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Protracted Course of Lymphocytic Choriomeningitis Virus WE Infection in Early Life: Induction but Limited Expansion of CD8$^+$ Effector T Cells and Absence of Memory CD8$^+$ T Cells$^\nabla$

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Viral infections in human infants frequently follow a protracted course, with higher viral loads and delayed viral clearance compared to viral infections in older children. To identify the mechanisms responsible for this protracted pattern of infection, we developed an infant infection murine model using the well-characterized lymphocytic choriomeningitis virus (LCMV) WE strain in 2-week-old BALB/c mice. In contrast to adult mice, in which viral clearance occurred as expected 8 days after infection, LCMV titers persisted for several weeks after infection of infant mice. LCMV-specific effector CD8$^+$ T cells were elicited in infant mice and fully functional on day 7 but rapidly waned and could not be recovered from day 12 onwards. We show here that this results from the failure of LCMV-specific CD8$^+$ T cells to expand and the absence of protective LCMV-specific memory CD8$^+$ T cells. Under these early life conditions, viral control and clearance are eventually achieved only through LCMV-specific B cells that contribute to protect infant mice from early death or chronic infection.

Early life is characterized by an increased vulnerability to infectious agents and particularly to viral pathogens. In fetuses or neonates, exposure to certain viruses (cytomegalovirus and hepatitis B virus) increases the risk of a chronic course of infection, as deficient host immune responses fail to achieve viral clearance. In infants, these noncytopathic viruses run protracted courses characterized by extended periods of viral excretion and/or higher viral titers than when they are acquired by older children (8, 23, 26, 48, 52, 55, 61). Cytopathic viruses that induce acute infections in immunologically mature hosts, such as influenza virus or respiratory syncytial virus, also follow a protracted course characterized by higher viral loads and several weeks of viral replication in infants (24, 49, 58). These infections, however, eventually resolve, reflecting delayed viral clearance rather than a persisting inability to eliminate the offending pathogen. The mechanisms that delay viral clearance in infants are not yet understood.

The inability to rapidly terminate an acute infection suggests that the immune response is not as effective in infants as in older children. Experimental studies have provided convincing evidence of the role of T-cell-mediated immunity and particularly CD8$^+$ T cells in the early control of primary viral infections. However, studies of infant T-cell responses to acute viral infections are scarce and mostly limited to CD4$^+$ T-cell responses (1, 34, 65). Viral exposure may induce CD8$^+$ T cells in utero or soon after birth, but their magnitude and quality appear to vary from one pathogen to another (reviewed in reference 37). CD8$^+$ T-cell responses are also reported after infant respiratory syncytial virus or influenza virus infection, although mostly at low levels (5 to 15% lysis) and/or in a minority of infants (12, 27, 28). Most importantly, infant T-cell responses have not been directly compared to those elicited in older antigen-naïve children or adults, such that the factors that support or limit the induction of CD8$^+$ T-cell responses capable of early viral clearance remain largely undefined (37).

During the last decade, neonatal animal models of infection and immunization have been developed to allow a better characterization of neonatal versus adult CD8$^+$ T-cell responses. They demonstrated that CD8$^+$ T-cell neonatal responses similar to those in adults may be readily elicited under certain experimental conditions (reviewed in references 2 and 62). This includes the use of low doses of live replicating viruses (59) or of specific antigen delivery systems, such as DNA vaccines (40, 81), virus-like particles (41), immunostimulating complexes (56), or adjuvants (33) that have in common the capacity to elicit strong CD4$^+$ Th1 neonatal responses. This suggested that induction of neonatal CD8$^+$ T-cell responses requires the help of strong CD4$^+$ Th1 cells, a condition frequently not met in early life due to a preferential polarization towards Th2 responses (reviewed in reference 2). However, antiviral CD8$^+$ T-cell neonatal responses similar to those of adults may even be elicited by synthetic microspheres that do not elicit strong Th1 responses (7, 57).

To address this discrepancy, we wished to study a murine model of viral infection with a murine pathogen that would follow an acute course in immunocompetent adults but a protracted course upon exposure in early life. We developed this model using the well-characterized lymphocytic choriomeningitis virus (LCMV) murine viral pathogen. It has been largely

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demonstrated that the infection of adult mice with LCMV strains, such as Armstrong or WE, results in a rapid viral multiplication that induces a massive expansion of antigen-specific $CD8^+$ T cells (11, 38, 47) essential for viral clearance, which occurs 7 to 10 days after infection (21, 22). It has also been well established that the age at infection influences the fate of LCMV infection, as intrauterine or perinatal (<24 h) infections lead to high-level lifelong virus persistence secondary to thymic infection and deletion of LCMV-reactive T cells (10, 31, 68). Contrasting its acute course in adults and persistent course in neonates, we show here that LCMV strain WE (LCMV-WE) infection in infant mice (experimentally defined here as 2-week-old mice) follows a protracted course characterized by an effective but delayed (≥4 weeks) viral clearance. This pattern does not result from the central deletion of LCMV-specific $CD8^+$ T cells, which are indeed elicited early after infection, but from their limited expansion, resulting in the absence of memory cells. In the absence of $CD8^+$ T cells, viral clearance is eventually achieved only through LCMV-specific B cells that protect infant mice from either death or chronic infection.

MATERIALS AND METHODS

Mice. BALB/c mice were purchased from IFFA CREDO (L'Arbresle, France) and kept under specific-pathogen-free conditions. $\mu MT^{-/-}$ BALB/c mice were a gift from Pascal Launois (WHO Immunology Research and Training Centre, Institute of Biochemistry, University of Lausanne, Switzerland). Breeding cages were checked daily, and the day of birth was recorded as the day the litter was found. Pups were kept with mothers until they were weaned at the age of 4 weeks. Adult mice were used at 8 to 16 weeks of age. Manipulations of mice were carried out according to Swiss and European guidelines, and all experiments were approved by the Geneva Veterinary Office.

LCMV, infection, and peptides. LCMV-WE was originally obtained as triple-plaque-purified stock from F. Lehmann-Grube (Heinrich-Pette Institut, Hamburg, Germany). Infection was performed by intravenous (i.v.) injection of 100 PFU of LCMV-WE. In some experiments, higher and lower doses were used as indicated. Virus titers of infected spleens, lymph nodes, kidneys, and brains were determined in a virus plaque assay as previously described (41). Virus titers are expressed as PFU per gram of organ. Peptide carrying the p118-132 $CD8^+$ sequence containing the immunodominant p118-126 peptide from the LCMV-WE nucleoprotein (LCMVnp118-126) (60) was synthesized by Neosystem (Strasbourg, France). Peptide carrying the subdominant peptide p283-291 $CD8^+$ sequence from the LCMV-WE glycoprotein (LCMVgp283-291) (71) was synthesized by the Proteomics Core Facility at the Faculty of Medicine (Geneva, Switzerland).

Quantification of cytotoxic responses. For ex vivo ^{51}Cr release assay, spleen cells from infected mice were incubated with LCMVnp118-132- or LCMVgp283-291-pulsed or unpulsed P815 target cells for 6 h. For ^{51}Cr release assay after 6 days of in vitro restimulation, cells were first cultured with irradiated spleen feeder cells in the presence of LCMVgp283-291 for 6 days before assessment in a ^{51}Cr release assay. The in vivo CTL assay was done as reported earlier (15). Splenocytes from naïve BALB/c mice were used as target cells and split into two equal populations. One was pulsed with 50 μg/ml of LCMVnp118-132 for 90 min at 37°C and then labeled with a high concentration (5 μM) of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Invitrogen, Carlsbad, CA). The other population was unpulsed and labeled with a low concentration of CFSE (0.5 μM). An equal number of cells from each population (3×10^7) was mixed together and adoptively transferred i.v. into naïve and LCMV-infected mice (adults and 2 weeks old). Twenty-four hours later, splenocytes were collected from recipient mice, erythrocytes were lysed, and cell suspensions were analyzed by fluorescence-activated cell sorting (FACS) (BD Biosciences, Mountain View, CA). Each population was distinguished by their respective fluorescence intensity. Assuming that the number of peptide-pulsed cells injected is equivalent to the number of non-peptide-pulsed cells injected, the percentage of killing of target cells in infected animals was calculated by using the following formula: $100 - [(\text{ratio infected}/\text{ratio uninfected}) \times 100]$ where the ratio is the percentage of cells expressing a low level of CFSE ($CFSE^{low}$)/percentage of cells expressing a high level of CFSE ($CFSE^{high}$).

