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Regulation of CD8$^+$ T Cells Undergoing Primary and Secondary Responses to Infection in the Same Host[1]

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Naive Ag-specific CD8$^+$ T cells expand, contract, and become memory cells after infection and/or vaccination. Memory CD8$^+$ T cells provide faster, more effective secondary responses against repeated exposure to the same pathogen. Using an adoptive transfer system with low numbers of trackable nontransgenic memory CD8$^+$ T cells, we showed that secondary responses can be comprised of both primary (naive) and secondary (memory) CD8$^+$ T cells after bacterial (*Listeria monocytogenes*) and/or viral (lymphocytic choriomeningitis virus) infections. The level of memory CD8$^+$ T cells present at the time of infection inversely correlated with the magnitude of primary CD8$^+$ T cell responses against the same epitope but directly correlated with the level of protection against infection. However, similar numbers of Ag-specific CD8$^+$ T cells were found 8 days postinfection no matter how many memory cells were present at the time of infection. Rapid contraction of primary CD8$^+$ T cell responses was not influenced by the presence of memory CD8$^+$ T cells. However, contraction of secondary CD8$^+$ T cell responses was markedly prolonged compared with primary responses in the same host mice. This situation occurred in response to lymphocytic choriomeningitis virus or *L. monocytogenes* infection and for CD8$^+$ T cell responses against multiple epitopes. The delayed contraction of secondary CD8$^+$ T cells was also observed after immunization with peptide-coated dendritic cells. Together, the results show that the level of memory CD8$^+$ T cells influences protective immunity and activation of naive precursors specific for the same epitope but has little impact on the magnitude or program of the CD8$^+$ T cell response. *The Journal of Immunology*, 2003, 170: 4933–4942.

In response to primary infection or vaccination, CD8$^+$ T cells undergo massive expansion followed by rapid contraction to memory levels (1). The magnitude of initial CD8$^+$ T cell expansion, which appeared to determine memory levels (2–4), was influenced at least in part by the number of naive precursors recruited into the response (5). Although the mechanisms that govern memory CD8$^+$ T cell formation remain elusive it seems that short-term Ag stimulation ($<$24 h) was sufficient to trigger a program of CD8$^+$ T cell expansion and subsequent contraction that resulted in generation of functional memory CD8$^+$ T cells (4–9). Prolonged infection and/or Ags display (after the first 24 h postinfection (p.i.)[3]) could increase memory CD8$^+$ T cell levels by recruiting more naive precursors into the primary response. However, experimental alteration in the duration of infection did not change the onset or rapid contraction in the Ag-specific CD8$^+$ T cell response to primary infection (4). In contrast, several recent studies with mouse models of viral infections and/or non-MHC transplantation Ags suggested that contraction in the secondary CD8$^+$ T cell response of immune hosts was prolonged compared with the rapid contraction observed after primary infection (10–13).

Under circumstances where the dose of infection or immunization resulted in incomplete recruitment of naive precursors into the primary response, immune hosts would contain both memory and naive T cells specific for the same pathogen Ags. In such cases, secondary CD8$^+$ T cell responses to re-infection could consist of both memory and previously naive pathogen-specific CD8$^+$ T cells, a situation that complicates interpretation of CD8$^+$ T cell contraction studies in immune mice. Previously, we described an adoptive transfer system to independently track the primary and secondary CD8$^+$ T cell responses in the same host after infection (4). In these studies, nuclear protein $(NP)_{118-126}$-specific memory CD8$^+$ T cells from lymphocytic choriomeningits virus (LCMV)-immune BALB/c (Thy1.2) hosts were transferred into naive BALB/c-Thy1.1 recipients. Recipient mice were challenged with recombinant *Listeria monocytogenes* (LM) that expressed a secreted fusion protein containing the $NP_{118-126}$ epitope (LM-NPs) (14). In this experimental setting both primary (Thy1.1) and secondary (Thy1.2) $NP_{118-126}$-specific CD8$^+$ T cell responses could be followed in a setting where memory CD4$^+$ T cell responses would not be stimulated. Under these conditions, contraction of the secondary CD8$^+$ T cell response was markedly prolonged compared with the primary response against the same epitope in the same host mice. This result occurred when the challenge dose of LM-NPs was varied or mice were treated with ampicillin 1 day p.i. with LM-NPs, suggesting that prolonged contraction of the secondary CD8$^+$ T cell response is programmed early after infection. Similar results were recently reported using a TCR-transgenic system to compare the contraction of primary and secondary CD8$^+$ T cell responses in the same host after viral infection (15). Thus, the adoptive transfer system with memory CD8$^+$ T cells provided a

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[3] Abbreviations used in this paper: p.i., postinfection; LM, *Listeria monocytogenes*; LCMV, lymphocytic choriomeningitis virus; LM-NPs, LM secreting $NP_{118-126}$ epitope; ICS, intracellular cytokine staining; DC, dendritic cell; BMDC, bone marrow-derived DC; LLO, listeriolysin O; NP, nuclear protein.

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powerful tool for analyses of primary and secondary responses in the same host after infection.

In this study we used the adoptive transfer model to determine the influence of different numbers of memory CD8$^+$ T cells on the magnitude of the naive CD8$^+$ T cell response and protection after infection. We also asked whether the delayed contraction of secondary CD8$^+$ T cell responses was observed in different models of infection (bacterial and/or viral), in the presence of memory CD4$^+$ T cell responses, and for multiple CD8$^+$ T cell epitopes. Finally, we asked whether the different programs of contraction in the primary and secondary CD8$^+$ T cell response were maintained after immunization with peptide-coated dendritic cells (DC) in the absence of infection.

Materials and Methods

Mice

BALB/c (H-2^d MHC, Thy1.2) mice were obtained from the National Cancer Institute (Frederick, MD). BALB/c Thy1.1 mice, obtained from R. Dutton (Trudeau Institute, Saranac Lake, NY), were maintained by brother-sister mating under specific pathogen-free conditions. Pathogen-infected mice were housed at the appropriate biosafety level. All mice were initially used at 8–16 wk of age.

Abs and peptides

The following mAbs were used: anti-IFN-γ-PE (clone XMG 1.2; eBioscience, San Diego, CA), anti-CD8-FITC or CyChrome (clone 53-6.7; BD PharMingen, San Jose, CA), αThy1.2 (clone 53-2.1; BD PharMingen), and anti-CD4-PE (clone L3T4; BD PharMingen). Synthetic peptides representing the defined LM epitopes (listeriolysin O $(LLO)_{91-99}$ and $p60_{217-225}$, H-2K^d-restricted (16, 17)) or LCMV ($NP_{118-126}$, H-2L^d-restricted (18)) were synthesized at the University of Iowa Protein Structure Facility (Iowa City, IA).

Bacteria and virus infection of mice

Virulent LM strain XFL303 (LM-NPs; LCMV $NP_{118-126}$-expressing) and attenuated *act*A-deficient LM-NPs were grown, injected, and quantified as previously described (14, 19). CFU per spleen and grams of liver were determined at the indicated days p.i. as described (20). Limit of detection was $<$100 CFU/organ. The Armstrong strain of LCMV (LCMV-Arm; 2×10^5 PFU/mouse, i.p.) was used as described (21). Virus titers in homogenates of spleen and liver were determined by plaque assay on VERO cells as described (14).

CD8$^+$ T cell enrichment

Splenocyte cell suspensions from naive and/or LCMV memory mice were enriched for CD8$^+$ T cells by negative selection (StemCell Technologies, Vancouver, British Columbia, Canada). Briefly, 8×10^7 splenic leukocytes/ml were labeled with a CD8$^+$ T cell enrichment mixture including Abs specific for B220, Gr-1, CD4, CD11b, TER119, and DX5, followed by conjugation of Ab-labeled cells to magnetic beads. CD8$^+$ T cells were obtained in the flow-through after passing the labeled cells through a magnetic separation column (Macs LS Separation Column; Miltenyi Biotec, Auburn, CA). The recovered cells were routinely $>$93% CD8$^+$ by flow cytometry. Recovered cells were washed three times in sterile 0.9% saline and transferred i.v. into mice 1 day before LCMV-Arm infection.

