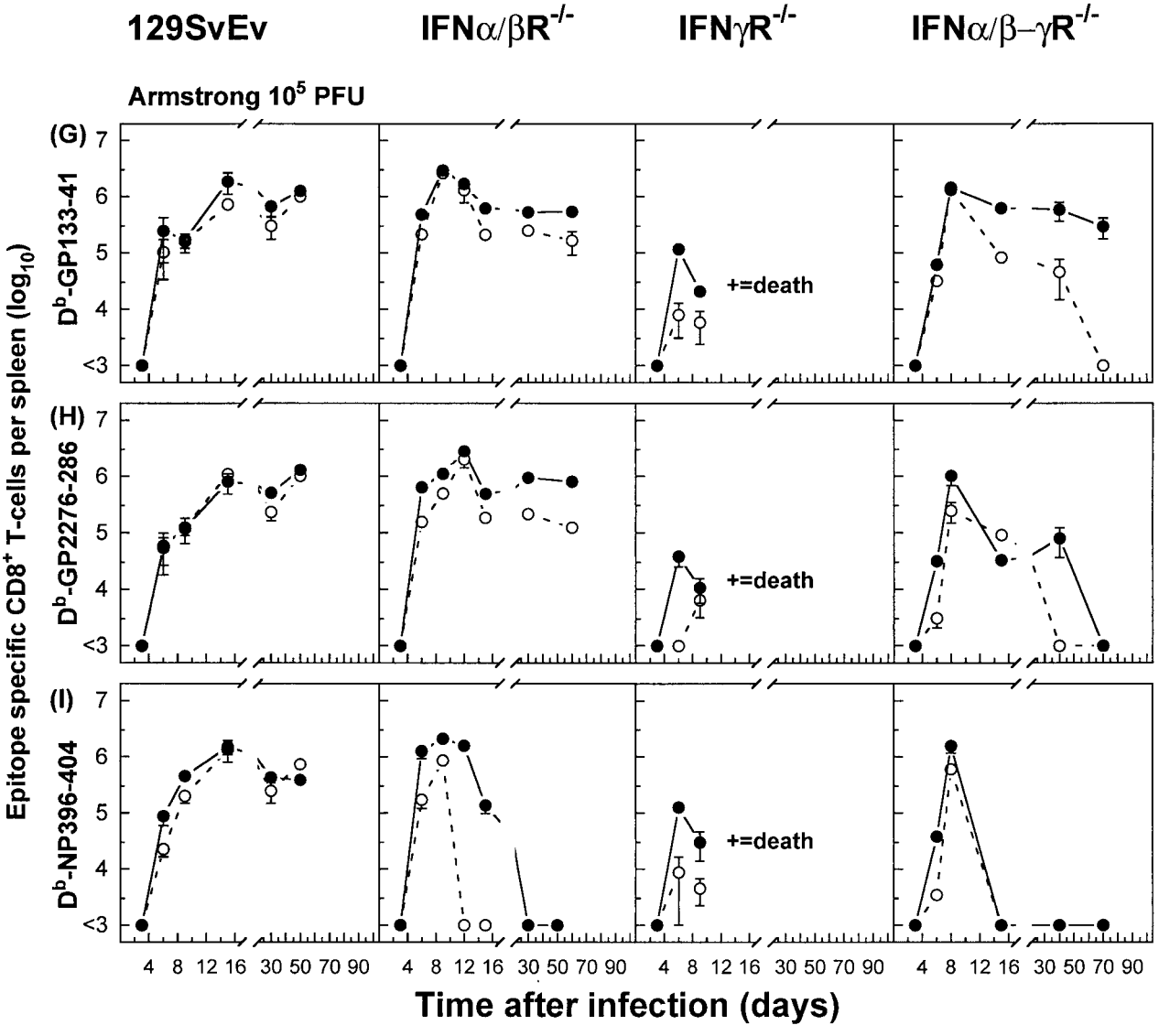

FIG. 4—*Continued.*

weeks delayed compared to 129/SvEv controls. Interestingly, $NP_{396–406}$- specific $CD8^+$ T cells fell below detectable levels by day 30, having become unresponsive by day 12 after infection.

