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Population Dynamics of Naive and Memory CD8 T Cell Responses after Antigen Stimulations In Vivo

Matthew D. Martin,* Stephanie A. Condotta,* John T. Harty, †,‡ and Vladimir P. Badovinac*†

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Memory CD8 T cells are the surviving progeny of relatively rare naive CD8 T cells that have been programmed to clonally expand upon encounter with cognate Ag presented by professional APCs (1–6). Only a small fraction (5–10%) of the responding cells present at the peak of the expansion phase survive to become memory cells (7–10). A number of studies have suggested that the protective capacity of primary memory CD8 T cells is dependent both on their absolute number and functional properties (1). Thus, controlling the quality and/or quantity of the developing memory CD8 T cell pool should represent an important goal in vaccine development.

Substantial progress has been made toward understanding the features that define primary memory CD8 T cells. In general, attributes of memory CD8 T cells such as increased representation (increase in numbers over naive CD8 T cell repertoire), changes in distribution (enhanced surveillance at potential sites of pathogen entry), longevity (long-term survival), and function (rapid killing and cytokine-producing abilities) have led to the assumption that memory CD8 T cells are qualitatively and quantitatively better than their naive counterparts (2, 4, 11, 12). All of these attributes are indeed important factors that contribute to the increased CD8 T cell-mediated resistance to infection in immune hosts. However, the extent to which the progeny of one memory CD8 T cell differs from the progeny of one naive CD8 T cell of the same specificity remains an unresolved and important question.

For instance, both naive and memory CD8 T cells are capable of exponential proliferation following Ag stimulations. Because memory CD8 T cells are present in higher numbers than naive cells they often, but not always, give rise to a higher number of secondary effectors compared with the number of primary effectors generated from the naive CD8 T cell pool. The ability of prime-boost protocols to increase memory CD8 T cell numbers is well documented (13–15). However, experiments that examine the proliferative potential of naive and memory CD8 T cells, although controlling for the numbers of precursor cells, have yielded conflicting results (16). Despite these caveats, it has been suggested that the numerical expansion capacity of memory CD8 T cells is the same or better compared with naive cells following Ag stimulation (16). Importantly, data that support this conclusion are complicated by the adoptive transfer with large numbers of naive TCR-transgenic (Tg) CD8 T cells, and our previous work showed that initial TCR-Tg cell precursor frequency dictates critical aspects of the CD8 T cell response to infection, including the magnitude of primary expansion (17). Additionally, primary and repeatedly stimulated (secondary, tertiary, quaternary) memory CD8 T cells differ substantially in their molecular signatures as well as in their functional attributes, including the ability to proliferate to new Ag stimulation (18, 19). Because loss of expansion capacity is correlated with the number of Ag encounters, the conclusion that primary memory CD8 T cells (on a per-cell basis) are capable of equal or greater Ag-driven proliferation compared with naive CD8 T cells warrants re-examination.

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Additionally, experiments examining the kinetics of primary and secondary CD8 T cell responses have noted a prolonged contraction phase of secondary compared with primary CD8 T cell responses, suggesting differential susceptibility to apoptosis between these populations (7, 18–21). However, the assumption that due to delayed contraction the ability to generate long-lived progeny (described in this study as “memory generation potential”) is greater for one primary memory cell compared with one naive CD8 T cell also remains unsupported.

To address intrinsic (cell-autonomous) differences between naïve and memory CD8 T cells that are not the result of differences in environmental factors, we used an experimental model in which both cell types were analyzed in the same host responding to infection or immunization. Two questions were asked in this study. First, to what extent does the expansion capacity of one naive CD8 T cell differ from one primary memory CD8 T cell upon Ag stimulation. Second, does memory generation potential in vivo differ between naive and primary memory CD8 T cells postinfection (p.i.).

Our results show that naive CD8 T cells undergo a higher magnitude of expansion than do memory CD8 T cells when analyzed on a per-cell basis in various models of infection and/ or immunization. Longitudinal analysis of primary and secondary CD8 T cell responses also revealed that memory generation potential of responding naive CD8 T cells is better than for responding primary memory CD8 T cells despite the differences in overall kinetics of both responses. Therefore, our data provide new insights into primary memory CD8 T cell function and will help further define the functional properties of primary memory CD8 T cells.