Adoptive transfer experiments

CD8$^+$ T cell-enriched splenocytes from naive or LM- or LCMV-immune BALB/c Thy1.2 mice were transferred at the indicated days p.i. into naive BALB/c Thy1.1 mice. Recipient mice were infected 1 or 2 days later with the indicated dose of LM or LCMV. At various days after challenge, recipient mice were euthanized and spleens were taken for analysis.

Generation of peptide-coated bone marrow-derived DC (BMDC)

CD11c$^+$ BMDC were generated as previously described (22). Briefly, RBC-depleted BALB/c (H-2^d) bone marrow was subjected to complement depletion after incubation with mAbs anti-CD8 (3.168), anti-I-A^d (34-5-3), anti-CD4 (RL172), anti-B220/CD45R (RA3-3A1/6.1), and anti-Ly6G (RB6-8C5). All mAbs were purified from culture supernatant by protein G (Amersham Pharmacia Biotech, Piscataway, NJ). Remaining cells were plated at 1×10^6/ml in RP10 supplemented with 1000 U/ml rGM-CSF (BD PharMingen) and 25 U/ml rIL-4 (PeproTech, Rocky Hill, NJ) and incubated for 5 days with 75% medium replacement every other day. LPS (500 ng/ml; Sigma-Aldrich, St. Louis, MO) was then added for 1–2 days to induce maturation, and 1 μM LLO_{91-99} peptide was added to cultures 3 h before harvest. Cells were then washed extensively before injection. These cells were also H-2L^{d+}, B7.1$^+$, B7.2$^+$, CD8a$^-$, I-A^{d+}, and CD11b$^+$. Based on the percentage of CD11c$^+$ cells (determined before injection), 2.5×10^5 mature BMDC were injected i.v. per mouse.

Quantification of Ag-specific CD8$^+$ T cells

The magnitude of the epitope-specific CD8$^+$ T cell response was determined by intracellular cytokine staining (ICS) for IFN-γ as previously described (23). Briefly, 20×10^6 splenocytes from an infected mouse were treated with ACK lysis buffer for 5 min at room temperature to remove RBC. Splenocytes were washed twice in RP10 and resuspend in the same medium in 1 ml. Cells (200 μl) were incubated for 6 h at 37°C with medium alone or with a synthetic peptide epitope in the presence of 1 μl/ml GolgiPlug (brefeldin A). Cells were washed twice in FACS buffer (PBS supplemented with 1% FCS and NaN_3) and were incubated with Ab directed against FcγRII/RIII (2.4G2), and CyChrome-labeled anti-CD8 (53-6.7, BD PharMingen) and FITC-labeled anti-Thy1.2 (53-2.1, BD PharMingen) on ice for 30 min. The cells were washed twice with FACS buffer then fixed and permeabilized by incubating for 15 min in 200 μl of Cytofix/Cytoperm solution. Cells were then washed twice in Perm/Wash solution and stained with PE-conjugated anti-IFN-γ (XMG 1.2; eBioscience) for 30 min on ice. Cells were washed twice in perm/wash solution and resuspended in 200 μl of FACS buffer before flow cytometry analysis.

List mode data were acquired on FACSCalibur flow cytometer (BD Biosciences) using CellQuest software. Typically, 100,000 events for splenocytes were acquired for analysis with Flow Jo software (Tree Star, San Carlos, CA).

Results

Influence of memory CD8$^+$ T cell level on expansion of naive CD8$^+$ T cells and protection after LCMV infection

Several studies have shown that transfer of increasing numbers of naive TCR-transgenic CD8$^+$ T cells into host mice before infection or immunization resulted in a corresponding reduction in the magnitude of the endogenous primary CD8$^+$ T cell response against the same epitope, suggesting competition for a limited number of APC (24, 25). In contrast, less is known about competition between naive and memory CD8$^+$ T cells specific for the same epitopes, and no experiments have been reported in non-TCR-transgenic systems. Using a system in which we transferred different numbers of Ag-specific memory CD8$^+$ T cells into naive mice (therefore preserving the pool of naive Ag-specific CD8$^+$ T cells), we determined the impact of memory cell numbers on the magnitude of the endogenous primary CD8$^+$ T cell response and the overall magnitude of the epitope-specific response. In addition, we determined how many memory CD8$^+$ T cells are required to reduce viral load after LCMV-Arm infection.

LCMV-Arm-immunized Thy1.2 BALB/c mice ($>$100 days p.i.) contained $\sim$10% of $NP_{118-126}$-specific CD8$^+$ T cells in the spleen as detected by intracellular IFN-γ staining (data not shown). We transferred different numbers of Thy1.2 splenocytes into Thy1.1 BALB/c mice (Fig. 1*A*), and 1 day later infected recipient as well as control groups of Thy1.1 BALB/c mice with 2×10^5 PFU of LCMV-Arm. Viral titers in the spleens were determined at day 3 p.i. (Fig. 1*B*). Mice that received 1×10^7 splenocytes ($\sim 8.5 \times 10^4$ $NP_{118-126}$-specific CD8$^+$ T cells) or more reduced viral titers by $\sim$300-fold in the spleen when compared with mice that did not receive memory CD8$^+$ T cells. In contrast, 10-fold fewer memory $NP_{118-126}$-specific CD8$^+$ T cells ($\sim 8.5 \times 10^3$) did not enhance viral clearance at day 3 p.i. (group 3, Fig. 1*B*). Similar results were also obtained in the lungs of the infected mice (data not shown). Thus, the level of protection depended on the number of memory CD8$^+$ T cells present at the time of LCMV infection. One potential complication to interpretation of these data was the presence of both memory CD4$^+$ T cells and B cells in the donor immune