Consistent with the data obtained from IFN-$\alpha/\beta R^{-/-}$ mice, IFN-$\gamma R^{-/-}$ mice infected with 10^6 PFU of CL 13 Armstrong retained functionally inactive $GP1_{33–41}$- and $GP2_{276–286}$-specific $CD8^+$ T cells at high levels but rapidly deleted $NP_{396–404}$-specific cells (Fig. 4A to C). In contrast, IFN-$\gamma R^{-/-}$ mice infected with 10^2 PFU of CL 13 Armstrong, which is cleared by day 15, developed high levels of tetramer-positive $CD8^+$ T cells which retained functionality and persisted for at least 90 days (Fig. 4D to F). Finally, IFN-$\gamma R^{-/-}$ mice injected with 10^5 PFU of Armstrong, which causes a lethal wasting disease in these mice, developed a T-cell response against the $GP1_{33–41}$, $GP2_{276–286}$, and $NP_{396–404}$ epitopes prior to death (Fig. 4G to I). However, while the percentages of D^b-peptide tetramer-positive $CD8^+$ T cells remained relatively high (up to 20%), the overall T-cell responsiveness of these mice was lower than that in the 129/SvEv controls due to a profound loss of splenocytes associated with the wasting syndrome.

Finally, analyses of the immune response in IFN-α/β-$\gamma R^{-/-}$ mice infected with CL 13 Armstrong (10^6 PFU) revealed that activated $CD8^+$ T cells were generated against all three epitopes examined in almost equal numbers (Fig. 4A to C). However, $GP1_{33–41}$-specific cells became progressively unresponsive by day 50 to 70 (some 20 to 40 days later than in IFN-$\alpha/\beta R^{-/-}$ mice) and were eventually deleted by day 90. Similarly, $GP2_{276–286}$- or $NP_{396–404}$-specific cells were deleted after a brief initial period of expansion. This pattern was repeated in IFN-α/β-$\gamma R^{-/-}$ mice infected with 10^2 PFU of CL 13 Armstrong or 10^5 PFU of Armstrong, with the exception that $GP1_{33–41}$-specific cells were not deleted once they had become nonresponsive (Fig. 4D to I).

Taken together, these results provide additional evidence for the inverse correlation between antigenic load and responsiveness of virus-specific $CD8^+$ T cells. The degree of T-cell

TABLE 1. Virus-specific cytotoxic activity in the spleens of mice deficient in type I, type II, or both type I and II IFN receptors, or in spleens 129/SvEv of control mice, following infection with 10^6 PFU of LCMV-Docile[a]

Day of infection	Dilution	% CTL activity specific to the indicated viral epitope							
		129/SvEv mice		IFN-α/βR$^{-/-}$ mice		IFN-γR$^{-/-}$ mice		IFN-α/β-γR$^{-/-}$ mice	
		GP1	NP	GP1	NP	GP1	NP	GP1	NP
6	1	71	54	81	61	55	47	82	43
	3	52	35	66	41	32	32	64	22
	9	28	16	39	19	14	12	33	8
9	1	31	17	69	98	28	7	46	15
	3	15	6	42	53	27	7	35	8
	9	7	2	22	3	21	6	10	4
15	1	2	5	12	15	1	1	27	13
	3	2	3	6	7	1	2	14	2
	9	2	2	2	3	2	1	6	2
30	1	2	3	4	3	5	4	4	5
	3	2	2	2	2	2	2	3	2
	9	2	2	2	2	1	1	2	2

[a] Splenocytes isolated on days 6, 9, 15, and 30 after infection 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, using MC57G cells pulsed with 10 μg of $GP1_{33–41}$ $NP_{396–404}$ peptide per ml. Restimulated splenocytes were resuspended in 1 ml of medium per culture well, and serial threefold dilutions of effector cells were performed. Lysis of untreated target cells was ≤5% at the highest effector cell/target cell ratio. Results are representative of those from three separate experiments.

activation, which probably determines the ratio of functional to nonfunctional antigen-specific T cells in the transition phase between acute and persistent infection, dictates whether the infection is resolved or not. In addition, the results are consistent with the concept that the relative contribution of mechanisms operating to silence antigen-specific T-cell responses (anergy or deletion) can vary depending upon the epitope specificity of cells. Thus, $NP_{396–406}$ tetramer-binding $CD8^+$ T cells are more sensitive to deletion than $GP2_{276–286}$-specific cells, whereas $GP1_{33–41}$-specific T cells are relatively resistant and are deleted only under conditions of long-term virus persistence.