Quantification of IFN-γ-producing LCMV-specific $CD8^+$ T cells by ELISPOT assay. The enzyme-linked immunospot (ELISPOT) assay for detection of peptide-specific gamma interferon (IFN-γ)-secreting T cells was performed essentially as described previously (44). For analysis of ex vivo cytokine secretion, splenocytes were incubated for 48 h in ELISPOT plates with interleukin 2 (IL-2) and 10^6 irradiated (3,000 rads) syngeneic splenocytes in the presence or absence of LCMVnp118-132 or LCMVgp283-291. The proportion of $CD8^+$ splenocytes was determined by flow cytometry before incubation in ELISPOT plates. The number of peptide-specific IFN-γ-producing cells was calculated by subtracting the number of IFN-γ-secreting cells cultured without peptide to that obtained with cells cultured with peptide. In one experiment, splenocytes were split in two populations and one was depleted of $CD4^+$ T cells. $CD4^+$ T-cell depletion was performed by using anti-CD4 antibody conjugated to phycoerythrin (BD Pharmingen, San Diego, CA) followed by antiphycoerythrin beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and sorted using an AutoMACS separator according to the manufacturer's instructions (Miltenyi Biotec). Then, CD4-depleted or nondepleted cells were added to ELISPOT plates. More than 95% of $CD4^+$ T cells were depleted as verified by FACS.

FACS staining. Single-cell suspensions were obtained from different organs from the infected animals. Specific staining of T-cell receptor (TCR) was performed by using dimeric major histocompatibility complex (MHC) class I-peptide complexes according to the manufacturer's instructions (BD Pharmingen). FACS staining was performed with the following antibodies: anti-CD8 (clone 53-6.7; BD Pharmingen), anti-CD44 (clone IM7; BD Pharmingen), and anti-CD62L (clone Mel-14; BD Pharmingen). Staining for annexin V-Cy5 was performed according to the manufacturer's instructions (BD Pharmingen). Cells were analyzed using the FACSCalibur (BD Biosciences) and CellQuest software (BD Immunocytometry Systems, San Jose, CA). An electronic gate was set on $CD8^+$ cells on acquisition. For intracellular IFN-γ analysis, 10^6 splenocytes were cultured in the absence or presence of LCMVnp118-126 peptide (2 μg/ml) for 30 h at 37°C and brefeldin A (Sigma) for the last 5 h. Following staining for surface antigens (CD8 and specific TCR), cells were stained for IFN-γ using the cytofix/cytoperm kit (BD Pharmingen) and anti-IFN-γ (clone XMG-1; BD Pharmingen).

In vivo proliferation. Adult and 2-week-old mice were injected intraperitoneally with 1 mg and 0.4 mg of bromodeoxyuridine (BrdU) (Sigma-Aldrich, Buchs, Switzerland), respectively, and were killed 12 h later. After cell preparation and counting, cells were stained for CD8, specific TCR, and BrdU (anti-BrdU antibody conjugated to fluorescein isothiocyanate) according to the BrdU Flow kit manual (BD Pharmingen).

Adoptive transfer experiments. Adult and 2-week-old mice were infected with 100 PFU of LCMV as described above. Four months later, spleen cells were harvested, and 2×10^7 cells were adoptively transferred i.v. to 2-week-old recipient mice. Six hours later, recipient mice were infected with 100 PFU of LCMV. Four days after infection, the spleens of recipient mice were recovered for evaluation of memory IFN-γ-producing $CD8^+$ T-cell responses by ELISPOT assay and for evaluation of virus titers by plaque assay.

Anti-NP and neutralizing antibodies. LCMV nucleoprotein (NP)-binding antibodies were detected by enzyme-linked immunosorbent assay against baculovirus-derived recombinant protein as previously described (5). The neutralizing antibodies against LCMV were detected in a focus reduction assay as previously described for LCMV (5).

Statistical analysis. Statistical differences between two groups were analyzed by the Mann-Whitney U test, and differences between multiple groups were analyzed by one-way analysis of variance followed by the Tukey multiple-comparison test. Differences with probability values of >0.05 were considered insignificant.

RESULTS

LCMV-WE infection follows a protracted course in infant mice. To study the patterns of viral infection and the role of $CD8^+$ T-cell responses in infant mice, we used LCMV-WE and BALB/c mice and selected an age of 2 weeks for infection. This age was chosen to avoid the risk of thymic infection (≤24 h) and the immune limitations that are notoriously associated with the neonatal (≤7 days) period and was experimentally defined. Indeed, 2-week-old BALB/c mice are capable of rais-

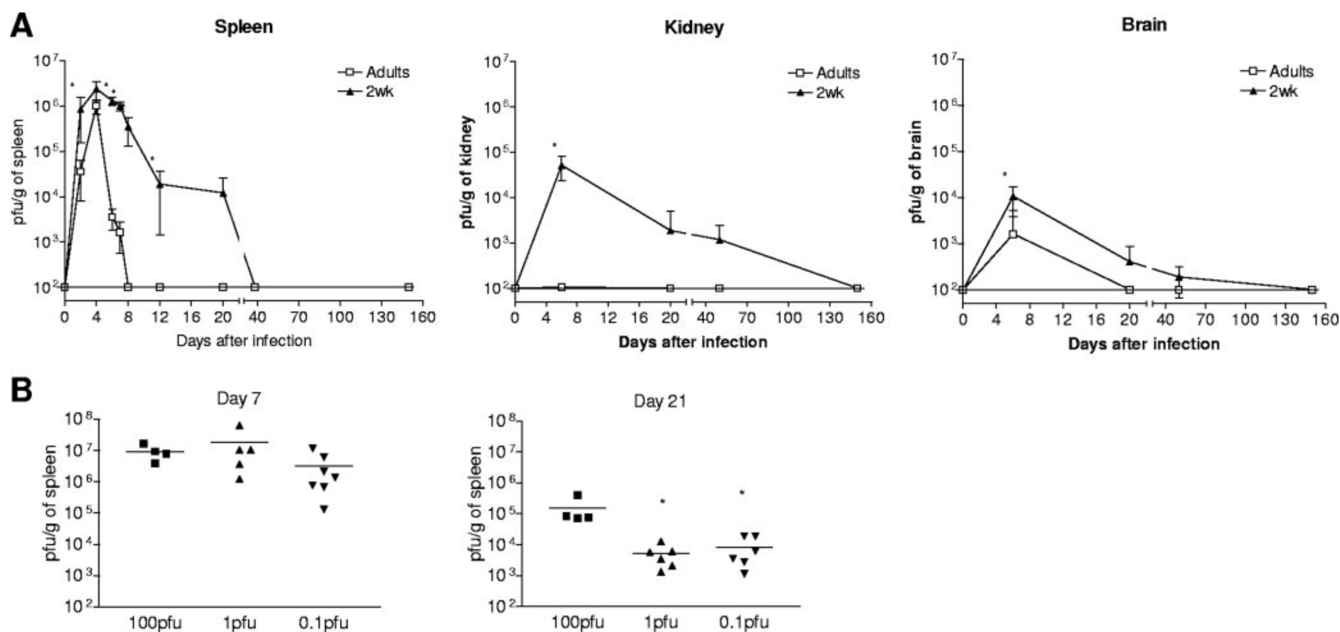

FIG. 1. Delayed viral clearance in infant mice. (A) Two-week-old (2wk) and adult mice were infected by i.v. injection of 100 PFU of LCMV-WE. The spleens, brains, and kidneys were removed from the mice at different time points after infection, and virus titer was determined by a plaque assay. Results are expressed as means ± standard deviations (error bars) obtained for two experiments of two to four representative experiments with at least four mice per group for each time point. Values that are significantly different from the value for adult mice ($P < 0.05$) are indicated with an asterisk. (B) Two-week-old mice were infected by i.v. injection of 100, 1, or 0.1 PFU of LCMV-WE. The spleens were removed from the mice 7 or 21 days after infection, and virus titer was determined by a plaque assay. Each symbol represents the value for one individual mouse, and the mean is represented by the short horizontal line. The results from one of two representative experiments are shown. Mean values that are significantly different from the mean value for 2-week-old mice infected with 100 PFU ($P < 0.05$) are indicated with an asterisk.

ing adult-like LCMV-specific $CD8^+$ T cells in response to peptide-loaded virus-like particles (41) or microspheres (57). In contrast to neonatal mice, 2-week-old mice raise adult-like $CD4^+$ Th1 cells in response to most antigen delivery systems (reviewed in reference 62 and our unpublished data). Two-week-old and adult (control) BALB/c mice were infected i.v. with 100 PFU of LCMV-WE, and viral infection course was determined in the spleen, brain, and kidneys of infected animals by quantifying virus titer at different time points after infection. Spleen virus titers were similar in adult and infant mice at the peak of viremia (day 4) but were reached earlier in mice infected at 2 weeks of age (Fig. 1A). As expected (21, 22), adult mice rapidly controlled LCMV-WE, and splenic viral clearance was achieved by day 8. LCMV-WE infection in the spleen followed a markedly different course in early life: the course of viremia was not only faster, but virus titers persisted at high levels ($>10^6$ PFU/g) until day 7 or 8 and significantly declined only during the second and third week after infection (Fig. 1A). Infection was eventually cleared from the spleens of infant mice, albeit with a marked delay, as LCMV became undetectable in all mice by day 40. A similar course of infection was observed in the lymph nodes (not shown). In the kidneys, LCMV-WE was again rapidly cleared in adult mice, whereas titers declined only during the third week following infant infection, reaching complete elimination only between day 50 and day 150. In the brain, LCMV-WE was transiently detected in only one of six adult mice at day 6, whereas the kinetics of viremia was similar to that in the kidneys following infant infection. The absence of viral persistence was further confirmed by the fact that injection of serum from mice infected 100 days earlier at 2 weeks of age (or as an adult) did not result in infection of naïve adult mice.