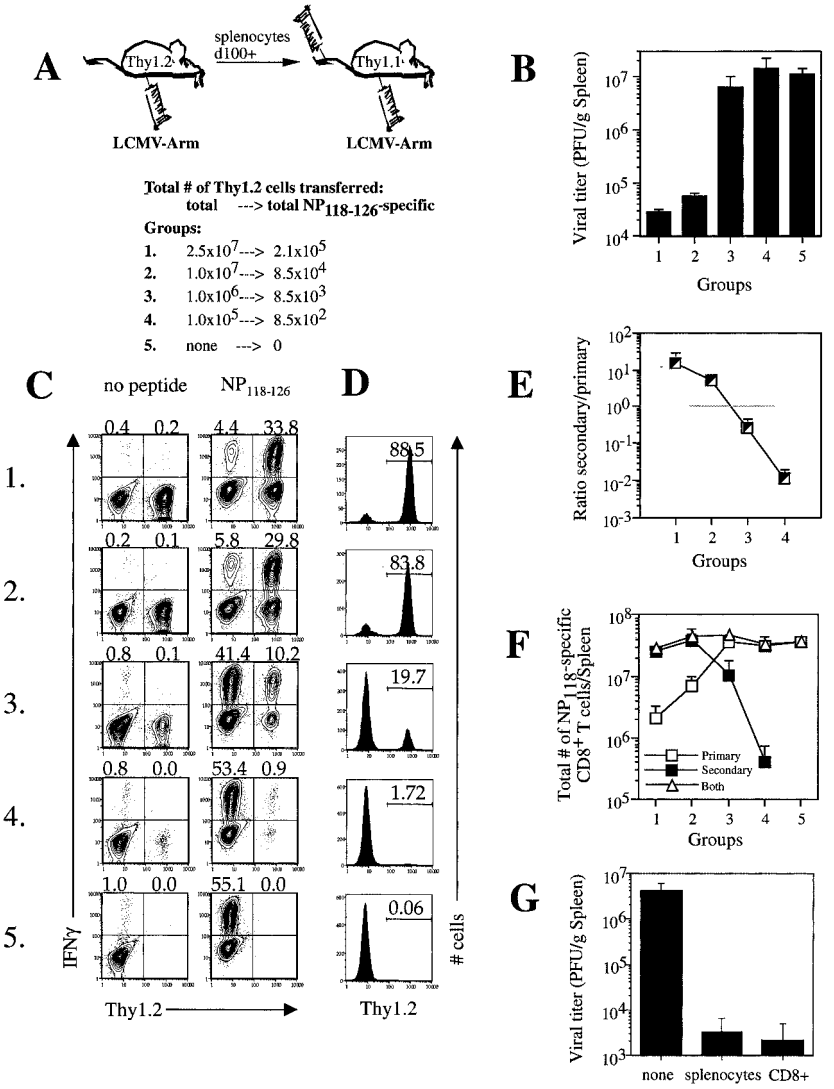

FIGURE 1. Visualizing primary and secondary $CD8^+$ T cell expansion in the same host after LCMV infection. *A*, Experimental design. Naive BALB/c Thy1.1 mice that received indicated number of splenocytes from LCMV infected memory (day 100+) BALB/c Thy1.2 mice were infected with LCMV-Arm (2×10^5 PFU/mouse). *B*, LCMV titer in the spleens at day 3 after LCMV challenge. *C*, $NP_{118-126}$-specific $CD8^+$ T detected by intracellular IFN-γ in representative mice on day 8 p.i. Numbers represent frequencies of Ag-specific $CD8^+$ T cells in the spleen. *D*, Frequencies of secondary $Thy1.2^+$ $NP_{118-126}$-specific $CD8^+$ T cells among all $NP_{118-126}$-specific $CD8^+$ T cells. *E*, Ratio ± SD (secondary/primary) is calculated using total numbers of $NP_{118-126}$-specific $CD8^+$ T cells detected in the spleens after LCMV challenge. *F*, Total number of Thy1.1 and Thy1.2 $NP_{118-126}$-specific $CD8^+$ T cells per spleen ($n = 3$/group). *G*, In a separate experiment, naive BALB/c mice that received unseparated or $CD8^+$ T cell-enriched (~95% purity) splenocytes from LCMV infected memory (day 100+) mice were infected with LCMV-Arm (2×10^5 PFU/mouse). Before transfer total number of $NP_{118-126}$-specific $CD8^+$ T cells was determined by tetramer staining, and both populations of cells contained 1×10^5 $NP_{118-126}$-specific $CD8^+$ T cells. LCMV titer in the spleens at day 3 after LCMV challenge is shown ($n = 3$/group)

splenocyte populations. However, transfer of ~10^5 $NP_{118-126}$-specific memory $CD8^+$ T cells as whole splenocytes or as enriched $CD8^+$ T cells resulted in similar (~3 $\log_{10}$) reduction in LCMV titers, indicating that $CD8^+$ T cells were sufficient for virus clearance by immune splenocytes in this assay (Fig. 1*G*). In addition, $>10^4$ but $<10^5$ $NP_{118-126}$-specific $CD8^+$ T cells per mouse were required to substantially reduce viral load at 3 days after infection with 2×10^5 PFU of LCMV-Arm.

We also compared the expansion of primary $NP_{118-126}$-specific $CD8^+$ T cells after LCMV infection in the presence of different numbers of memory $NP_{118-126}$-specific $CD8^+$ T cells. Thy1.2 expression was used to distinguish the contribution of primary (Thy1.1) and secondary (Thy 1.2) $CD8^+$ T cells in the pool of $NP_{118-126}$-specific $CD8^+$ T cells at day 8 after viral infection (Fig. 1, *C* and *D*). Primary $NP_{118-126}$-specific $CD8^+$ T cell responses were detected in all groups of mice although the contribution of those cells inversely correlated with the numbers of memory $CD8^+$ T cells transferred (Fig. 1, *E* and *F*). Plotting the ratio between frequencies of secondary vs primary $CD8^+$ T cells from various groups suggested that ~10,000 memory $CD8^+$ T cells per mouse would result in similar numbers of primary and secondary $CD8^+$ T cells at day 8 after LCMV infection (Fig. 1*E*, gray line). Strikingly, the total numbers of $NP_{118-126}$-specific $CD8^+$ T cells were essentially identical in all groups of mice despite different numbers of memory $CD8^+$ T cells before the infection (Fig. 1*F*).

These data demonstrate that the level of memory $CD8^+$ T cells directly correlated with protection and inversely correlated with recruitment of naive precursors in the same host mice. Thus, some level of competition exists for activation of naive and memory $CD8^+$ T cells of the same specificity. However, memory cell numbers that represent 100- to 1000-fold increases over the estimated endogenous naive precursor pool (26) and provide significant protection, as judged by reduction in viral load, did not prevent a substantial response from the endogenous naive precursors. Thus, secondary responses to infection could be composed of both primary and memory $CD8^+$ T cells if naive precursors are present at the time of rechallenge. Finally, equivalent $CD8^+$ T cell expansion was seen in the spleen at 8 days p.i. despite the number of LCMV-specific memory $CD8^+$ T cells at the time of infection. Thus, at least in the LCMV model, the total number of epitope-specific cells generated after infection is regulated and independent of the number of memory cells that contribute to the response.

Kinetics of primary and secondary $CD8^+$ T cell responses after LCMV infection

In our previous study, we generated memory $NP_{118-126}$-specific $CD8^+$ T cells by LCMV infection of BALB/c mice, transferred these cells into BALB/c-Thy1.1 recipients, and challenged with LM-NPs to elicit primary and secondary $CD8^+$ T cell responses in the same host (4). In this experimental setting, which revealed

prolonged contraction of the secondary CD8$^+$ T cell response, the only secondary response generated was specific for $NP_{118-126}$, and no secondary CD4$^+$ T cell responses or responses to other CD8$^+$ T cell epitopes would be expected (14). To determine whether the kinetics of contraction would be altered by 1) concurrent secondary T cell responses (both CD4$^+$ and CD8$^+$) against other Ags or 2) the number of memory cells at the time of infection, we used a system where memory T cells were generated by LCMV infection, transferred to naive recipients, and restimulated by challenge with LCMV. Thy1.1 BALB/c mice received 0.8×10^6 or 8×10^6 splenocytes from LCMV immune BALB/c Thy1.2 mice before LCMV challenge. The total number of $NP_{118-126}$-specific CD8$^+$ T cells transferred was 1.2×10^4 or 1.2×10^5 per mouse (Fig. 2*A*).

The level of memory $NP_{118-126}$-specific CD8$^+$ T cells at the time of infection again influenced the magnitude of expansion of naive $NP_{118-126}$-specific CD8$^+$ T cells (Fig. 2). When ~10,000 memory cells were present at the time of challenge, the expansion of newly activated endogenous $NP_{118-126}$-specific CD8$^+$ T cells was similar to the expansion of memory cells (18.3% of CD8$^+$ T cells compared with 20.5%) (Fig. 2, *B* and *C*). This number is consistent with the estimate from the data in Fig. 1*E*. However, this result was unexpected in light of recent data showing that transfer