$CD8^+$-T-cell effector functions are lost in mice with a protracted persistent infection. The results described above revealed a close association between virus persistence and exhaustion (anergy and/or deletion) of the virus-specific $CD8^+$-T-cell repertoire. However, under certain conditions of infection (e.g., 129/SvEv mice infected with 10^6 PFU of LCMV-Docile), permanent persistence of infection was not observed and the virus was eventually cleared from the host. This occurred despite the absence of functional $CD8^+$ T cells specific to dominant epitopes, as determined by intracellular IFN-γ staining of restimulated MHC-tetramer-binding cells. It is possible in this case that resolution of infection may be mediated by an altered physiological pattern of T-cell response, such as synthesis of alternate cytokines, which would not be detected in these experiments. In addition, the tetramer-MHC-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). Thus, it is possible that functional $CD8^+$ T cells specific to dominant or even subdominant epitopes, which escape T-cell exhaustion at the onset of infection and persist at low levels, are capable of subsequently mounting an effective antiviral response and go on to clear the viral infection.

To address these possibilities, intracellular staining was performed on splenocytes from 129/SvEv mice infected with LCMV-Docile (10^6 PFU). No IFN-γ-secreting $CD8^+$ T cells specific to subdominant epitopes ($GP1_{92–101}$ and $NP_{205–212}$) were detectable, and as previously reported (71), the cells did not exhibit altered patterns of cytokine production. Thus, tumor necrosis factor alpha, IL-2, IL-4, or IL-10 was not detectable by intracellular cytokine staining after peptide stimulation in vitro (data not shown). Next, as an independent test of functional activity, we measured the ability of splenocytes from these mice to develop cytotoxic activity towards the virus or individual peptide epitopes following stimulation in vitro. This analysis was extended to include IFN-α/βR$^{-/-}$, IFN-γR$^{-/-}$, or IFN-α/β-γR$^{-/-}$ mice and infection with CL 13 Armstrong (10^6 PFU). $CD8^+$-T-cell responses to virus or to dominant ($GP1_{33–41}$, $GP2_{276–286}$, $NP_{396–406}$, or $GP1_{34–43}$ [Table 1 and data not shown]) or subdominant ($GP1_{92–101}$ or $NP_{205–212}$ [data not shown]) epitopes were detectable in mice with protracted infection during the initial phase but not in the later phase. Consistent with the IFN-γ staining data, splenocytes from 129/SvEv mice infected with CL 13 Armstrong (10^6 PFU) initially exhibited substantial cytotoxic activity, almost lost their cytotoxic activity by day 15, but later (by day 30) regained a fully functional phenotype (data not shown). To further address the functional behavior of virus-specific T cells, parallel studies were conducted on mice infected with 10^2 PFU of Docile or CL 13 Armstrong. As evident from Table 2, the magnitude of CTL activity obtained following in vitro restimulation with virus-infected macrophages as antigen-presenting cells correlates with the IFN-γ staining data. Thus, cytotoxic activity was detected in the initial phase of infection in both virus-mouse combinations but was later lost in mice with chronic infection, while cytotoxic activity was sustained at high levels in animals that eliminate the virus in the acute phase. 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. This is true for all situations of chronic infection except 129/SvEv mice infected with

TABLE 2. Virus-specific cytotoxic activity in the spleens of mice deficient in type I, type II, or both type I and II IFN receptors, or in spleen of 129/SvEv control mice, following infection with 10^2 PFU of LCMV-Docile or CL 13 Armstrong[a]

Day of infection	Dilution	% CTL activity specific to the indicated LCMV strain							
		129/SvEv mice		IFN-α/βR$^{-/-}$ mice		IFN-γR$^{-/-}$ mice		IFN-α/β-γR$^{-/-}$ mice	
		Docile	CL 13	Docile	CL 13	Docile	CL 13	Docile	CL 13
6	1	43	43	49	58	47	52	18	31
	3	24	26	18	36	30	30	9	13
	9	8	10	2	12	14	12	3	7
9	1	70	70	14	28	70	65	44	55
	3	48	49	6	15	50	41	23	30
	9	28	22	2	6	29	16	10	11
20	1	75	63	3	4	ND[b]	70	3	2
	3	59	38	1	1	ND	41	1	1
	9	32	16	1	1	ND	16	1	0

[a] Splenocytes isolated on days 6, 9, and 20 after infection were stimulated in vitro with virus-infected macrophages 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, using virus-infected MC57G target cells. Restimulated splenocytes were resuspended in 1 ml of medium per culture well, and serial threefold dilutions of effector cells were performed. Lysis of untreated target cells was ≤5% at the highest effector cell/target cell ratio. Results are representative of those from three separate experiments.
[b] ND, not determined.

CL 13 Armstrong (10^6 PFU), where no complete extinction of antiviral function (cytotoxicity or IFN-γ production) was observed.