Materials and Methods

**Mice, bacteria, and virus infections**

C57BL/6 (B6) Thy1.1/1.2 or CD45.1/CD45.1 mice were obtained from the U.S. National Cancer Institute. OT-I TCR-Tg mice (Thy1.1/1.2 and Thy1.1/1.1) were previously described (19, 22). Pathogen-infected mice were housed in the appropriate biosafety conditions. All mice were used at 6–10 wk age. All animal protocols followed approved Institutional Animal Care and Use Committee protocols. The virulent *Listeria monocytogenes* strain expressing OVA257 (VirL. *monocytogenes*-OVA) and the attenuated actA-deficient *L. monocytogenes* strain expressing OVA257 (AttL. *monocytogenes*-OVA) were grown, injected i.v., and quantified i.v., and quantified as described (19, 23, 24). Vaccinia virus expressing the OVA257 peptide (VacV-OVA) has been described previously (25).

**Dendritic cell immunizations**

Splenic dendritic cells (DCs) were isolated after s.c. injection of B6 mice with 5 × 10^7 B16 cells expressing Flt3 ligand (Flt3L) as previously described (26). When tumors were palpable (5 × 5 mm), mice were injected with 2 μg LPS (Sigma-Aldrich) i.v. to mature the DCs. Spleens were harvested 16 h later and were digested with DNase and collagenase for 20 min at 37°C/5% CO2 with shaking. Spleen pieces were smashed through a nylon cell strainer (70 mm) to generate a single-cell suspension, RBCs were lysed, and splenocytes were resuspended in 2 parts 10% FCS RPMI 1640 to 1 part B16-Flt3L–conditioned medium plus rGM-CSF (1000 U/ml) plus 2 μM OVA257–264 and incubated 2 h at 37°C/5% CO2. Splenocytes were washed three times and CD11c+ cells were isolated using anti-CD11c microbeads (Miltenyi Biotec). The purity and activation status of DCs were determined on a per-cell basis in various models of infection and/or stimulation. Seconed, does memory generation potential in vivo differ between naive and primary memory CD8 T cells postinfection (p.i.).

**Adoptive transfer experiments and isolation of lymphocytes from tissues**

OT-I cells were obtained from peripheral blood (PBL) samples of 2- to 3-mo-old naïve OT-I mice. Contaminating memory phenotype (CD44+CD11a+Vα2+Vβ5+) OT-I cells were always <5%. To generate primary OT-I CD8 T cells for adoptive transfer experiments, 1 × 10^6 naïve Thy1.1/1.2 or Thy1.1/1.1 OT-I cells were transferred into Thy1.2 recipients 1 d before VacV-OVA (3 × 10^6 PFU/mouse; i.p.) infection. For cotransfer of naïve and memory OT-I cells, naïve OT-I T cells were obtained from PBL of TCR-Tg OT-I mice and mixed at the indicated ratios with memory OT-I cells obtained from the spleens of VacV-OVA–immunized mice at various time points p.i. The ratio of naïve to memory OT-I cells in master mix used for adoptive transfer was verified by flow cytometry before transfer. For adoptive transfer of endogenous OVA257–specific primary memory CD8 T cells, splenocytes from VacV-OVA–immunized mice (day 113 postinfection) were used. OVA257–specific CD8 T cells (CD45.2; 5 × 10^6) were transferred into naïve B6 (CD45.1) mice 1 d before Att L. *monocytogenes*-OVA infection.

For isolation of lymphocytes from secondary lymphoid organs and tertiary tissues, samples of blood were obtained by retro-orbital puncture before tissue removal. Anesthetized mice were then perfused through the left ventricle with cold PBS for 1–2 min and tissues were collected. Single-cell suspensions from liver, lung, spleen, lymph nodes, and bone marrow were washed with PBS before staining (27).

**Abs and peptides**

The following mAbs from eBioscience with the indicated specificity and with appropriate combinations in fluorochromes were used: CD8 (clone 53-6.7), Thy1.1 (OX-7 or HIS51), Thy1.2 (53-2.1), CD45.2 (104), CD127 (A7R34), CD62L (MEL-14), CD27 (LG.7P9), CD122 (SH4), anti-BrdU (PKB-1) and appropriate isotype controls.