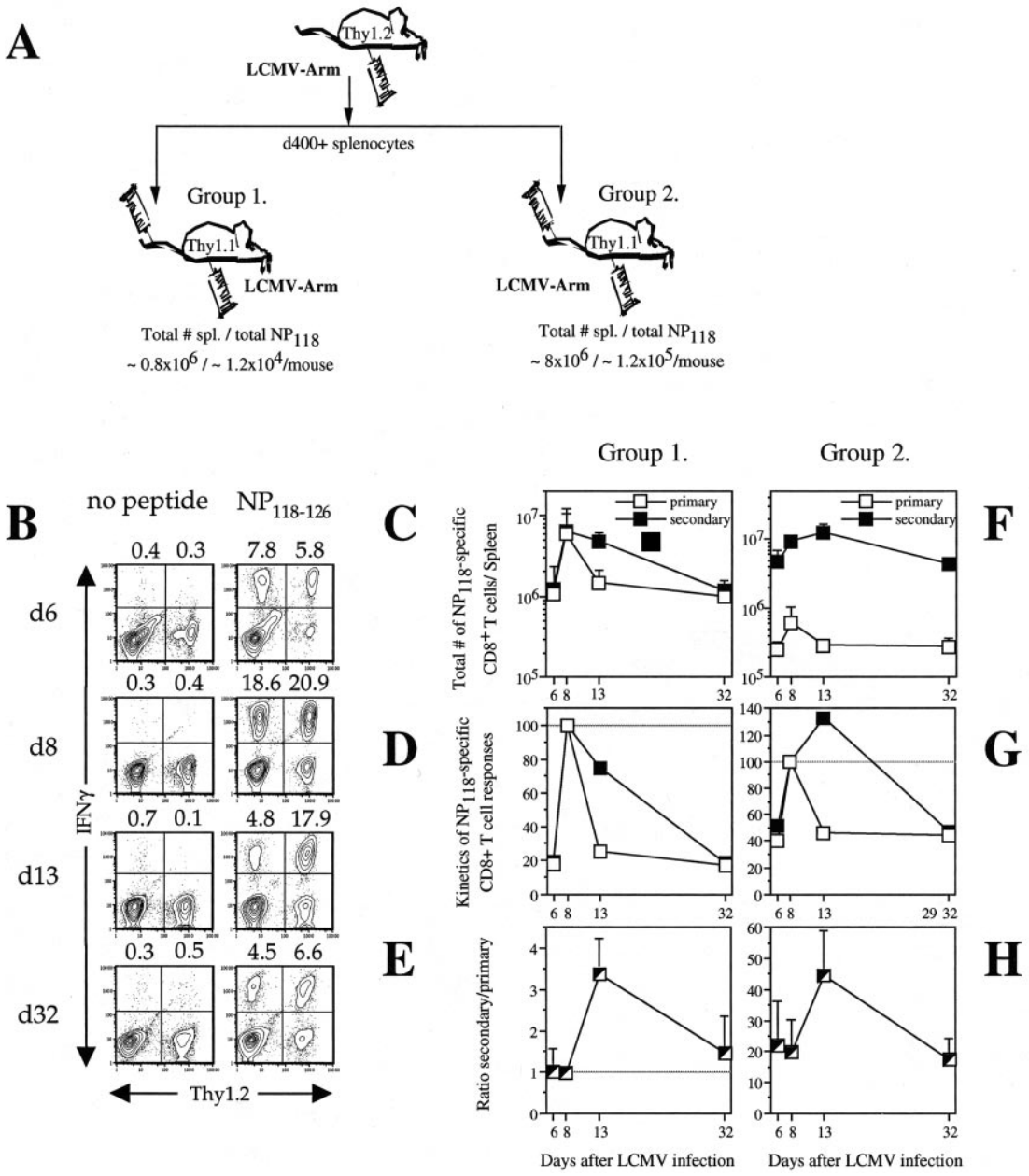

FIGURE 2. Kinetics of primary and secondary CD8$^+$ T cell responses after LCMV infection. *A*, Experimental design. Naive BALB/c Thy1.1 mice that received 0.8×10^6 (group 1, *B–E*) or 8×10^6 (group 2, *F–H*) splenocytes from LCMV infected memory (d400$^+$) BALB/c Thy1.2 mice were infected with LCMV-Arm (2×10^5 PFU/mouse). The total number of $NP_{118-126}$-specific cells transferred is indicated. *B*, Frequencies of primary Thy1.2$^-$ and secondary Thy1.2$^+$ $NP_{118-126}$-specific CD8$^+$ T cells from representative mice (group 1) at various days p.i. *C* and *F*, Total number of primary and secondary $NP_{118-126}$-specific CD8$^+$ T cells per spleen at indicated days p.i. ($n = 3$/group). *D* and *G*, Normalized kinetics $NP_{118-126}$-specific CD8$^+$ T cell responses in the spleen. The peak of the response (day 8 p.i.) is presented as 100%. *E* and *H*, Ratio ± SD (secondary/primary) is calculated using total numbers of $NP_{118-126}$-specific CD8$^+$ T cells detected in the spleens of individual mice at indicated days after LCMV challenge.

of <1000 naive TCR-transgenic cells before LCMV infection resulted in similar expansion of endogenous and TCR-transgenic cells specific for the same epitope (26). When 10× more memory cells (~100,000/mouse) were present at the time of LCMV infection, the magnitude of expansion in the primary $NP_{118-126}$-specific $CD8^+$ T cell response was reduced by >10-fold, although still detectable (Fig. 2*F*). These data support the notion that competition between memory and naive $CD8^+$ T cells can occur in vivo and influence the composition of the $CD8^+$ T cell response.

Despite the different magnitudes of primary $CD8^+$ T cell expansion in the two groups, contraction was rapid in both, with 60–80% loss of Ag-specific $CD8^+$ T cells from day 8 to 13. The contraction of secondary (memory) $CD8^+$ T cells in the same 5-day period was much less pronounced, with either no decrease (group 2) or <25% decrease (group 1) (Fig. 2, *D* and *G*). Thus, the program of contraction in $CD8^+$ T cells that are responding to infection for the second time was prolonged after viral infection in an experimental setting where the magnitude of expansion varied and secondary $CD4^+$ T cell responses were likely to occur. One caveat to this interpretation is the lack of formal proof that a secondary response of $CD4^+$ T cells was generated. Currently, no strong $CD4^+$ T cell epitopes from LCMV or LM are identified in the BALB/c system. However, we are routinely able to detect both memory $CD4^+$ and $CD8^+$ T cell responses after transfer in the LM model of infection of B6 mice (data not shown), thus the experimental design clearly permits both responses.

To formally exclude the influence of in vitro manipulation during the transfer process as the cause for the delayed contraction of secondary $CD8^+$ T cells after LCMV infection, we transferred naive $CD8^+$ T cell-enriched Thy1.2 splenocytes into naive Thy1.1 BALB/c mice (Fig. 3*A*). Recipient mice were infected with LCMV-Arm 1 day later and the $CD8^+$ T cell response was analyzed on days 8 and 15 p.i. The contraction kinetics of both Thy1.1 and Thy1.2 primary responses in the same host were rapid (Fig. 3, *B–D*). Therefore, in vitro manipulation does not impose an inevitable program of delayed contraction on the transferred T cell population. Based on these data we conclude that the program of contraction of primary and memory $CD8^+$ T cells in the same host is independent of the starting number or magnitude of expansion of each population after infection.

Kinetics of primary and secondary $CD8^+$ T cell responses after LM infection

The previous work from our laboratory and others focused on contraction of primary and secondary $CD8^+$ T cell response to single, dominant epitopes from LCMV that are recognized after infection of BALB/c or B6 mice (4, 15). To determine whether prolonged contraction of secondary $CD8^+$ T cell responses can be generalized for multiple epitopes and to bacterial infections, we used LM infection of BALB/c mice, in which multiple $H\text{-}2^d$ MHC class I-restricted $CD8^+$ T cell epitopes are defined (27).