Contribution of CD4$^+$ T cells and neutralizing antibodies in clearance of a protracted infection in the absence of functional virus-specific CD8$^+$ T cells. CD8$^+$ T cells are critical for clearance of LCMV infection, and their depletion results in persistence of virus infection. However, under certain conditions, as described above, virus was eventually cleared from mice with protracted infection, although functional CD8$^+$ T cells were undetectable. Evidence for CD4$^+$ T cells and neutralizing antibody contributing to the resolution of LCMV infection has been obtained in several experimental settings and may provide an explanation for the eventual clearance of virus following a protracted infection. Note that CD4$^+$-T-cell help is essential for virus-specific antibody (IgM or IgG) production and for maintaining specific CD8$^+$-T-cell responses (41).

To test this hypothesis, neutralizing antibody titers were measured in the four strains of mice during chronic infection with Docile or CL 13 Armstrong (10^6 PFU). The data presented in Table 3 suggest a correlation between the neutralizing antibody titer and resolution of the infection. Thus, higher levels of antibody activity observed in 129/SvEv mice were associated with eventual clearance, by day 70 of the infection, with LCMV-Docile, while the lower neutralization titers in IFN-α/βR$^{-/-}$, IFN-γR$^{-/-}$, or IFN-α/β-γR$^{-/-}$ mice were insufficient for controlling chronic infection. Similar results were obtained for infection with 10^6 PFU of CL 13 Armstrong (data not shown). Next, the contribution of CD4$^+$ T cells and the antibody response in clearance of the chronic infection was tested by depletion of CD4$^+$ T cells from 129/SvEv mice infected with 10^6 PFU of LCMV-Docile. As evident from Fig. 5, virus was not cleared when mice were treated with antibody against CD4 at the time of infection. This protracted infection was associated with poor antiviral antibody response (>99% reduction of antibody levels detected by ELISA compared to untreated controls and no detectable neutralizing antibody activity) (data not shown). In contrast, depletion of CD4$^+$ T cells on day 20 after infection, when virus-specific CD8$^+$ T cells became unresponsive, did not inhibit antibody production, and sera contained antiviral antibody activity equal to or greater than that from controls as detected by ELISA or plaque neutralization assay. Virus titers declined with kinetics similar to those for non-antibody-treated controls, falling below the threshold of detection by day 50 and remaining undetectable for a period of around 2 months. However, at the end of this period (day 100), virus in the spleen reemerged and replicated to high titers. In addition, CD4$^+$-T-cell depletion at the time of infection did not significantly affect expansion of virus-specific CD8$^+$ T cells that exhibited a fully functional phenotype in the initial phase of infection, but these CD8$^+$ T cells progressively lost function and persisted at slightly (two- to fivefold) lower levels compared to controls for several weeks before rapidly declining to below detectable levels at between days 80 and 100

TABLE 3. Kinetics of the neutralizing antibody response in mice deficient in type I, type II, or both type I and II IFN receptors compared to their 129/SvEv congenic controls following infection with a high dose of LCMV-Docile[a]

Mouse strain	Neutralizing antibody titer ($\log_2$, 10)[b] at the following time after infection (days):				
	15	30	50	70	90
129/SvEv	1.3 ± 0.3	2.0 ± 0.0	3.5 ± 0.5	5.0 ± 1.0	5.0 ± 0.5
IFN-α/βR$^{-/-}$	1.5 ± 0.5	1.5 ± 1.5	2.8 ± 0.3	3.0 ± 1.0	2.2 ± 1.2
IFN-γR$^-$	1.0 ± 0.0	2.8 ± 0.3	1.5 ±t0.5	3.0 ± 0.0	3.0 ± 0.0
IFN-α/β-γR$^{-/-}$	1.5 ± 0.5	1.3 ± 0.3	2.3 ± 0.3	2.5 ± 1.5	2.2 ± 0.8

[a] IFN-α/βR$^{-/-}$, IFN-γR$^{-/-}$, IFN-α/β-γR$^{-/-}$, or 129/SvEv mice were infected with 10^6 PFU of LCMV-Docile. Sera were analyzed for neutralizing antibody activity as described in Materials and Methods.
[b] Data are means and standard errors of the means for three to five mice.