**FIGURE 1.** Naïve CD8 T cells undergo a higher magnitude of expansion than do memory CD8 T cells after *L. monocytogenes* infection. A, Experimental design. Naive Thy1.1/1.1 OT-I cells (1 × 10^6) were mixed with an equal number of primary memory Thy1.1/1.2 OT-I (day 200+ after primary VacV-OVA infection) and injected into naïve B6 Thy1.2/1.2 recipients. Mice were challenged 24 h later with Att L. *monocytogenes*-OVA (5 × 10^5 CFU/mouse; i.v.). B, Representative plots showing the primary (Thy1.1/1.1) and secondary (Thy1.1/1.2) OT-I responses at the indicated days postinfection. Numbers inside the plots indicate the percentage of primary or secondary OT-I CD8 T cells in PBL, and numbers below the plots indicate the ratio of primary to secondary OT-I cells at the indicated days postinfection. C, Kinetic analysis of OT-I cells in PBL. Data are presented as the percentage of primary or secondary OT-I cells in PBL (mean ± SEM for five mice per group). D, The ratio of primary to secondary OT-I cells at the indicated days p.i. Dots represent individual mice, solid lines indicate the mean, and the dashed line indicates the starting ratio of naïve and primary memory OT-I cells in the mix used for adoptive transfer before infection. The data are representative of at least three independent experiments. **p < 0.01.
Quantification of CD8 T cell responses and detection of BrdU uptake and activated caspase-3/7 in Ag-specific CD8 T cells

OT-I cell responses in PBL and tissues were monitored by FACS analysis for Thy1.1-positive CD8 T cells. Thy1.2 expression was used to discriminate between primary and secondary responses in the same host. Endogenous OVA257-specific CD8 T cells were detected by K\textsuperscript{b}-OVA\textsubscript{257} tetramers as previously described (28). To determine the rate of proliferation of Ag-specific T cells during the expansion phase, mice were i.p. injected with 2 mg BrdU on days 4, 5, or 6 after \textit{Att L. monocytogenes}-OVA infection and given 0.8 mg/ml BrdU in drinking water for an additional 24 h. PBL was collected and splenocytes were isolated and surface stained for CD8 and Thy1.1, followed by fixation and permeabilization procedures as recommended in the BrdU flow kit (BD Biosciences). Anti-BrdU mAb was used for intracellular staining to detect BrdU uptake. To determine the proliferation rate of Ag-specific CD8 T cells during the memory maintenance phase, mice were i.p. injected with 2 mg BrdU and given 0.8 mg/ml BrdU in drinking water for an additional 8 d. BrdU uptake was determined as described above. For detection of activated caspase-3 and -7 in Ag-specific CD8 T cells, splenocytes were first surface stained for CD8, Thy1.1, and