LM immunized Thy1.2 BALB/c mice (>100 days p.i. with *act*A-deficient LM-NPs) were used as splenocyte donors. These donor mice contained ~0.3%, 0.07%, and 0.4% of LLO_{91-99}-, $p60_{217-225}$-, and $NP_{118-126}$-specific $CD8^+$ T cells, respectively, in the spleen as detected by intracellular IFN-γ staining (data not shown). We transferred ~1.5×10^7 immune splenocytes (~1.5×10^4 LLO+p60 plus NP-specific $CD8^+$ T cells) into naive BALB/c Thy1.1 mice and subsequently challenged these mice and control mice with virulent LM-NPs (~1 LD_{50}). In this experiment, we were able to detect primary ($Thy1.2^-$) and secondary ($Thy1.2^+$) $CD8^+$ T cell expansion in the same host for all three epitopes (Fig. 4). In addition, the low levels of memory cells were sufficient to provide protection because all of the splenocyte-recipient mice survived to day 7 p.i. compared with 25% survival in mice that did not receive immune splenocytes. Thus, under conditions where relatively few memory cells were transferred, we were able to detect substantial, and nearly equivalent (Fig. 4*C*) expansion of both primary and secondary Ag-specific $CD8^+$ T cell responses in the same host.

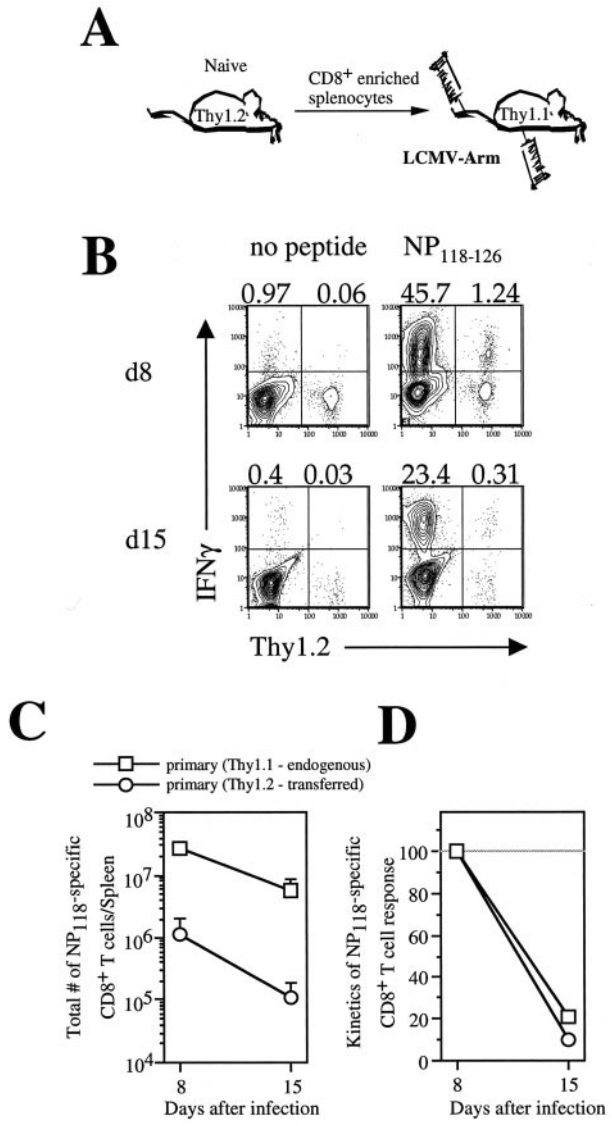

FIGURE 3. Kinetics of endogenous and transferred primary $CD8^+$ T cell responses after LCMV infection. *A*, Experimental design. $CD8^+$ T cell-enriched population of splenocytes (~94% pure) from naive BALB/c Thy1.2 mice were transferred (8×10^6) into naive BALB/c Thy1.1 a day before LCMV-Arm infection. *B*, Frequencies of endogenous $Thy1.2^-$ and transferred $Thy1.2^+$ $NP_{118-126}$-specific $CD8^+$ T cells from representative mice at day 8 and 15 p.i. C, Total number of Thy1.1 and Thy1.2 $NP_{118-126}$-specific $CD8^+$ T cells per spleen ($n = 3$). *D*, Normalized kinetics $NP_{118-126}$-specific $CD8^+$ T cell responses in the spleen. Day 8 p.i. is presented as 100%.

We next determined the kinetics of primary vs secondary Ag-specific $CD8^+$ T cell responses in the same host after LM challenge. Naive BALB/c Thy1.1 mice were used as recipients of splenocytes derived from day 100-plus LM-NPs immune Thy1.2 mice. In repeated experiments, which included transfer up to 2.5×10^7/splenocytes, 10 ± 2% of transferred cells were found in the spleen (data not shown). Similar results were described recently for transfer of naive TCR-transgenic cells (26). After transfer of 1.2×10^7 splenocytes/mouse in the experiment presented in Fig.

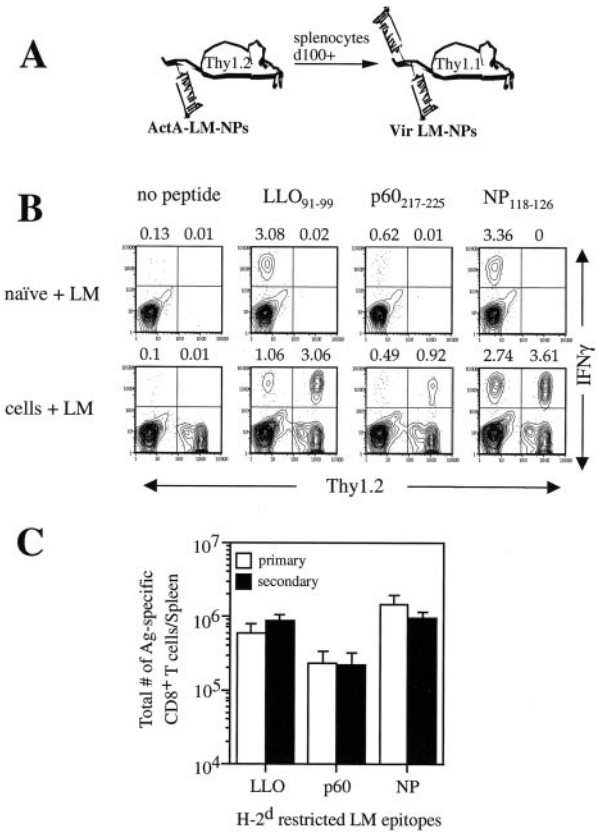

FIGURE 4. Visualizing primary and secondary CD8$^+$ T cell responses in the same host after LM infection. BALB/c Thy1.2 mice were infected with $\sim 1 \times 10^6$ ($\sim$0.1 LD_{50}) of $NP_{118-126}$-expressing *act*A-deficient LM (*act*A-LM-NPs) and used as donors >100 days postinitial challenge. BALB/c Thy1.1 mice that received splenocytes (1.5×10^7) from Thy1.2 LM-infected memory mice as well as control group of mice (*A*) were challenged with 10^4 (1 LD_{50}) of virulent LM-NPs. B, LLO_{91-99}, $p60_{217-225}$, and $NP_{118-126}$-specific CD8$^+$ T detected by intracellular IFN-γ in representative mice on day 7 p.i. Numbers represent frequencies of Ag-specific CD8$^+$ T cells in the spleen. *C*, Total number of Thy1.1 and Thy1.2 Ag-specific CD8$^+$ T cells per spleen ($n = 4$). Survival at day 7 p.i. was 25% (1 of 4) and 100% (17 of 17) for naive and recipient mice, respectively.

5, $\sim$8% of transferred cells were found in the spleen. In this population, <1% of the CD8$^+$ T cells were specific for LLO_{91-99}, $p60_{217-225}$, and $NP_{118-126}$ as determined by ICS for IFN-γ before transfer (Fig. 5*A*). The total number of Ag-specific CD8$^+$ T cells (LLO+p60 plus NP) transferred was $<10^4$ cells. Two days after adoptive transfer, recipient and control mice were infected with 6×10^3 of Vir LM-NPs (Fig. 5, *A* and *B*). Because the level of infection was reduced below the LD_{50} all mice survived throughout the course of the experiment.