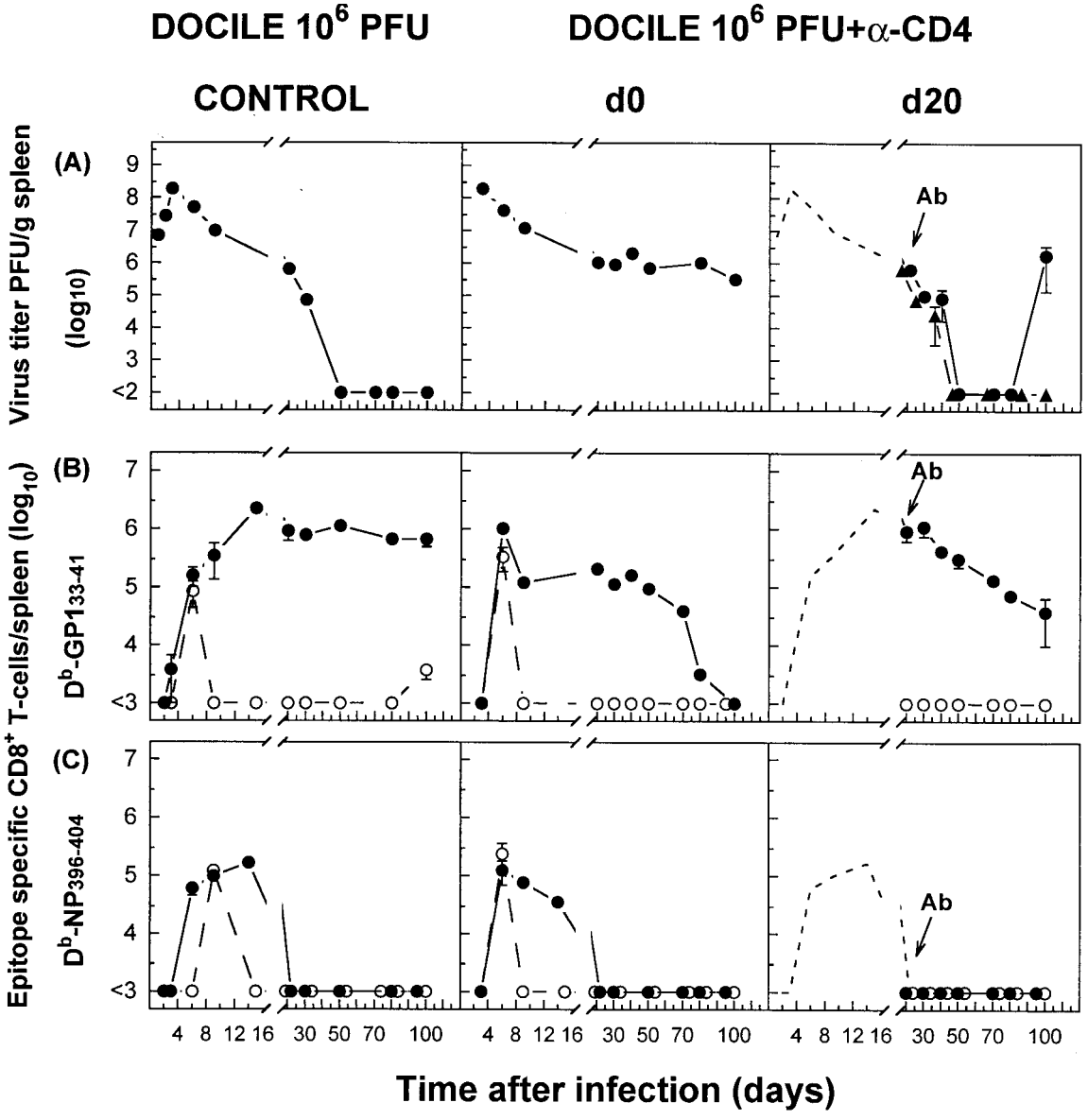

FIG. 5. A critical role for $CD4^+$ T cells in clearance of a protracted viral infection via regulation of virus-specific antibody response in the absence of functional virus-specific $CD8^+$ T cells. Kinetics of virus replication and antigen-specific $CD8^+$-T-cell responses in the spleens of 129/SvEv mice infected with 10^6 PFU of LCMV-Docile and depleted of $CD4^+$ or $CD8^+$ T cells are shown. 129/SvEv untreated infected mice were used as controls. (A) Virus titers in spleens were measured at the time points indicated following treatment with antibody (Ab) against CD4 (●) at the time of infection (day 0 [d0]) or on day 20 after infection (d20). In a separate group mice were treated with antibody against CD8 (▲). Data are means and standard errors of the means for three to five mice. (B and C) Total numbers of $GP1_{33–41}$ or $NP_{396–404}$ epitope-specific $CD8^+$ T cells in $CD4^+$-T-cell-depleted or control animals were measured by staining with H-$2D^b$-peptide tetramers (●) or intracellular IFN-γ production (○) following stimulation of cells with viral epitope peptide to determine the functional responsiveness of these cells. The kinetics of $GP1_{33–41}$ or $NP_{396–404}$ peptide-specific $CD8^+$ T cells in control mice are indicated as broken lines in the right-hand panels. Data are means and standard errors of the means for three to five mice.