FIGURE 2. Naive CD8 T cells undergo a greater magnitude of expansion than do memory CD8 T cells in response to infections or noninfectious immunization. A, Experimental design. Naive Thy1.1/1.1 OT-I cells (1 \times 10\textsuperscript{5}) were mixed with an equal number of primary memory Thy1.1/1.2 OT-I cells (day 200+ after primary VacV-OVA infection) and injected into naive B6 Thy1.2/1.2 recipients. Mice were challenged 24 h later with \textit{Att L. monocytogenes}-OVA (5 \times 10\textsuperscript{3} or 5 \times 10\textsuperscript{4} CFU/mouse; i.v.), \textit{Vir L. monocytogenes}-OVA (5 \times 10\textsuperscript{2} CFU/mouse; i.v.), VacV-OVA (3 \times 10\textsuperscript{5} PFU/mouse; i.p.), or DC-OVA (1 \times 10\textsuperscript{6} cells/mouse; i.v.). B, Dot plot showing the mix of naive and memory OT-I cells used for adoptive transfer. Numbers inside the plot indicate the percentage of naive OT-I (Thy1.1/1.1) or primary memory OT-I cells (Thy1.1/1.2). C, Kinetic analysis of OT-I cells in PBL at the indicated days p.i. or immunization. Data are presented as the percentage of primary or secondary OT-I cells in PBL (mean \textpm SEM for five mice per group). D, The ratio of primary to secondary OT-I at the indicated days after infection or immunization. Dots represent individual mice, solid lines indicate the mean, and the dashed line indicates the starting ratio of naive and memory OT-I cells before infection. E, Experimental design. Endogenous primary memory OVA\textsubscript{257}-specific CD8 T cells (day 113 after primary VacV-OVA infection) were transferred (5 \times 10\textsuperscript{3} cells/mouse) into naive B6 CD45.1/CD45.1 recipients 1 d before \textit{Att L. monocytogenes}-OVA (5 \times 10\textsuperscript{6} CFU; i.v.) challenge. F, Representative plots showing the mix and memory OT-I cells used for adoptive transfer. Numbers inside the plot indicate the percentage of naive OT-I (Thy1.1/1.1) or primary memory OT-I cells (Thy1.1/1.2). G, Kinetic analysis of OT-I cells in PBL at the indicated days p.i. Data are presented as the percentage of primary or secondary OT-I cells in PBL (mean \textpm SEM for five mice per group). These data are representative of two to three similar and independent experiments. *p = 0.01–0.05, **p \leq 0.01.
Thy1.2. Samples were then incubated with the fluorescent labeled inhibitor of caspases reagent, which binds to activated caspases, at 37°C and 5% CO₂ for 60 min as recommended in the Vybrant FAM caspase-3 and -7 assay kit (Invitrogen, Carlsbad, CA).

Statistical analysis

Statistical significance was assessed using the two-tailed t test with a confidence interval of >95%. Data are presented as means (±SEM) unless otherwise stated in the figures.

Results

Naive CD8 T cells undergo a higher magnitude of expansion than do memory CD8 T cells after L. monocytogenes infection

Naive and primary memory CD8 T cells are characterized by their ability to undergo vigorous expansion in numbers upon Ag encounter. The prevailing assumption is that memory CD8 T cells due to their increased frequencies, tissue distribution, and function are both qualitatively and quantitatively superior to their naive counterparts in responding to Ag. However, whether memory CD8 T cells have an enhanced proliferation capacity compared with naive cells is still an unresolved question (reviewed in Ref. 16). In experiments where the magnitude of the expansion of primary and secondary CD8 T cell responses were compared in different hosts, it was shown that memory CD8 T cells have an equal or increased ability to proliferate and accumulate in vivo (21, 29, 30). Because multiple factors can influence the expansion of naive and/or primary memory CD8 T cells (e.g., precursor frequencies, type of Ag stimulation, duration of infection and inflammation, phenotype of responding CD8 T cells) (1, 2, 31–34), we developed an experimental model to address the proliferative potential of both naive and memory CD8 T cell populations in the same host environment (Fig. 1A). The model includes: 1) the adoptive transfer of physiologically relevant numbers of naive CD8 T cells; and 2) co-transfer of primary memory CD8 T cells into the same recipients to control for differences in the in vivo environment (e.g., duration of infection and inflammation).

We adoptively co transferred 1 × 10³ naive TCR-Tg OT-I CD8 T cells (Thy1.1/1.1) with the same number of primary memory OT-I cells (Thy1.1/1.2) and subsequently infected recipient mice (naive B6 mice; Thy1.2/1.2) with a sublethal dose (0.1 LD₅₀) of Att L. monocytogenes-OVA (Fig. 1A, experimental design). Recall potential of memory CD8 T cell populations changes with time after initial stimulation (33), and to ensure that memory CD8 T cells used in the experiments were capable of vigorous secondary expansion, primary memory OT-I cells were obtained from donor mice that had been infected >200 d before. To determine the magnitude of expansion of each population we analyzed the percentage of primary or secondary effector OT-I cells in the PBL from days 5 to 7 after challenge (Fig. 1B, 1C). Interestingly, the data showed that the proliferation capacity of naive OT-I (1° responders) is greater than the capacity of memory OT-I (2° responders) after bacterial challenge (Fig. 1C). Importantly, a higher ratio of primary to secondary effectors was seen in each individual mouse examined at days 6 and 7 p.i. (Fig. 1D). Therefore, these results suggest that the magnitude of the expansion of naive CD8 T cells is higher than memory CD8 T cells when analyzed on a per-cell basis in the same host after bacterial infection.