To directly address the role of the viral infectious dose in the different fates of infant and adult LCMV-WE infections, BALB/c mice were infected i.v. with 100 PFU, 30 PFU, 10 PFU, 1 PFU, or even 0.1 PFU. Across this dose range, peak viral titers ($>10^6$ PFU/g) were similar on day 4 in infant and adult mice (not shown). Delayed viral clearance was observed in infant mice under all experimental conditions tested (Fig. 1B and data not shown). On day 7, LCMV-WE was present at similarly high titers in the spleens of mice infected at 2 weeks of age with 100, 1, or even 0.1 PFU, although titers were more heterogeneous in mice exposed to very low doses of LCMV-WE. A protracted course was also observed regardless of the infectious dose, as LCMV-WE was still detected during the third week of infection (Fig. 1B). Thus, LCMV-WE does induce a classical acute infection in adult BALB/c mice but a protracted infection in infant 2-week-old BALB/c mice, even upon exposure to a very low viral load.

Induction and early waning of LCMV-specific $CD8^+$ T-cell effectors in infant mice. The essential role of $CD8^+$ T cells in the control of acute LCMV infections prompted their study in LCMV-WE-infected adult and infant mice. $CD8^+$ T-cell responses were first evaluated at different time points after infection by the ex vivo quantification of splenic $CD8^+$ T cells producing IFN-γ against the LCMV nucleoprotein immuno-

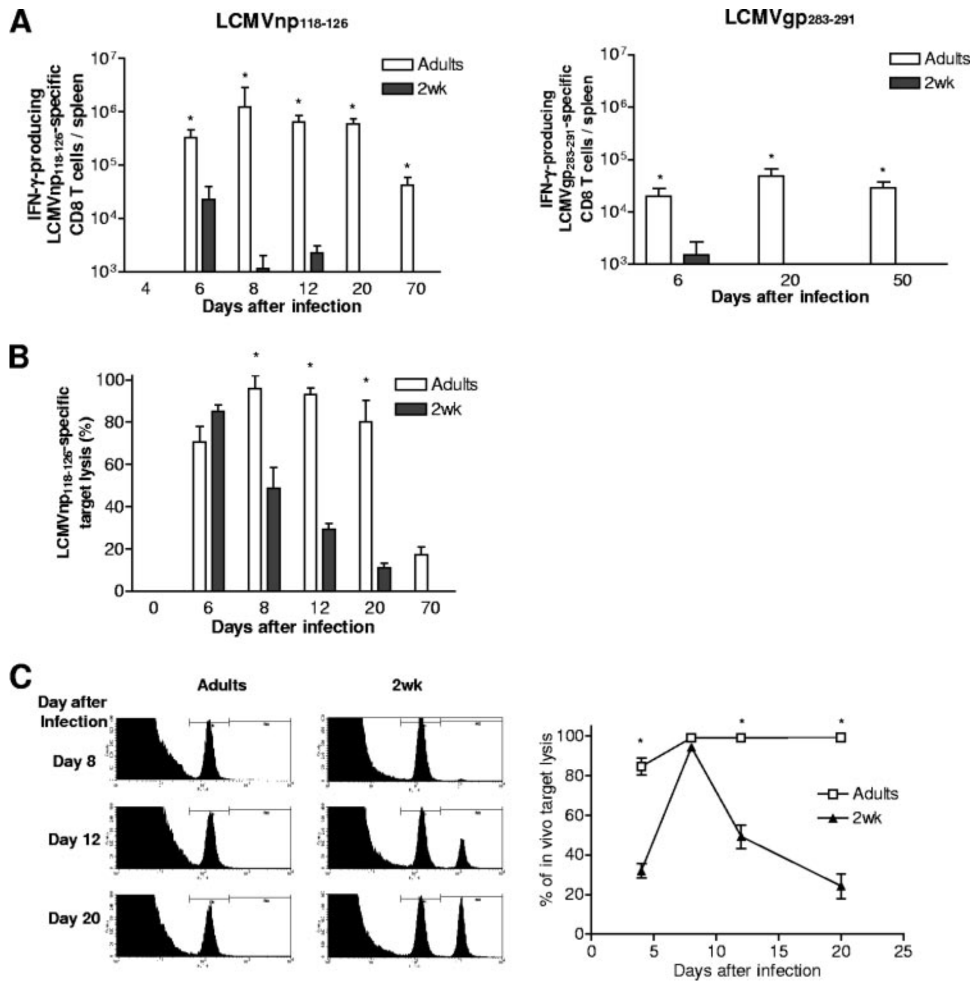

FIG. 2. $CD8^+$ T-cell responses are induced but not maintained in infant mice. Two-week-old (2wk) and adult mice were infected by i.v. injection of 100 PFU of LCMV-WE. (A) ELISPOT assays were performed by incubating spleen cells (removed at different time points) with LCMVnp118-126 or LCMVgp283-291 and irradiated splenocytes as feeders for 48 h. Results are expressed as means plus standard deviations (error bars) obtained in two representative experiments out of two to four independent experiments, with three or four mice per group for each time point. Values that are significantly different from the value for 2-week-old mice ($P < 0.05$) are indicated with an asterisk. (B) Ex vivo CTL assays were performed by incubating spleen cells (removed at different time points) with LCMVnp118-126-pulsed or -unpulsed P815 target cells for 6 h. Specific lysis (at a ratio of 25 effectors to 1 target cell) was calculated as described in Materials and Methods. Results are expressed as means plus standard deviations (error bars) obtained in two representative experiments out of four experiments, with three or four mice per group for each time point. Values that are significantly different from the value for 2-week-old mice ($P < 0.05$) are indicated with an asterisk. (C) For in vivo CTL assays, two subsets of target cells (unpulsed $CFSE^{low}$ splenocytes and LCMVnp118-126-pulsed $CFSE^{high}$ splenocytes) were injected at different time points after infection. Twenty-four hours later, the in vivo elimination of target cells was assessed by FACS. FACS panels show results from one mouse in each group for each time point representing five mice per group and per time point. In vivo-specific lysis kinetic results are plotted in a graph representing means ± standard deviations (error bars) obtained in one representative experiment of two independent experiments, with four to seven mice per group for each time point. Values that are significantly different from the value for 2-week-old mice ($P < 0.05$) are indicated with an asterisk.

dominant peptide (LCMVnp118-126) and the glycoprotein subdominant epitope (LCMVgp283-291). In adult infected mice, LCMVnp118-126-specific IFN-γ-producing T cells were first detected ex vivo on day 6 and rapidly increased to reach a peak on days 8 to 20 (Fig. 2A). As expected, $CD4^+$ T-cell depletion prior to incubation with the LCMVnp118-126 peptide did not reduce the number of IFN-γ-producing T cells, indicating the contribution of $CD8^+$ T cells (data not shown). The adult $CD8^+$ T-cell response followed the expected contraction phase shortly after viral clearance but remained at a significant level ($\sim 40 \times 10^3$ IFN-γ-producing cells/spleen) until at least day 70. In mice infected at 2 weeks of age, LCMVnp 118-126-specific IFN-γ-producing $CD8^+$ T cells were reproducibly detected ex vivo on day 6, albeit at a 14-fold-lower frequency ($\sim 23 \times 10^3$ versus 330×10^3 IFN-γ-producing cells/spleen, respectively) than in adults (Fig. 2A). Unexpectedly, however, this IFN-γ response waned between days 8 and 12, and ex vivo IFN-γ-producing $CD8^+$ T cells returned to levels below detection after day 20. As expected, the subdominant IFN-γ response against LCMVgp283-291 epitope was markedly lower in adult mice than the immunodominant IFN-γ response (day 20, 50×10^3 versus 600×10^3 IFN-γ-producing cells/spleen, respectively) at each time point tested. In infant mice, LCMVgp283-291-specific IFN-γ responses followed a

similar pattern as that observed for the immunodominant NP epitope, with a peak on day 6.