after infection. Similarly, mice depleted of $CD4^+$ T cells on day 20 after infection exhibited only a moderate reduction (two- to fivefold) in levels of functionally inactive $GP1_{33–41}$-specific $CD8^+$ T cells that persisted for 2 to 3 weeks before their levels progressively declined. This occurred despite virus clearance by day 50 with kinetics comparable to those for infected untreated controls. Depletion of $CD8^+$ T cells on day 20 after infection had no effect on virus clearance, with virus falling to undetectable levels by day 50 associated with high levels of LCMV-specific antibody (data not shown). In conclusion, these data support an important role for $CD4^+$ T cells and antibody-producing B cells in resolution of long-term LCMV infection, especially in situations where $CD8^+$-T-cell activity is lost during the initial phase of infection.

DISCUSSION

In this work, we studied the down-regulation of the virus-specific $CD8^+$-T-cell response (clonal exhaustion) during per-

sistent infection of adult mice, with particular emphasis on examining the contribution of IFNs, which play a large part in containment of virus growth in the early course of infection. The data obtained support the hypothesis that virus load in the early phase of infection is a critical factor in the effectiveness of the T-cell response and that the production of IFNs can critically regulate the delicate balance between viral replication and host immune control in favor of viral dissemination, resulting in exhaustion of virus-specific $CD8^+$ T cells and persistent infection. The main conclusions of this study are that an inverse correlation exists between antigen persistence and responsiveness of virus-specific T cells and that distinct programs of activation or tolerance (functional unresponsiveness and/or physical elimination of antigen-specific cells) are operative during acute and chronic virus infections. (i) During the course of acute infection (which lasts only about 2 weeks), $CD8^+$ T cells expand and differentiate into effector cells, which mediate viral clearance. Fully functional, antigen-specific memory $CD8^+$ T cells are maintained throughout the life of the host at relatively high levels. (ii) During chronic infection with protracted persistence of virus, antigen-specific $CD8^+$ T cells initially exhibiting antiviral function lose this function and persist in this state for several weeks. However, after virus levels decline below threshold levels, functional $CD8^+$ T cells become detectable again. The origin of these cells is unknown, but they may be anergic virus-specific T cells, which have regained their function in the antigen-free environment, or newly selected virus-specific T cells emerging from the antigen-free thymus. (iii) During permanent persistence of infection, activated antigen-specific cells progressively lose their function and either persist indefinitely in this state or are deleted in the late stages of infection. A further distinction in the last two scenarios is that anergy followed by physical deletion of virus-specific $CD8^+$ T cells can operate to silence certain epitope-specific $CD8^+$ T cells irrespective of whether permanent or time-limited persistence of virus infection is observed. Thus, $NP_{396–406}$ tetramer-binding $CD8^+$ T cells are more sensitive to deletion than $GP2_{276–286}$-specific cells, whereas $GP1_{33–41}$-specific T cells are relatively resistant and are deleted only under conditions of long-term virus persistence. Finally, the data concur with and provide further experimental evidence for the concept that an appropriate balance between cellular and humoral components of the immune system to avoid host disease caused by immunopathology and prevent immune escape variations of the pathogen is a feature of a successful immune response with definitive viral clearance. Thus, failure of $CD8^+$ T cells to rapidly clear the virus due to their functional exhaustion may lead to a protracted viral persistence. In this situation, $CD4^+$ helper T cells and neutralizing antibody produced by B cells can become the principal arm of antiviral defense, and viral clearance can be achieved at a later stage of infection. Long-term persistence of infection can occur if the effectiveness of both arms of the immune response is lost or drastically reduced. Furthermore, an imbalance between viral spread and the host immune response can lead to host death, due to excessive activation of $CD8^+$ T cells resulting in lethal immune pathology. This has been observed in our experiments with IFN-$\gamma R^{-/-}$ mice under conditions of infection where rapid viral clearance or viral persistence cannot be readily achieved.