The presence of memory CD8$^+$ T cells did not prevent substantial expansion of endogenous Ag-specific CD8$^+$ T cells after LM infection, which reached similar levels as observed in control groups of mice (Fig. 5, *C*, *D*, *F*, *G*, *I*, and *J*). Interestingly, immunodominance hierarchies between epitopes were preserved in primary and secondary CD8$^+$ T cell responses, suggesting that precursor frequencies of both naive and memory CD8$^+$ T cells may be important determinants of CD8$^+$ T cell expansion. Finally, the overall kinetics of the primary Ag-specific CD8$^+$ T cell response were similar whether or not memory cells were present at the time of LM infection (Fig. 5, *E*, *H*, and *K*).

The secondary response of transferred LLO_{91-99}, $p60_{217-225}$, or $NP_{118-126}$ memory CD8$^+$ T cells exhibited significantly delayed contraction in the spleen in comparison to rapid contraction of the primary response (Fig. 5, *D*, *G*, and *J*) where $\sim$60–80% of Ag-specific CD8$^+$ T cells were lost between day 7 and day 12. The contraction of secondary (memory) CD8$^+$ T cells in the same 5-day period was much less pronounced, with either no decrease (p60) or <30% decrease (LLO, NP) (Fig. 5, *E*, *H*, and *K*). Thus, contraction of secondary CD8$^+$ T cell responses was protracted for all LM epitopes analyzed, suggesting coordinate regulation of secondary CD8$^+$ T cell contraction.

Although LLO_{91-99}, $p60_{217-225}$, and $NP_{118-126}$ are thought to be dominant epitopes in this system, the complexity of the CD8$^+$ T cell response to LM is unknown. The *Listeria* genome contains >2800 genes (28). Among them, $\sim$60 represent secreted proteins that by definition could serve as suitable targets for MHC class I-restricted CD8$^+$ T cell priming. Also, nonsecreted LM proteins may prime CD8$^+$ T cell responses (14), thus the potential number of epitopes may be quite large. We used the adoptive transfer of memory cells to ask whether we can account for the Ag specificity of the Thy1.2$^+$/CD8$^+$ T cells undergoing secondary response to infection.

Before infection, the spleen of recipient mice contained $\sim 8 \times 10^4$ Thy1.2$^+$/CD8$^+$ cells and $\sim 2 \times 10^5$ Thy1.2$^+$/CD8$^-$ cells (primarily CD4$^+$ T cells) (Fig. 6*B*). LLO+p60 plus NP-specific CD8$^+$ T cells represent <1% ($\sim 8 \times 10^2$) of the Thy1.2$^+$/CD8$^+$ T cells in the spleen (Fig. 6*A*). At day 29 p.i., the number of Thy1.2$^+$/CD8$^+$ T cells had increased 15-fold to $>10^6$ per spleen. At the same time, LLO+p60+NP-specific CD8$^+$ T cells represented $\sim$50% (5×10^5) of Thy1.2$^+$/CD8$^+$ T cells. Because the number of Thy1.2$^+$/CD8$^+$ T cells increased by $\sim 9 \times 10^5$, these results suggest that the LLO+p60+NP epitopes account for $\sim$50–60% of LM-specific CD8$^+$ T cells in the secondary response. Therefore, a substantial expansion of Ag-specific CD8$^+$ T cells of "unknown" specificity occurred that equaled the expansion of LLO+p60+NP-specific CD8$^+$ T cells. Because the immunodominance hierarchy was preserved throughout the course of infection, this small proportion of unknown LM-specific CD8$^+$ T cells were presumably specific for subdominant epitopes such as $p60_{449-457}$ and mpl_{84-92} (27). Together, these data suggest that there may be a few strong, as yet undefined, LM-derived CD8$^+$ T cell epitopes. Alternatively, a large number of weak epitopes that stimulate undetectable primary CD8$^+$ T cell responses may contribute to the overall number of Ag-specific CD8$^+$ T cells in the secondary response. Based on the complexity of the LM genome, we favor the latter possibility.

Finally, CD4$^+$ T cells are activated after LM infection as well, although much less is known about the Ag specificity of CD4$^+$ T cells in H-2^d mice (29). Nevertheless, in contrast to the substantial expansion of transferred CD8$^+$ T cells ($\sim$15-fold), the total number of Thy1.2$^+$/CD8$^-$ T cells found at day 29 p.i. was only increased by 50% ($\sim$1.5-fold) more than was the number found immediately following transfer (Fig. 3*B*). Additional experiments will be required to resolve contribution of Ag-specific CD4$^+$ T cells during the course of infection in these mice.

Kinetics of primary and secondary CD8$^+$ T cell responses after DC immunization

The reason for the differences in contraction of Ag-specific CD8$^+$ T cells responding for the first or second time to infection are unknown. One fundamental distinction between these populations is the stringent requirement for Ag presentation by mature DC, with high levels of costimulatory molecule expression, for activation of naive T cell responses (30). In contrast, the same costimulatory molecules appear dispensable for activation of memory T cell responses, which may be triggered by interaction with any cell