To determine whether this limitation of infant $CD8^+$ T-cell responses only or preferentially affected IFN-γ production, cytotoxic T-lymphocyte (CTL) responses were quantified ex vivo using a chromium release assay (Fig. 2B). The frequency of LCMVgp283-291-specific responses was too low to allow ex vivo detection, and CTL responses to this subdominant epitope could be detected only in adult mice following in vitro restimulation (data not shown). In adult mice, strong LCMVnp118-126-specific CTL responses were observed ex vivo between days 6 and 20 after infection and remained detectable after the contraction phase until the last time point assessed (day 70) (Fig. 2B and data not shown). In mice infected at 2 weeks of age, LCMVnp118-126-specific CTL responses similar to those of adults were observed on day 6 and on day 8 (Fig. 2B). However, these responses waned rapidly (day 12) and returned to levels below the detection level from day 20 onwards. This transient cytotoxicity pattern was confirmed in vivo by the quantification of LCMVnp118-126-pulsed $CFSE^{high}$ and control $CFSE^{low}$ target cells injected at different time points after infection. In adult mice, CTL activity was already at the highest level on day 4 and maintained until day 20 after infection (Fig. 2C). Mice infected at 2 weeks of age also cleared LCMVnp118-126-pulsed target cells in vivo. However, their cytotoxic capacity was delayed, as on day 4, it was significantly lower than in adult controls (Fig. 2C). A marked increase of the cytotoxic capacity was observed between day 4 and day 8, at which the elimination of LCMVnp118-126-pulsed target cells reached adult levels. However, this CTL capacity was rapidly lost and became barely detectable on day 20 (Fig. 2C). Thus, functional $CD8^+$ T-cell responses are elicited in infant mice early in the course of LCMV-WE infection. However, their induction is followed by an early waning of effector T cells, the decline of IFN-γ-producing cells on days 8 to 12 shortly preceding that of cytotoxic T cells on days 12 to 20.

Early waning of infant anti-LCMV $CD8^+$ T cells. This protracted course of infection with progressive exhaustion of $CD8^+$ T cells was reminiscent of that observed upon infection of immunocompetent adult mice with LCMV clone 13 (19, 76). The hypothesis of functional exhaustion was thus assessed by tracking splenic LCMVnp118-126-specific $CD8^+$ T cells using dimeric MHC class I-LCMVnp118-126 staining. As expected, the majority of dimeric MHC class I-LCMVnp118-126-positive $CD8^+$ T cells produced IFN-γ (73.8% ± 6.1% versus 3.2% ± 1.2% in the presence or absence of peptide, respectively), confirming staining specificity. In adult infected mice, LCMVnp118-126-specific $CD8^+$ T cells were detected from day 6 onwards (Fig. 3A). There was a marked expansion phase during the first 2 weeks after infection, the proportion of LCMVnp118-126-specific $CD8^+$ T cells reaching 30% of total $CD8^+$ T cells on day 12. As expected, the number of LCMVnp118-126-specific $CD8^+$ T cells declined during the contraction phase but persisted at significant levels (10% of total $CD8^+$ T cells) up to the last time point assessed (Fig. 3A and B). In mice infected at 2 weeks of age, LCMVnp118-126-specific $CD8^+$ T cells were also detected from day 6 onwards (Fig. 3A). However, this $CD8^+$ T-cell response failed to expand between days 6 and 12, and LCMV-specific $CD8^+$ T cells remained at least 30-fold lower than in adults at all times (for example, 7×10^4 versus 300×10^4, respectively, on day 7), including after viral clearance (Fig. 3A and B and data not shown).

To exclude the possibility that infant LCMVnp118-126-specific $CD8^+$ T cells had migrated to organs other than the spleen, specific $CD8^+$ T cells were quantified in other secondary lymphoid organs (Fig. 3B). In adult mice, LCMVnp118-126-specific $CD8^+$ T cells were recovered in all compartments assessed both early (day 7 [not shown]) and late (day 50 [Fig. 3B]) after infection. In mice infected at 2 weeks of age, low numbers of LCMVnp118-126-specific $CD8^+$ T cells were recovered on day 7 in the spleen and bone marrow (not shown). Thereafter, however, these cells remained below the levels in the spleen, lymph nodes, and bone marrow of adult mice (Fig. 3B). Similar results were obtained in the blood (not shown). Thus, the protracted course of LCMV-WE infection in infant BALB/c mice does not reflect the persistence of functionally exhausted $CD8^+$ T cells but their physical disappearance.

The maintenance of the peptide-specific $CD8^+$ T-cell pool reflects the balance between their proliferation rate and their degree of apoptosis (3). The proportion of apoptotic LCMVnp 118-126-specific $CD8^+$ T cells was measured by annexin V staining between days 6 and 12, i.e., during the expansion phase of adult $CD8^+$ T cells (Fig. 3C). This proportion was modest and similar regardless of the age at infection, declining from 4% to <0.1% between day 6 and day 12 (Fig. 3C). The rate of proliferation of LCMVnp118-126-specific $CD8^+$ T cells was quantified 6 days after infection by BrdU injection, and the assessment of BrdU incorporation in LCMVnp118-126-specific $CD8^+$ T cells was performed 8 h later. The proliferation rates of LCMV-specific $CD8^+$ T cells were similar in mice infected as adults and mice infected at 2 weeks of age (Fig. 3D) and significantly higher than in naïve control mice (data not shown). Thus, the disappearance of LCMVnp118-126-specific $CD8^+$ T cells in early life does not appear to reflect either an excess death rate or an intrinsic limitation of the proliferative capacity of $CD8^+$ T cells.

Reducing the viral inoculum fails to restore the expansion of infant antiviral $CD8^+$ T cells. Neonatal exposure (<7 days of age) to adult viral inocula of a murine leukemia virus inhibits the generation of $CD8^+$ T-cell responses that may be restored by lowering the viral inoculum (59). To assess whether this applies to our model of infant infection, 2-week-old BALB/c mice were infected i.v. with 30 PFU instead of 100 PFU. Mice were bled on day 7 to quantify LCMVnp118-126-specific IFN-γ-producing $CD8^+$ T cells and sacrificed on day 12 to assess splenic CTL responses ex vivo. Reducing the dose of LCMV-WE to 30 PFU did not restore either IFN-γ-producing $CD8^+$ T cells or cytolytic effector cells (Fig. 4A). The infectious dose was thus further reduced to 1 PFU of LCMV-WE, which was still sufficient to infect 2-week-old mice (Fig. 1B) and induce detectable (although more heterogeneous) IFN-γ responses on day 7 (Fig. 4B). However, reducing the viral inoculum to 1 PFU failed to restore day 12 to 20 responses (Fig. 4B). A further reduction of the viral dose to 0.1 PFU was still sufficient to elicit viral replication (Fig. 1B) but triggered fewer $CD8^+$ T-cell responses (Fig. 4B).

The failure of expansion of anti-LCMV $CD8^+$ T cells results in the absence of generation of memory CD8 T cells. A protracted course of infection is observed in immunocompetent