A vital question concerns the mechanisms and factors that determine the nature and kinetic patterns of functional inactivation and/or physical deletion of virus-specific $CD8^+$ T cells during persistent infection. Down-regulation of the virus-specific $CD8^+$-T-cell response is the first critical step for survival and persistence of the virus in the host, but $CD4^+$ helper T cells are also susceptible to anergy or physical deletion as reported previously (51, 65). According to these earlier reports, antigen-specific $CD4^+$ T cells persist for several weeks and retain a functional phenotype in C57BL/6 mice infected with a high dose of LCMV-Docile, which causes permanent viral persistence, but they progressively become unresponsive, and eventually a fraction are physically deleted. Furthermore, the process of exhaustion proceeds with much slower kinetics, in that anergic $CD4^+$ T cells become detectable at around day 70, compared to virus-specific $CD8^+$ T cells, which develop an anergic phenotype by day 15. As $CD4^+$ helper T cells are central regulators for the B-cell response, exhaustion of virus-specific $CD4^+$ T cells at this late time may affect the production of neutralizing antibody, which represents a critical component of the host immune system for control of persistent LCMV infection (6, 17, 53, 57). The concept that acute or persistent viral infection (protracted or permanent) is dictated by the degree and kinetics of down-regulation of the virus-specific immune response can explain the different outcomes of the viral infection in this study. As virus-specific $CD8^+$ T cells in mice with LCMV persistence become functionally inactive or deleted in the early stages of infection, the eventual clearance of infection seen in mice with protracted viral persistence may involve intervention of antigen-specific $CD4^+$ T cells and antibody production. Three roles for virus-specific $CD4^+$ T cells can be considered. Either (i) virus-specific $CD4^+$ T cells are critically involved in induction and differentiation of $CD8^+$ T cells, for example, by secretion of cytokines (such as IL-2) or increasing expression of costimulatory molecules (e.g., B7-1 and B7-2) on antigen-presenting cells (32), (ii) $CD4^+$ T cells are involved in the virus clearance process by regulating neutralizing antibody production, or (iii) they function by direct inhibition of virus replication via secretion of antiviral factors (e.g., IFN-γ or tumor necrosis factor alpha) (6, 25, 26, 53, 57). A major role for $CD4^+$ T cells in the induction and differentiation of $CD8^+$ T cells can be excluded, as our data indicate that depletion of $CD4^+$ T cells at the time of infection has no significant effect on the kinetics of proliferation or functional inactivation of virus-specific $CD8^+$ T cells during the acute phase of infection. However, it remains possible that $CD4^+$ T cells do have a function in the maintenance of $CD8^+$-T-cell activation and influence the longevity of the $CD8^+$-T-cell population once it has become anergic. Our data most favor a role for $CD4^+$ T cells in initiation of the virus-specific antibody response responsible for eventual clearance of LCMV infection, as elimination of $CD4^+$ T cells inhibits antiviral antibody production. The fact that $CD4^+$-T-cell depletion on day 20 after infection, when functional virus-specific $CD8^+$ T cells are not detectable, does not prevent clearance of virus infection and can no longer affect virus-specific antibody production provides further support for an antibody-mediated role in the clearance of a protracted viral persistence. Note that the induction and differentiation of virus-specific B cells are advanced on day 20 after infection and may no longer be dependent on $CD4^+$-T-cell help. Consistent with this, we have

reproducibly observed moderate levels of neutralizing antibody in IFN-deficient mice infected with a high dose of LCMV-Docile, while 129/SvEv control mice exhibited significantly higher antibody levels, having cleared the infection. However, we should keep in mind that the virus-specific antibody response is auxiliary in the virus clearance process and that inactivation of $CD8^+$ T cells is the crucial step for virus persistence. This view is consistent with studies by us and others (9, 16, 37, 66) showing that $CD4^+$ T cells are critical only in threshold situations where complete exhaustion of $CD8^+$ T cells normally does not occur and thus virus resolution proceeds with delayed kinetics. Finally, a vital question concerns the mechanisms allowing virus to reemerge in the spleens of infected mice that were depleted of $CD4^+$ T cells by day 20 after infection. One plausible explanation for this could be that neutralizing antibody escape variants are selected at late times in the course of infection as a result of immune pressure and are able to persist in the host. Analyses to test this hypothesis are under way.