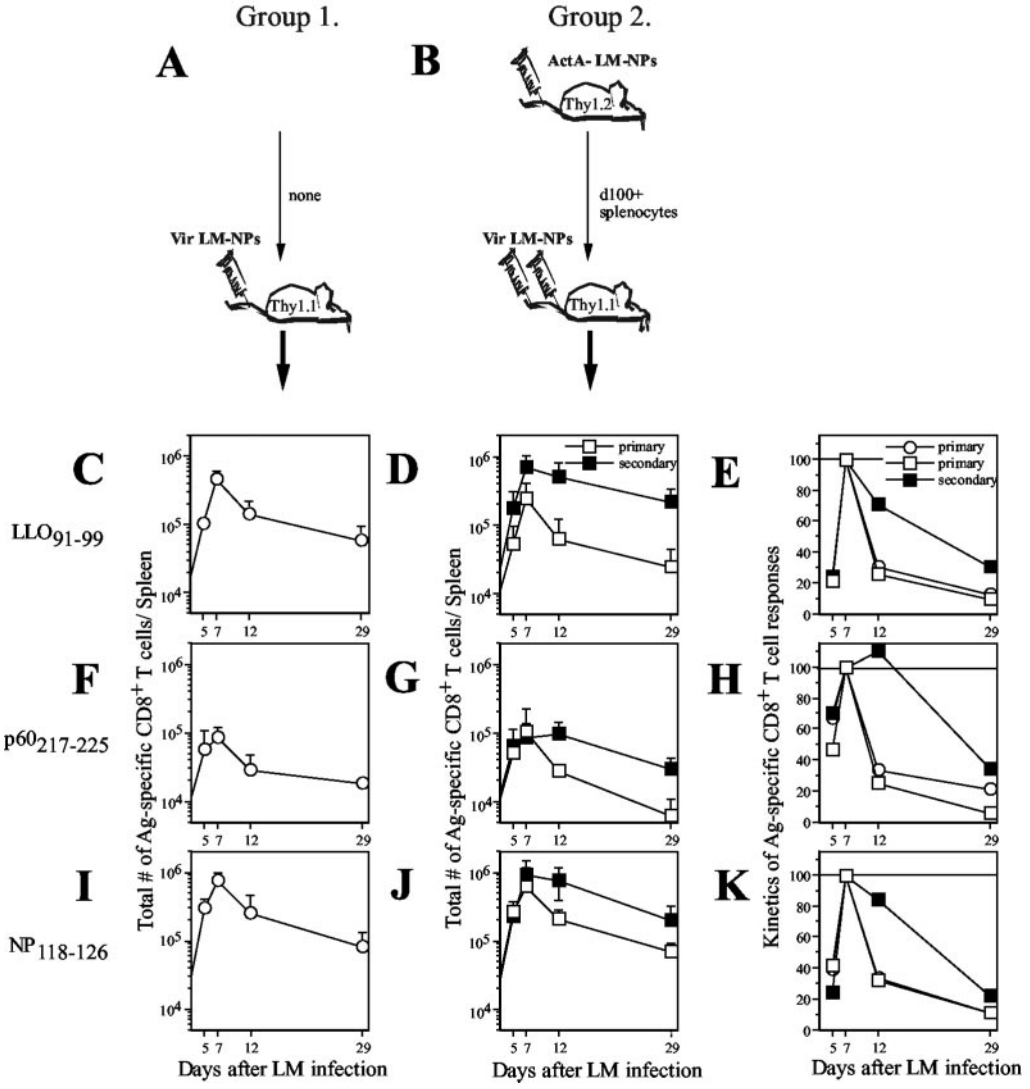

FIGURE 5. Kinetics of primary and secondary $CD8^+$ T cell responses after LM infection. *A* and *B*, Experimental design. BALB/c Thy1.2 mice were infected with $\sim 1 \times 10^6$ ($\sim$0.1 LD_{50}) *act*A-LM-NPs and used as donors >100 days after initial challenge. BALB/c Thy1.1 mice that received splenocytes (1.2×10^7) from Thy1.2 LM-infected memory mice (group 2, *B*) as well as control group of mice (group 1, *A*) were challenged with 6×10^3 ($\sim$0.5 LD_{50}) of virulent LM-NPs. Total number of LLO_{91-99}- (*C* and *D*), $p60_{217-225}$- (*F* and *G*), and $NP_{118-126}$-specific (*I* and *J*) $CD8^+$ T cells per spleen at indicated days p.i. (n = 3/group). Normalized kinetics of LLO_{91-99}- (*E*), $p60_{217-225}$- (*H*), and $NP_{118-126}$-specific (*K*) $CD8^+$ T cell responses in the spleen. The peak of the response (day 7 p.i.) is presented as 100%. Both groups of mice cleared infection by day 7 (limit of detection $\sim$100 CFU) with 100% survival.

that presents the appropriate peptide-MHC complex (1). Thus, it is possible that the nature of the APC dictates the program of contraction. To address this issue, we activated primary and secondary $CD8^+$ T cell responses in the same host by immunization with peptide-coated DC (22) and followed the kinetics of contraction.

Thy1.2 BALB/c splenocytes (containing $\sim$4000 LLO_{91-99}-specific $CD8^+$ T cells) from LM-immune mice were transferred into naive Thy1.1 mice (Fig. 7*A*). One day later, the recipient mice were immunized with 2.5×10^5 LLO_{91-99}-coated $CD11c^+$ DC (22). In repeated experiments, the peak of the expansion of LLO_{91-99}-specific $CD8^+$ T cells, for primary and secondary responses, was day 5 postimmunization (Fig. 7). The total numbers of primary $CD8^+$ T cells declined 5-fold from the peak of the response by day 12 postimmunization. In contrast, the number of secondary $CD8^+$ T cells was only reduced 2-fold over the same 7-day period (Fig. 7*D*). Thus, delayed contraction of secondary $CD8^+$ T cell responses was observed after DC immunization in the absence of infection. These data suggest that the program of rapid contraction, as seen with primary $CD8^+$ T cell responses, was not imposed solely by DC-mediated activation.

Discussion

In this report, we employed an adoptive transfer system to dissect the relationships between $CD8^+$ T cells responding for the first or second time to infection in the same host. We showed that expansion, but not contraction, of primary Ag-specific $CD8^+$ T cells after infection was influenced by the levels of pre-existing memory $CD8^+$ T cells. However, the overall magnitude of the Ag-specific $CD8^+$ T cell response was similar at 8 days after LCMV infection, no matter the starting number of memory cells or their contribution to the antiviral response. We also extended our previous findings (4) and those of Grayson et al. (15) to show that delayed contraction in the secondary $CD8^+$ T cell response was observed in nontransgenic systems, after viral or bacterial infections, against multiple epitopes and was not influenced by the numbers of memory

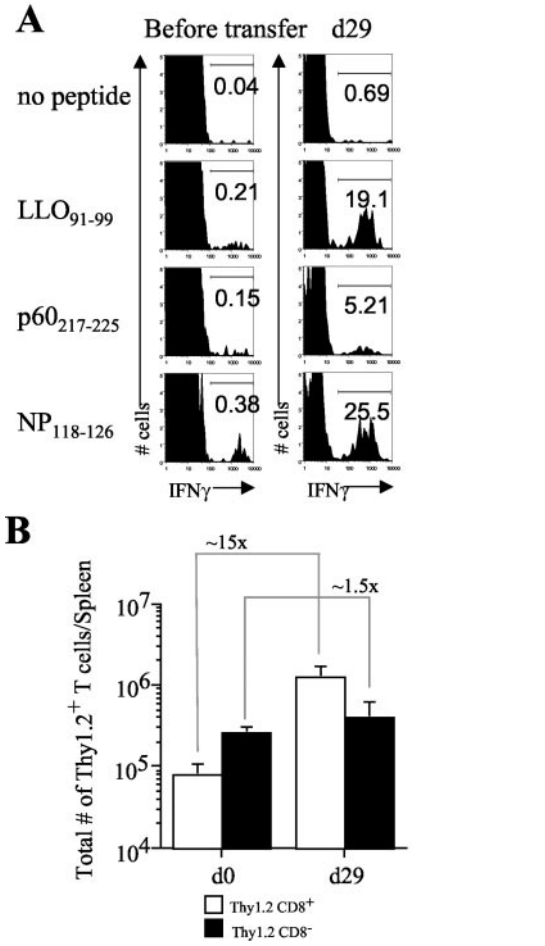

FIGURE 6. Expansion of transferred T cells after LM infection. *A*, Frequencies of Thy1.2$^+$ Ag-specific CD8$^+$ T cells detected before transfer and at day 29 after LM infection. *B*, Total number of Thy1.2$^+$/CD8$^+$ and Thy1.2$^+$/CD8$^-$ cells after transfer and at day 29 after LM infection. Numbers represent fold increase in total numbers from day0 to d29 p.i. Ratio of Thy1.2$^+$/CD8$^-$ vs Thy1.2$^+$/CD8$^+$ at day 0 and day 29 was 3.2 and 0.3, respectively.