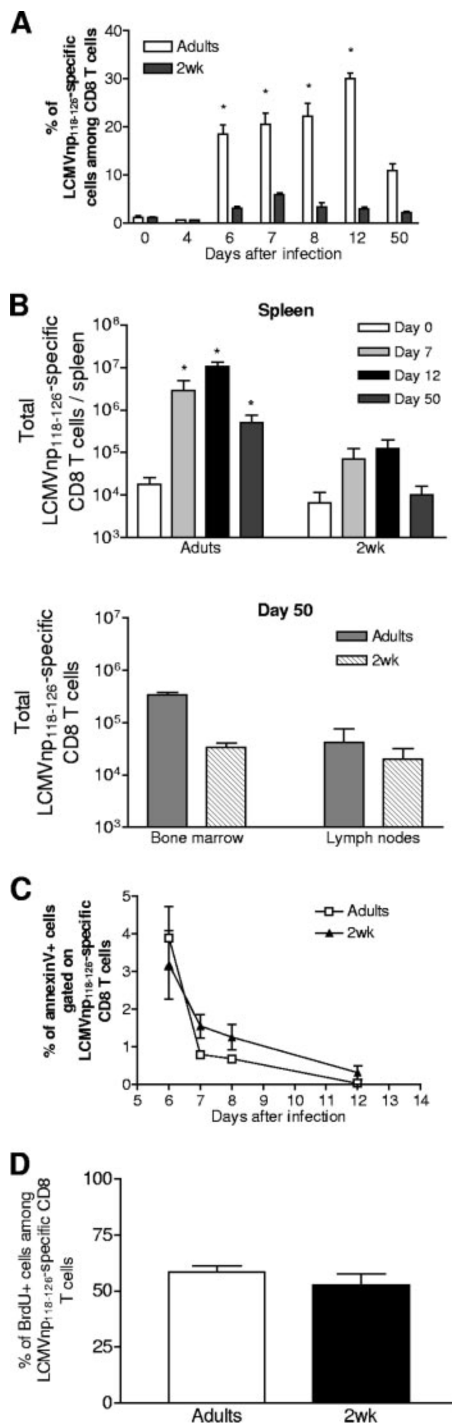

FIG. 3. Failure of LCMVnp118-126-specific $CD8^+$ T cells to expand in response to infection. Two-week-old (2wk) and adult mice were infected by i.v. injection of 100 PFU of LCMV-WE. (A) Spleens were removed at different time points after infection, and FACS staining of LCMVnp118-126-specific $CD8^+$ T cells was performed as described in Materials and Methods. Results are expressed as mean percentages plus standard deviations (error bars) of LCMVnp118-126-specific $CD8^+$ T cells among total $CD8^+$ T cells obtained in two experiments, representative of three independent experiments, with three to seven mice per group for each time point. Values that are significantly different from the value for 2-week-old mice ($P < 0.05$) are indicated with an asterisk. (B) Spleens were removed 0, 7, 12, and 50 days after infection (top); the lymph nodes (inguinal, axial, and mesenteric) and bone marrow were removed 50 days after infection (bottom), and FACS staining of LCMVnp118-126-specific $CD8^+$ T cells was performed as described in Materials and Methods. Results are expressed as means plus standard deviations (error bars) of the total number of LCMVnp118-126-specific $CD8^+$ T cells per organ obtained in two experiments, representative of three independent experiments, with three or four mice per group for each time point. Values that are significantly different from the value for 2-week-old mice ($P < 0.05$) are indicated with an asterisk. (C) Spleens were removed at different time points after infection, and FACS annexin-V staining of LCMVnp118-126-specific $CD8^+$ T cells was performed as described in Materials and Methods. Results are expressed as mean percentages ± standard deviations (error bars) of annexin V-positive (annexinV+) LCMVnp118-126-specific $CD8^+$ T cells among total LCMVnp118-126-specific $CD8^+$ T cells obtained in one experiment, with four to seven mice per group for each time point. (D) Five days after infection, infant and adult mice were injected intraperitoneally by BrdU as described in Materials and Methods. Eight hours later, spleens were removed, and cells were stained for BrdU, CD8, and specific TCR. Results are expressed as mean percentages plus standard deviations (error bars) of BrdU-positive (BrdU+) cells among LCMV-specific $CD8^+$ T cells obtained in one experiment, representative of two independent experiments, with five to seven mice per group.

adult mice infected with very high doses of LCMV-A22.2b (54, 70, 71) or with LCMV clone 13 (19, 42, 51). During these infections, immunodominant CD8 T cells are lost, while other clones are maintained, a restoration of their effector function being observed after viral clearance, which does not occur when virus persists indefinitely (19, 71) and/or upon adoptive transfer (36). To confirm that these patterns are different from the situation reported here in mice infected at 2 weeks of age by a normal dose of LCMW-WE, we attempted to highlight the presence of LCMV-specific memory $CD8^+$ T cells. The low number of LCMVnp118-126-specific $CD8^+$ T cells elicited in infant mice (Fig. 3) precluded direct evaluation of their memory markers. However, naïve infant mice raised under specific-pathogen-free conditions express memory markers on only <5% of total $CD8^+$ T cells (Fig. 5A, day 0), allowing the use of total memory $CD8^+$ T cells as surrogates for LCMV-specific memory cells. Memory-associated markers on total $CD8^+$ T cells were thus tracked in blood following LCMV-WE infection of 2-week-old and adult control mice. In adult mice, $CD44^{high}$ memory $CD8^+$ T cells markedly increased from day 7 to day 20 after LCMV exposure and returned to baseline levels 2 months after infection (Fig. 5A). This pattern mirrored the kinetics of total LCMVnp118-126-specific $CD8^+$ T cells (Fig. 3A). As previously described (77), $CD62L^{low}$ effector memory $CD8^+$ T cells appeared earlier (day 8 versus day 20) and reached higher numbers than $CD62L^{high}$ central memory $CD8^+$ T cells did. We failed to detect either effector or central memory $CD8^+$ T cells in infant mice at any time following infection (Fig. 5A). The number of total memory $CD8^+$ T cells increased after day 50 in mice infected at 2 weeks of age but remained similar to that of noninfected age-matched control mice (not shown). Similar results were obtained in the spleen and nodes (not shown).

Memory $CD8^+$ T cells could be present in mice infected in infancy at levels insufficient for their direct detection by FACS staining. Their function was thus assessed through secondary viral challenge. To avoid the inhibitory influence of neutraliz-

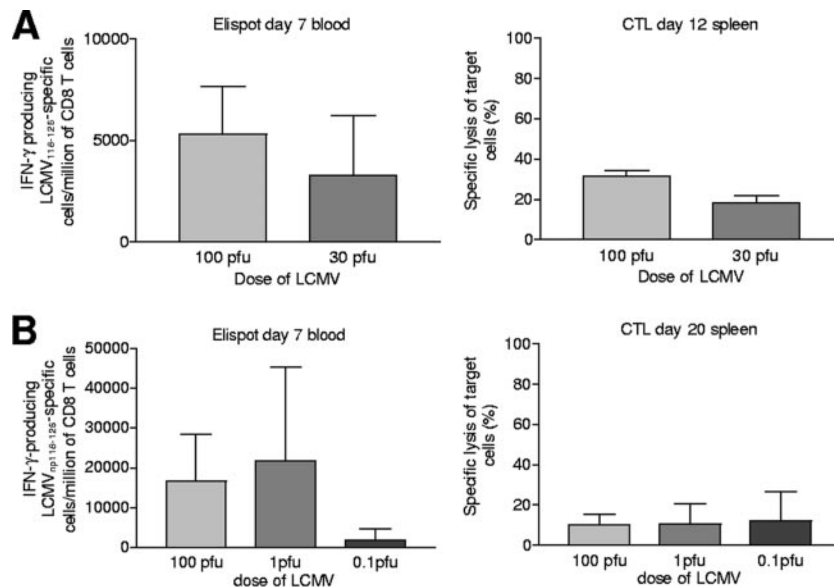

FIG. 4. Lower viral load did not restore infant antiviral CD8 T-cell responses. (A) Two-week-old mice were infected by i.v. injection of 100 or 30 PFU of LCMV-WE. Mice were bled 7 days after infection, and ELISPOT assays were performed by incubating blood cells with LCMVnp118-126 and irradiated splenocytes as feeders for 48 h. Mice were killed 12 days after infection, and the spleens were removed from the mice. Ex vivo CTL assay was performed by incubating spleen cells with LCMVnp118-126-pulsed or nonpulsed P815 target cells for 6 h. Specific lysis (at a ratio of 25 effectors to 1 target cell) was calculated as described in Materials and Methods. Results are expressed as means plus standard deviations (error bars) obtained in one experiment, representative of two independent experiments, with five to seven mice per group. (B) Two-week-old mice were infected by i.v. injection of 100, 1, or 0.1 PFU of LCMV-WE. Mice were bled 7 days after infection, and ELISPOT assays were performed by incubating blood cells with LCMVnp118-126 and irradiated splenocytes as feeders for 48 h. Mice were killed 20 days after infection, and spleens were removed. Ex vivo CTL assay was performed by incubating spleen cells with LCMVnp118-126-pulsed or nonpulsed P815 target cells for 6 h. Specific lysis (at a ratio of 25 effectors to 1 target cell) was calculated as described in Materials and Methods. Results are expressed as means plus standard deviations (error bars) obtained in one experiment, representative of two independent experiments, with four or five mice per group.

ing antibodies, mice were infected as adults or at 2 weeks of age, and their splenocytes were harvested 150 days later for adoptive transfer into 2-week-old recipient mice. These recipient mice were challenged 6 h later, and their memory $CD8^+$ T-cell responses against LCMVnp118-126 and LCMVgp283-291 epitopes were assessed by ELISPOT assays on day 4, i.e., before primary responses reach sufficient levels to be detected ex vivo (Fig. 2A). As expected, IFN-γ-producing $CD8^+$ T cells remained undetectable on day 4 in mice adoptively transferred with naïve control cells (Fig. 5B). LCMVnp118-126-specific IFN-γ-producing $CD8^+$ T cells were readily recalled in 2-week-old mice adoptively transferred with adult immune cells, T cells to the subdominant LCMVgp283-291 epitope remaining below the detection level (Fig. 5B), which was already sufficient to control viral replication on day 4 (Fig. 5C). This indicates that the early life microenvironment does not inhibit the function of adult immunodominant LCMV-specific memory $CD8^+$ T cells. A direct correlation was observed between the magnitude of $CD8^+$ T-cell responses in individual adult mice (Fig. 5B) and viral control (Fig. 5C). In contrast, IFN-γ-producing $CD8^+$ T cells against both epitopes remained undetectable in infant recipient mice transferred with splenocytes of adult mice that had been previously infected at 2 weeks of age (Fig. 5B), which was associated with high viral titers on day 4 (Fig. 5C). These high viral titers did not reflect the transfer of persistently infected cells, as no virus was detected in the spleens of recipient mice that were not infected after transfer. Similar results were obtained when adoptive transfers were performed with lymph node cells compared to spleen cells (not shown). Thus, the failure of $CD8^+$ T cells to expand in early life after LCMV infection results in the failure of the generation of memory $CD8^+$ T cells, resulting in an enhanced vulnerability to secondary viral challenge.