It is well recognized that the interaction between T cells and antigen-presenting cells can result in T-cell activation, anergy, or deletion, depending on the level of T-cell receptor (TCR) engagement (30, 33, 62). Likewise, the levels of TCR engagement can trigger a spectrum of biological responses, such as cytotoxicity or cytokine secretion (31). In this situation, different factors, such as the activation state of T cells, the dose of antigen, and the presence or absence of costimulation, have been shown to play a role in determining the outcome of the T-cell response. Clearly, the complexity of the molecular interactions between virus-infected antigen-presenting cells and T cells makes it difficult to analyze the relative contributions of these factors to the final outcome. However, the following scenarios, based on the central concept that the duration of antigen stimulation determines the fate of T cells during persistent LCMV infection, may help to shed light on the phenomenon of T-cell exhaustion. The extent of T-cell activation can be influenced by the abundance of viral antigen presented and by the affinity of the TCR for the given MHC-peptide complex. LCMV-specific $CD8^+$ T cells recognize the dominant epitope peptide ($GP1_{33–41}$, $GP2_{276–286}$, or $NP_{396–404}$) presented by $H\text{-}2D^b$ molecules on virus-infected cells. One possible scenario is that the density of the peptide-MHC complexes on infected cells in persistently infected mice differs for individual epitopes. Consequently, $CD8^+$ T cells specific for individual peptides may not be similarly activated, and therefore the fate of these cells could be differentially regulated. Evidence against this is provided in a recent report, where estimation of peptide density on LCMV-infected MC57G cells revealed that $GP1_{33–41}$ was present at $\approx$1,000 copies/cell, $NP_{396–404}$ was present at 160 copies/cell, and $GP2_{276–286}$ was present at 90 copies/cell (22). Note that $NP_{396–406}$-specific $CD8^+$ T cells are more sensitive to deletion than $GP2_{276–286}$-specific cells, whereas $GP1_{33–41}$-specific T cells are relatively resistant to deletion in chronically infected mice. However, the fact that differential regulation of viral gene expression may occur in different cell types or that different amounts of the viral glycoprotein and nucleoprotein are produced in different stages of the viral replication cycle leaves this possibility open. Consistent with this, it has been found that nucleoprotein accumulated in large amounts in different cell types in persistently infected mice (50). Alternatively, the range of T-cell responses may result from differences in TCR affinity and dissociation rates of epitope-specific T cells. In this case, even if viral peptides are presented in similar densities on infected cells, the responding T cells may undergo different activation programs and consequently succumb to different fates (anergy versus physical deletion). It is possible that sustained signaling from high levels of antigen in the absence of appropriate costimulation could explain the functionally inactive phenotype of T cells. Alternatively, it has been suggested that clonal exhaustion is a consequence of a targeted infection of professional antigen-presenting cells (dendritic cells), rendering these critical accessory cells targets for destruction by an antiviral immune response (13, 55). The facts that induction of virus-specific T cells ($CD8^+$ and $CD4^+$) proceeds normally and that some of these cells persist for many weeks while others are deleted might argue more for a TCR-specific regulated process rather than a deficit in stimulation of T cells by accessory cells. However, infection of dendritic cells in chronically infected mice may disrupt the production of cytokines critical for survival and activation of virus-specific T cells. This is an attractive possibility that will be addressed in detail in ongoing studies. Finally, despite large viral loads at the onset of infection of IFN-$\alpha/\beta R^{-/-}$ mice with 10^5 PFU of Armstrong and the resultant delay in clearance of virus, $CD8^+$ T cells never entirely lost their antiviral function. This suggests that inactivation of IFN-mediated responses may differentially affect the pattern of infection, as determined by spread of the infection in different cell types and/or by the rate of virus replication per cell, depending on the virus isolate. Analyses to explore these possibilities are under way.

The concepts developed above to explain the different patterns of virus infection in this study rely solely on the function of IFNs in regulating virus-specific T-cell responses through modulation of viral replication and spread. However, IFNs have been proposed to regulate T-cell homeostasis (expansion, immunodominance, hierarchies, death phase, and memory phase of T cells) through pathways independent of their antiviral function (27). Our data are largely consistent with the view that the principal function of IFNs in LCMV infection is via direct control of virus spread; however, there are observations that may be better interpreted in the context of direct regulation of T-cell responses by IFNs. Thus, development of the wasting disease and subsequent death of IFN-$\gamma R^{-/-}$ mice infected with 10^2 PFU of LCMV-Docile in this study, or with LCMV-Traub in studies by others (48), suggest that IFN-γ deficiency can result in impaired silencing of virus-specific $CD8^+$-T-cell responses and thus exacerbation of immune-mediated pathology. More-detailed analyses are required to determine the mechanisms of IFN-γ-mediated regulation of antigen-specific T-cell homeostatic under different conditions of infection with LCMV.

Resolving the issue of why the immune system ultimately fails to control some viral infections requires a comprehensive analysis of several factors and mechanisms that regulate both replication and spread of the virus and cellular and humoral host immune responses. The studies presented here clearly document that viral infections do not simply either terminate by rapid viral clearance or persist following collapse of virus control but that there are several possible outcomes lying be-

tween these extreme situations, including the possibility of a balanced long-standing coexistence of virus and active T-cell immune surveillance as reported previously (7, 64).

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