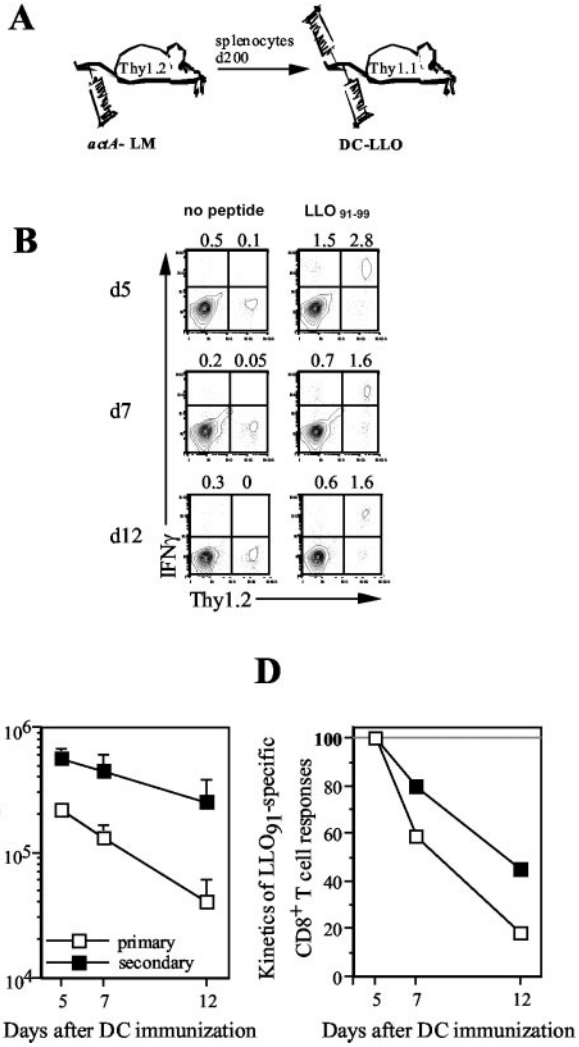

FIGURE 7. Kinetics of primary and secondary CD8$^+$ T cell responses after DC immunization. *A*, Experimental design. BALB/c Thy1.2 mice were infected with $\sim 1 \times 10^6$ ($\sim$0.1 LD_{50}) *act*A-LM and used as donors >200 days after initial challenge. BALB/c Thy1.1 mice that received splenocytes (1.2×10^7) from Thy1.2 LM-infected memory mice were immunized with 2.5×10^5 LLO_{91-99}-coated DC. *B*, Frequencies of primary Thy1.2$^-$ and secondary Thy1.2$^+$ LLO_{91-99}-specific CD8$^+$ T cells from representative mice at various days postimmunization. *C*, Total number of Thy1.1 and Thy1.2 LLO_{91-99}-specific CD8$^+$ T cells per spleen (n = 3–5). *D*, Normalized kinetics LLO_{91-99}-specific CD8$^+$ T cell responses in the spleen. Day 5 postimmunization is presented as 100%. One of two similar experiments is shown.

CD8$^+$ T cells at the time of infection. Finally, differences in contraction of primary and secondary CD8$^+$ T cells in the same host were preserved, even in the absence of infection after peptide-coated DC immunization. Thus, delayed contraction of secondary CD8$^+$ T cell responses appeared to be a generalizable feature of the adaptive immune response.

The number of memory CD8$^+$ T cells required for defense against infection will depend on the pathogen, the level of infection, and the capability of the host to fully use all CD8$^+$ T cell effector mechanisms.[4] In this study, we show that $>8.5 \times 10^3$ but $<8.5 \times 10^4$ memory CD8$^+$ cells/mouse substantially decreased viral load at day 3 p.i. with a challenge dose of LCMV-Arm that was cleared from naive mice by day 8–10 p.i. (31, 32). Approximately 1.5×10^4 memory CD8$^+$ T cells protected mice after challenge with 1 LD_{50} of LM. Interestingly, 10,000 Ag-specific memory CD8$^+$ T cells in the whole animal falls below the level of detection for current techniques for direct ex vivo enumeration of T cells (e.g., tetramer staining and/or ICS). Thus, even low numbers of memory cells can provide measurable protection against infection. However, our data confirmed the widely held notion that more memory cells provide better protection.

Adoptive transfer of memory CD8$^+$ T cells before infection reduced the magnitude of the endogenous primary response (precursor recruitment) in a dose-dependent fashion. It has been suggested from studies of naive TCR-transgenic cells that competition for APC might influence the magnitude of the Ag-specific CD8$^+$ T cell responses against the same or even different specificities (24, 25). Recently, the relevance of the crowding effect for APC by CD8$^+$ T cells of different specificity has been challenged. In elegant experiments, Probst et al. (33) showed that transfer of an increasing number of naive TCR-transgenic cells can decrease and eventually suppress the expansion of endogenous CD8$^+$ T cells of the same specificity after viral and/or DC immunization. In contrast, CD8$^+$ T cell responses to other epitopes were not decreased,

[4] K. Nordyke Messingham, V. P. Badovinac, and J. T. Harty. Deficient anti-listerial immunity in the absence of perforin can be restored by increasing memory CD8$^+$ T cell numbers. *Submitted for publication.*

suggesting that competition for APC by $CD8^+$ T cells is not functionally important during induction of antiviral responses. In addition to competition for APC memory, $CD8^+$ T cells might decrease the availability of MHC class I-peptide complexes on the APC (epitope stripping) and therefore prevent the priming of naive Ag-specific $CD8^+$ T cells (34). Finally, the presence of memory $CD8^+$ T cells at the time of infection could decrease the number of APC by direct killing of infected cells (35). However, it is interesting to note that a substantial endogenous primary response was seen even in mice that received the largest dose of memory $CD8^+$ T cells. It remains to be determined whether transfer of higher numbers of memory cells will completely eliminate the endogenous primary response to the same epitope as is seen after transfer of large numbers of naive TCR-transgenic $CD8^+$ T cells (24, 33). If not, competition between memory and naive cells with the same Ag specificity may not be driven simply by equilibrium dynamics.

Recently, attempts were made to estimate naive $CD8^+$ T precursor frequencies by transfer of graded numbers of naive TCR-transgenic T cells followed by infection of mice (24, 26, 36). Using this approach, Blattman et al. (26) suggested that there may be several hundred naive $CD8^+$ T cells specific for the LCMV-derived gp33–41 epitope before infection. In this study we show that ~10-fold higher levels of memory $CD8^+$ T cells/mouse than reported for naive gp33–41-specific $CD8^+$ T cells (in the range of several thousand for LM and ten thousand for LCMV derived epitopes) expanded to similar levels as naive $CD8^+$ T cells of the same specificity after LCMV and/or LM infections. Immunodominance hierarchies between LM-derived epitopes were preserved in primary and secondary $CD8^+$ T cell responses, suggesting that precursor frequencies of memory, and likely also naive $CD8^+$ T cells, were critical determinants of $CD8^+$ T cell expansion. The reason(s) why more memory than naive TCR-transgenic $CD8^+$ T cells (26) were required to generate similar expansion to the endogenous response after LCMV infection is unknown. In both studies, ~10% of the injected Ag-specific $CD8^+$ T cells could be found in the spleen, suggesting that differential homing or survival would not account for the results. However, it has been reported that total number of memory $CD8^+$ T cells actually decrease in the spleen early after reinfection (27) (V. P. Badovinac and J. T. Harty, unpublished observations). To the best of our knowledge similar findings have not been reported for naive Ag-specific $CD8^+$ T cells. This initial drop in memory cell numbers could account for the large number of memory cells required to generate equivalent numbers of primary and secondary effector cells.

The mechanisms that determine the rate of contraction after primary and secondary $CD8^+$ T cell responses remain to be defined. Our results with DC immunization suggested that the contraction program was not imposed by interaction with a specific APC. In addition, delayed contraction of the secondary $CD8^+$ T cell responses was observed when the experimental system was designed to eliminate (4) or include concurrent responses (this study) by other Ag-experienced cells ($CD4^+$ T cells for example). Recently, Grayson et al. (15) used a similar approach with TCR-transgenic $CD8^+$ T cells and showed that contraction of secondary gp33–41-specific $CD8^+$ T cells is delayed compared with primary gp33–41-specific $CD8^+$ T cells in the same host after LCMV infection. They showed that differences in the contraction between primary and secondary $CD8^+$ T cells were associated with increased apoptosis of primary effector cells compared with the secondary effector cells (15). Importantly, these investigators ruled out the possibility that delayed contraction of secondary $CD8^+$ T cells resulted from increased proliferation of these cells (15). In conjunction with our results demonstrating that contraction of both primary and secondary $CD8^+$ T cell responses was programmed by early events after infection (4), we favor the possibility that prolonged contraction was hardwired into the population of cells that were selected into the primary memory pool. Experiments are currently underway to address this hypothesis.

Our results suggest that depending on the memory levels secondary $CD8^+$ T cell responses may or may not be comprised of cells responding for both the first and second time to infection. This situation may have important impact on vaccine design and emerging strategies of prime-boost vaccination (1). It remains to be determined how prolonged contraction of secondary $CD8^+$ T cell response benefits the host. Perhaps secondary infection with a pathogen is perceived as an additional level of danger, requiring high levels of $CD8^+$ T cells for a prolonged period. In turn this may contribute to increased numbers of tissue resident $CD8^+$ T cells that are poised for rapid response to recurrent infections with the same pathogen.

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