Contribution of antibodies to protection against LCMV-WE infection in early life. Viral clearance is eventually achieved by day 40 in mice infected at 2 weeks of age (Fig. 1) despite the lack of expansion/persistence of $CD8^+$ T effector T cells and the lack of induction of memory $CD8^+$ T cells, suggesting that other effector mechanisms eventually contribute to control infection. As previously reported in adult mice, neutralizing antibodies were detected only from day 30 onwards in the serum of mice infected at 2 weeks of age (Fig. 6A), i.e., after achievement of viral clearance. Total LCMV-NP-binding antibodies appeared earlier, i.e., during the second week after infection, in both age groups (Fig. 6B). Remarkably, neutralizing and anti-NP antibodies reached significantly higher titers in mice infected in early life than in adults (Fig. 6A and B), an observation reminiscent of that observed in $CD8^+$ T-cell-depleted mice (5). To directly investigate the contribution of antiviral B cells to infant LCMV infection, $\mu MT^{-/-}$ BALB/c mice were infected with LCMV-WE (100 PFU) at 2 weeks of age or as adults (controls). The influence of age on the $CD8^+$ T-cell response in $\mu MT^{-/-}$ mice was similar to that in wild-type (WT) mice. Adult IFN-γ-producing $CD8^+$ T cells peaked at day 7, efficiently controlled viral replication, and persisted at day 20 (Fig. 6C and D). Again, IFN-γ-producing $CD8^+$ T cells

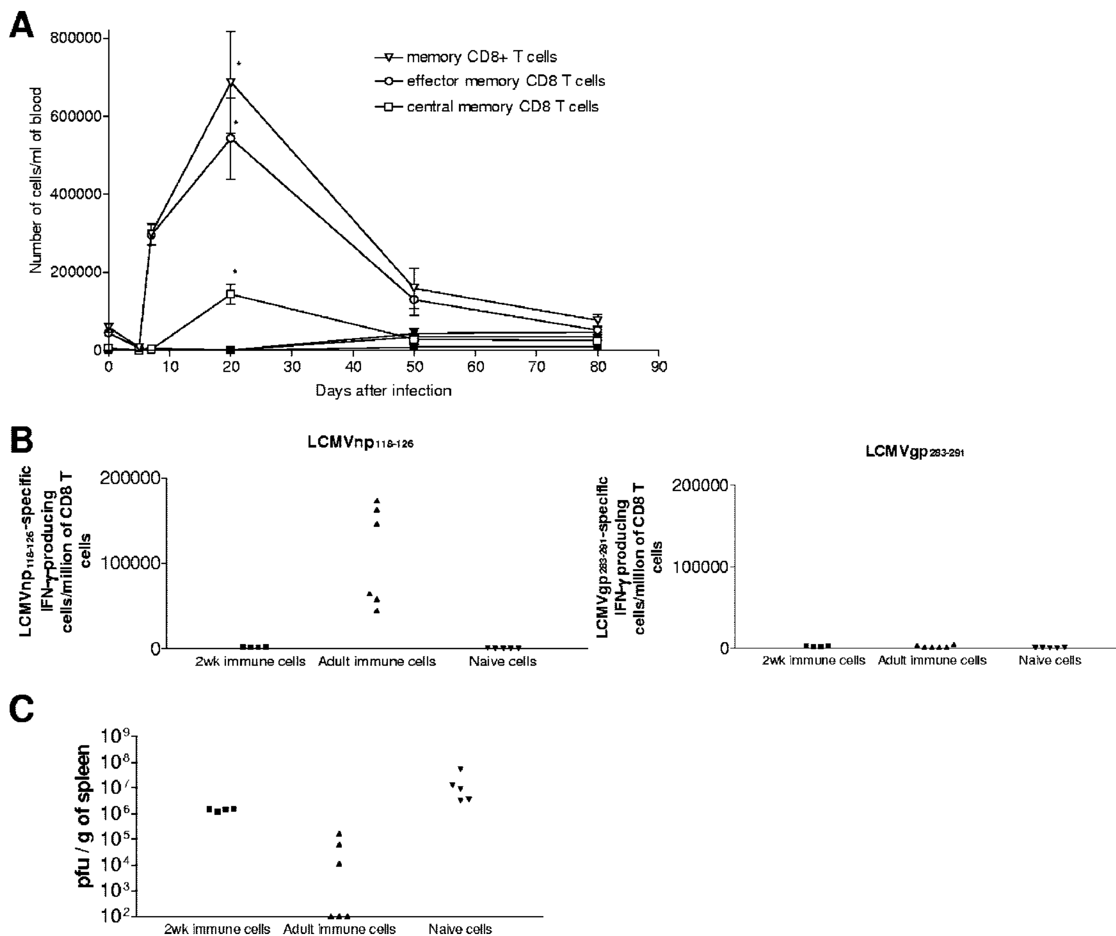

FIG. 5. Absence of elicitation of memory $CD8^+$ T cells in infant mice infected with LCMV. (A) Two-week-old (black symbols) and adult mice (white symbols) were infected by i.v. injection of 100 PFU of LCMV-WE and were bled at different time points after infection. FACS staining of different subsets of memory $CD8^+$ T cells was performed as described in Materials and Methods. Results are expressed as means ± standard deviations (error bars) obtained in one experiment, representative of two independent experiments, with four to seven mice per group. Values that are significantly different from the value for 2-week-old mice ($P < 0.05$) are indicated with an asterisk. (B and C) Two-week-old mice (2wk) were adoptively transferred with spleen cells from mice infected 150 days before as adults or from mice infected as infants. Six hours after the transfer, mice were infected with 100 PFU of LCMV-WE. Mice were killed 4 days after challenge, and spleens were removed. ELISPOT assay (B) was performed by incubating spleen cells with LCMVnp118-126 or LCMVgp283-291 and irradiated splenocytes as feeders for 48 h. Symbols show the values for individual mice obtained in one experiment, representative of two independent experiments, with four or five mice per group. Virus titer was determined by a plaque assay (C). Symbols show the values for individual mice, obtained in one experiment, representative of two independent experiments, with four or five mice per group.

in $\mu MT^{-/-}$ mice infected at 2 weeks of age were numerous on day 7 (4,000 per million $CD8^+$ T cells; Fig. 6C and D) but had disappeared by day 20 and failed to control viral replication. Unexpectedly, 60% of $\mu MT^{-/-}$ mice infected at 2 weeks of age succumbed during the second week after infection, a mortality rate which was never observed either in adult $\mu MT^{-/-}$ or WT infant mice. In the few surviving $\mu MT^{-/-}$ mice, LCMV-WE persisted until the last time point assessed (day 70 [Fig. 6D]). Thus, B cells contribute to the clearance of LCMV-WE in mice infected at 2 weeks of age and eventually terminate a protracted course of infection, reducing the susceptibility of young mice to early death or persistent infection.

DISCUSSION

Viral clearance or persistence is determined by a critical balance between virus-specific immune responses and virus replication patterns. Numerous studies have documented that immunocompetent adult mice elaborate a massive $CD8^+$ T-cell response that clears LCMV within 1 week (11, 20, 47, 74) and elicits a functional T-cell memory protecting against secondary challenge, whereas neonatal exposure may result in persistent infection. In contrast to these two patterns, we report here that LCMV-WE follows a protracted course in 2-week-old mice, in which $CD8^+$ T cells are elicited but fail to expand and thus generate memory $CD8^+$ T cells that control infection.

The neonatal and early life period is notoriously characterized by antiviral responses different from those elicited in immunologically mature adult hosts. The protracted pattern of infant LCMV infection first described here is however different from the chronic LCMV infection that results from neonatal exposure, which leads to thymic infection and to the central

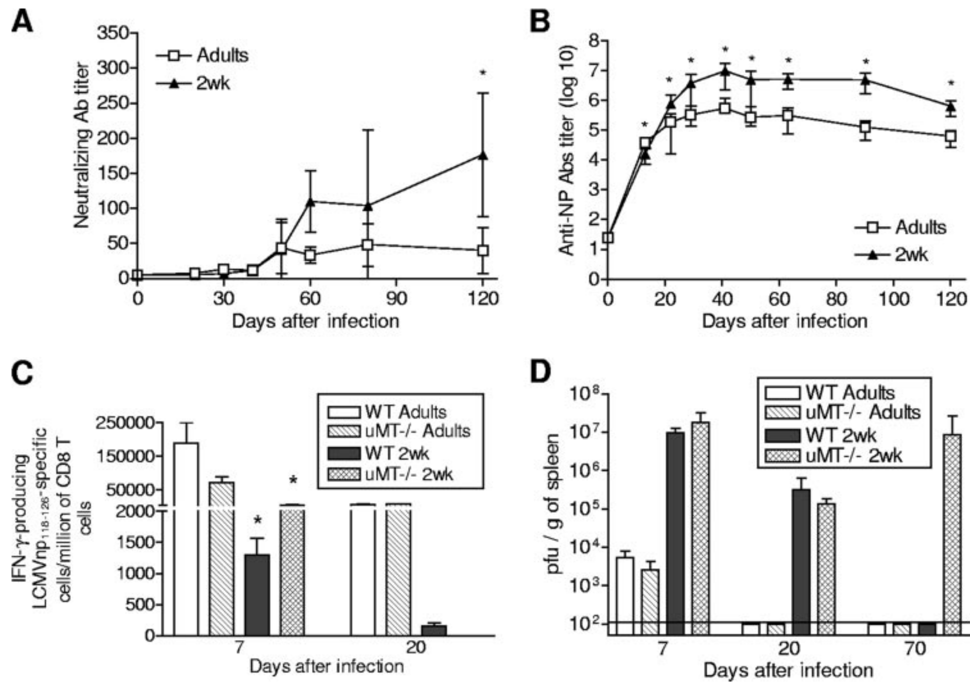

FIG. 6. Contribution of antibodies to the protection of infant mice following LCMV infection. (A and B) Two-week-old (2wk) and adult mice were infected by i.v. injection of 100 PFU of LCMV-WE and were bled at different time points after infection. (A) The presence of neutralizing antibodies (Ab) in sera was tested by a modified plaque assay as described in Materials and Methods. Results are expressed as means ± standard deviations (error bars) obtained in two independent experiments, with four to seven mice per group. Values that are significantly different from the value for adult mice ($P < 0.05$) are indicated with an asterisk. (B) The presence of LCMV-NP-binding antibodies (Anti-NP Abs) in sera was quantified by enzyme-linked immunosorbent assay as described in Materials and Methods. Results are expressed as means ± standard deviations (error bars) obtained in three independent experiments, with 4 to 12 mice per group. Values that are significantly different from the value for adult mice ($P < 0.05$) are indicated with an asterisk. (C) Two-week-old and adult $\mu MT^{-/-}$ mice (uMT−/−) (and their WT controls) were infected by i.v. injection of 100 PFU of LCMV-WE. Seven and 20 days after infection, ELISPOT assay was performed by incubating spleen cells with LCMVnp118-126 and irradiated splenocytes as feeders for 48 h. Results are expressed as means plus standard deviations (error bars) obtained in two independent experiments, with three to five mice per group for each time point. Values that are significantly different from the value for WT adult mice ($P < 0.05$) are indicated with an asterisk. (D) Spleen virus titer was determined by a plaque assay. The horizontal line indicates the detection level. Results are expressed as means ± standard deviations (error bars) obtained in two independent experiments, with three to five mice per group for each time point.

deletion of LCMV-specific $CD8^+$ T cells (10, 31, 50). As the infectivity of thymocytes is markedly reduced after the first 24 h of life (66), central deletion of LCMV-specific $CD8^+$ T cells does not occur in 2-week-old mice, in which fully functional $CD8^+$ T cells are elicited within 1 week after exposure. Another major difference is that viral clearance is eventually achieved more than 4 weeks after infection of 2-week-old mice, whereas infection persists indefinitely after neonatal exposure. This protracted infection pattern also does not reflect the limitation of LCMV replication reported in mice with immature spleen marginal zones (46), another hallmark of early life (67), as similarly high LCMV titers are generated on day 4 in the spleens of adult or infant mice. Last, this protracted pattern is different from the high-dose neonatal tolerance phenomenon that results from neonatal infection with high viral doses, as (i) fully functional LCMV-specific T $CD8^+$ cells are indeed elicited on days 6 to 8 after LCMV infection and (ii) reducing the viral inoculum to as low as 1 or 0.1 PFU of LCMV-WE does not restore adult-like $CD8^+$ T-cell response patterns (Fig. 4). Thus, the protracted course run by LCMV-WE in mice infected at 2 weeks of age is different from the previously described patterns of neonatal infections.

$CD8^+$ T-cell responses are induced with some delay in mice infected at 2 weeks of age compared to adults, as illustrated by weaker responses on day 4 (Fig. 2C). However, this is followed by a strong wave of $CD8^+$ effector T cells that appear to be recruited normally, function normally (IFN-γ production and cytotoxic capacity), and die at the same rate as adult cells, after a few days of effector function. This suggests that "nothing is wrong" with infant $CD8^+$ T cells, which show intrinsic properties similar to those of adult cells on a per cell basis. This is in accordance with the conclusions of numerous neonatal and early life vaccine studies (40, 41, 56, 81). These studies have demonstrated that 1-week-old mice raise adult-like responses to immunodominant LCMV peptides coated on synthetic microspheres, indicating a functional T-cell repertoire. Nevertheless, infant antiviral $CD8^+$ T-cell responses elicited by infection with LCMV-WE fail to achieve early viral clearance and to expand in order to generate immune memory cells.

The protracted pattern of infant LCMV infection that we described here is clearly different from the previously reported chronic patterns of infection. The infection of 2-week-old mice with LCMW-WE does not lead to an infection that persists indefinitely, which is different from the pattern elicited in adult mice with a high dose of LCMV-Docile (51, 83) or in adult CD4-deficient mice with LCMV clone 13 (19). Infection with

high doses of LCMV clone 13 results in an intense viral replication maintained in the kidneys and brain at high levels ($>10^5$ PFU/g) for more than 50 days, which does not resolve before 100 days postinfection (42, 76). In contrast, virus titers are already very low ($\leq 10^3$ PFU/g) by day 50 in the brain and kidneys of infant mice infected with LCMV-WE (Fig. 1A), indicating a better control of infection. Thus, the protracted LCMV infection pattern in mice infected at 2 weeks of age is different from the chronic persistent infections in immunocompetent or immunodeficient adult mice described previously.

The course of infection by LCMV-WE in infant mice is somehow reminiscent of the protracted course observed when adult immunocompetent mice are exposed to high doses of specific LCMV strains (19, 42, 51, 70, 82). This elicits primary $CD8^+$ T-cell responses to immunodominant epitopes that are initially robust but subsequently lost through a functional inactivation process successively affecting specific cytokine secretion and cytotoxic function (20, 45, 70, 76, 80, 82). Remarkably, IFN-γ production and cytotoxic capacity are lost much earlier (day 12) in infant mice infected with LCMV-WE than in adult mice exposed to LCMV clone 13 (day 30) (19). The early life primary $CD8^+$ T-cell response to 1 to 100 PFU or LCMV-WE is also similar to that reported in adult mice infected with a very high dose (at least 10^5 PFU [36]) of LCMV-WE. In these adult mice, intense antigen exposure results in a massive induction of $CD8^+$ T-cell responses that reach normal values on day 5 or 6 but subsequently plateau and are lost within a few weeks (13, 83). This pattern is thought to reflect the exhaustion of the primary immune response capacity of adult mice by high viral doses. The magnitude of early antiviral $CD8^+$ T-cell responses has been shown to vary inversely with viral inoculum (35). Lower inocula (1 or 0.1 PFU) of LCMV did not enhance the level of the $CD8^+$ T-cell response in infant mice (Fig. 4). Thus, the first conclusion of our observations is that the primary immune response capacity of 2-week-old mice may be exhausted by low (100 PFU) or even very low (1 PFU) doses of LCMV-WE that are far from reaching those doses (10^5 PFU [36]) that exceed the capacity of immunologically mature hosts. As the magnitude of the $CD8^+$ response is inversely proportional to the tempo and magnitude of viral replication (35), limitations of early life innate responses and/or $CD4^+$ T cells could fail to rapidly limit viral replication and adversely affect the expansion of $CD8^+$ T cells. This hypothesis is supported by the observation of higher viral titers on day 2 in infant mice than those in adult mice (Fig. 1).

Major differences between $CD8^+$ T-cell responses elicited in 2-week-old mice by LCMV-WE and adult mice exposed to high doses of LCMV concern immunodominance and memory $CD8^+$ T-cell responses. Chronic infections in adult mice skew immunodominance, allowing the preferential induction of subdominant T cells (76). This was not observed in infant mice, as responses to a dominant epitope and a subdominant epitope followed the same pattern of transient induction followed by disappearance. The subsequent reappearance of cytokine production by virus-specific $CD8^+$ T cells (19, 75) is also not observed following viral clearance in infant mice (Fig. 2A), and memory $CD8^+$ T cells are not elicited at any time point (Fig. 5A). Adoptive transfer experiments did not reveal the existence of any protection (Fig. 5C) or of LCMV-specific memory $CD8^+$ T cells against the dominant or subdominant epitope tested, excluding a contribution of CTLs directed against subdominant epitopes to virus clearance. Thus, the massive recruitment of CTLs into effector cells successfully elicited by the infection is followed in infant mice by their functional exhaustion and their peripheral clonal deletion, which purges the T-cell repertoire of LCMV-specific $CD8^+$ T cells. It is tempting to postulate that such permanent changes in the T-cell repertoire could contribute to the long-term influence of the early life environment on the risks and patterns of certain autoimmune diseases (43).

Why do LCMV-specific $CD8^+$ T cells elicited in 2-week-old mice fail to expand during the second week after infection? The expansion of a specific T-cell clone depends on the frequency of its precursors, its pattern of activation, its proliferative capacity, and its rate of apoptosis. The proliferative capacities of infant and adult $CD8^+$ T cells in response to infection are similar (Fig. 3D), and their levels of expression of the CD25 IL-2 receptor (data not shown) (17) are also similar. LCMVnp118-126-specific $CD8^+$ T cells also expand normally in response to immunization with inert antigen delivery systems presenting LCMV epitopes (41, 57). Although the rapid clearance of apoptotic cells in vivo may lead to the underestimation of apoptosis by ex vivo annexin staining, the rate of apoptosis was similar in both age groups, which is in accordance with the low levels of expression of proapoptotic genes by LCMV-specific $CD8^+$ T cells during the early stages of infection (82). In addition, numerous studies have demonstrated that $CD8^+$ T cells elicited in the neonatal period by specific antigen delivery systems are intrinsically similar to those induced in adult hosts (40, 41, 56, 81). Thus, the differences between normal early life $CD8^+$ T-cell responses to immunization and protracted $CD8^+$ T-cell responses to LCMV-WE infection suggest that the viral infection overwhelms the early life microenvironment, which fails to provide the signals required to support the massive $CD8^+$ T-cell expansion, persistence, and differentiation required to rapidly terminate an LCMV-WE infection.

Early life $CD8^+$ T-cell responses to LCMV-WE are supported by a large number of factors, among which dendritic cells (DCs), interferons, and $CD4^+$ T cells are the most likely candidates. The priming of LCMV-specific $CD8^+$ T cells is crucially dependent on DCs (53), and $CD8\alpha^+$ DCs are the only subset that activates naïve $CD8^+$ T cells exposed to LCMV (6). $CD8\alpha^+$ DCs are present at numbers similar to those in adults from the age of 7 days onwards (63), such that access to antigen-bearing $CD8\alpha^+$ DCs is unlikely to limit the induction of neonatal T-cell responses. Neonatal murine DCs competently induce adult-like $CD8^+$ T-cell responses to numerous vaccine antigens (reviewed in reference (2), including to the LCMV peptide used in this study (41, 57). However, the DC activation pattern could differ during early life and adult LCMV infections, resulting in differences in the production of interferons. Type I interferons play a critical role in $CD8^+$ T-cell expansion during LCMV infections (32, 51). Acutely infected IFN-α/β receptor$^{-/-}$ adult mice display delayed viral clearance and loss of effector functions of their $CD8^+$ T cells (51), and neonatal treatment with Fl3 ligand increased both type I interferon and antiviral resistance (73). The generation of LCMV-specific CTLs is also dependent on constitutively produced IFN-γ (79). Thus, the production of IFN-α/β and/or

IFN-γ by infected neonatal and infant DCs could be sufficient to support neonatal vaccine responses but nevertheless insufficient to support a rapid induction and efficient expansion of $CD8^+$ T-cell responses to infection. The higher LCMV titers observed in infant mice on day 2 (Fig. 1A), a time point considered as reflecting innate responses (39, 73), and the weaker CTL responses observed on day 4 (Fig. 2C) support this hypothesis.

Inadequate $CD4^+$ T-cell help may also impair $CD8^+$ T-cell responses. Although $CD4^+$ T cells are dispensable for the early control of acute LCMV infection in adult mice (4, 30), they are necessary to maintain effector responses and elicit and maintain long-term memory anti-LCMV $CD8^+$ T cells (30, 64). They support the emergence of $CD127^{high}$ $CD8^+$ T cells (18), which eventually constitute the $CD8^+$ T-cell memory pool (29). Accordingly, $CD4^+$ T cells play a major role in preventing the $CD8^+$ T-cell exhaustion that occurs upon adult exposure to high viral doses of LCMV (4). During chronic infection, their dysfunction occurs early (day 9) (9), as $CD4^+$ T cells lose their faculty to produce IL-2 (19, 20). It is thus tempting to attribute a role to $CD4^+$ T cells in the deficient expansion/maintenance/differentiation of infant LCMV-specific $CD8^+$ T cells. Again, although the immune response capacity of 1- to 2-week-old-mice is sufficient to raise adult-like $CD4^+$ T-cell responses in response to immunization (40), it could be overwhelmed when confronted with a challenge as massive as an LCMV infection. Adult LCMV-specific $CD4^+$ T cells produce only Th1-like cytokines (72), such as IFN-γ (51, 78) and IL-2 (69), which directly support $CD8^+$ T-cell responses. The preferential polarization of early life LCMV-specific $CD4^+$ T cells towards Th2 responses, a hallmark of early life responses (reviewed in reference 2), could thus compromise $CD8^+$ T-cell activation/expansion and consequently the induction/maintenance of memory $CD127^{high}$ $CD8^+$ T cells. An infant LCMV infection model is currently being adapted to C57BL/6 mice, in which the immunodominant LCMV-specific $CD4^+$ T-cell epitopes have been identified, to address the respective contributions of DC and $CD4^+$ T-cell responses to the protracted course run by LCMV in early life. Preliminary observations confirm that a protracted course of infection following infection in early life is also observed in $H\text{-}2^b$ C57BL/6 mice.

Regardless of the environmental factors that directly or indirectly contribute to limit infant $CD8^+$ T-cell expansion, they prevent antiviral $CD8^+$ T cells from achieving viral clearance. We therefore postulated that antibodies might contribute to LCMV clearance in early life, despite their minor role in adult hosts (4). LCMV-WE infection generated significantly higher titers of LCMV-neutralizing antibodies in infants than in adults (Fig. 6), a phenomenon previously observed in $CD8^+$ T-cell-depleted, infected adult mice (4, 14). B cells were indeed found to contribute to the control of LCMV infection in infant mice, as infant $\mu MT^{-/-}$ mice fail to achieve viral clearance, which results in either death or chronic infection (Fig. 6). $CD8^+$ T-cell responses were normal in adult $\mu MT^{-/-}$ mice, excluding a major antigen-presenting cell role for B cells in the priming of adult LCMV-specific $CD8^+$ T-cell effectors (16). This lethal/chronic pattern of LCMV-WE infection in infant $\mu MT^{-/-}$ mice may however result from the conjunction of the absence of B cells and of the neonatal limitations discussed above, such as those affecting $CD4^+$ T cells, as limitations of $CD4$ responses affect $\mu MT^{-/-}$ mice (25). As strong B-cell responses also promote T-cell activities, the neonatal limitations of B cells and $CD4^+$ cells may both contribute to the protracted course of infection observed in infant mice. Whether the failure of the expansion/differentiation of infant virus-specific $CD8^+$ T cells identified here similarly applies to other viral pathogens and to human infants, prolonging the course of certain viral infections (24, 49, 58), is certainly worth considering.

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