Naive CD8 T cells expand in numbers more than memory CD8 T cells in response to infections or noninfectious immunization

The magnitude of naive CD8 T cell responses varies depending on factors such as the type, route, and dose of infection as well as inflammation present during the infection (1, 12, 31). Additionally, it has been recently suggested that inflammation also controls the magnitude of expansion and differentiation of secondary CD8 T cell responses, but that naive and memory CD8 T cells might differ in their susceptibility to inflammatory cues in the environment (27, 35). Therefore, we first wanted to examine whether our initial findings seen after Att L. monocytogenes-OVA infection could be extended to additional types and doses of bacterial and viral infections as well as noninfectious priming conditions with DCs (Fig. 2A). Equal numbers of naive and memory OT-I cells (day 225 p.i.) were cotransferred into naive B6 recipients followed by immunization with Att L. monocytogenes-OVA (5 × 10⁶ or 5 × 10⁴ CFU/mouse), a virulent strain of L. monocytogenes-OVA (5 × 10⁷ CFU), VacV-OVA, or DCs coated with OVA peptide (DC-OVA) (Fig. 2A, experimental design). The starting ratio of naive to memory OT-I cells was determined before adoptive transfer in a master mix, which was diluted for injection of ~1000 of each OT-I population (Fig. 2B). The input ratio was later used to determine how the Ag-driven proliferation changes the numbers of primary and secondary CD8 T cells responding to in vivo Ag stimulation.

Importantly, when analyzed on a per-cell basis the overall magnitude of expansion of naive CD8 T cells was greater than primary memory CD8 T cells in all groups of mice (Fig. 2C, 2D). However, the degree of difference in the magnitude of expansion between primary and secondary CD8 T cells depended on the specific immunization. The magnitude of expansion of the primary and secondary CD8 T cell responses were the most similar p.i. with a virulent strain of L. monocytogenes, whereas differences between primary and secondary CD8 T cell responses were more pronounced after priming in an environment where systemic inflammation was substantially reduced (DC-OVA group; Fig. 2C, 2D) (13, 31). To determine whether the latter result was due to the systemic inflammation, mice containing equal numbers of naive and primary memory OT-I cells were immunized with DC-OVA with or without coinfection with L. monocytogenes that does not express the OVA epitope (Fig. 3A, experimental design). Again, naive CD8 T cells expanded substantially more than did memory CD8 T cells after immunization with DCs alone. However, in-

FIGURE 3. Systemic inflammation influences the expansion potential of primary memory CD8 T cells. A. Experimental design. Naive Thy1.1/1.1 OT-I cells (1 × 10³) were mixed with an equal number of primary memory Thy1.1/1.2 OT-I cells (day 350+ after primary infection) and injected into naive B6 Thy1.1/1.2 recipients. Mice were immunized 24 h later with DC-OVA (2 × 10⁵ cells/recipient) in the presence or absence of Att L. monocytogenes coinfection (5 × 10⁵ CFU/mouse). B. Graph showing the ratio of primary to secondary OT-I cells in PBL at the indicated days after DC-OVA immunization in the presence or absence of infection. C. The ratio of primary to secondary OT-I cells in the indicated organs at day 7 after immunization. The dotted lines in B and C indicate the ratio of naive to primary memory OT-I cells used in the adoptive transfer mix. A representative experiment out of three similar and independent experiments is shown.
ducing systemic inflammation by concurrent *L. monocytogenes* infection eliminated the differences in expansion between naive and memory CD8 T cells (Fig. 3B). Similar data were obtained in secondary lymphoid organs and in peripheral tissues of mice, indicating that the observations made in the blood are consistent throughout the host (Fig. 3C).

Finally, we determined whether differences in expansion potential between monoclonal TCR-Tg naive and memory CD8 T cells can be observed with polyclonal (endogenous) CD8 T cell responses. To address this question $5 \times 10^5$ OVA$_{257}$-specific CD8 T cells (CD45.2) generated by VacV-OVA infection were transferred into naive B6 (CD45.1) mice before Att *L. monocytogenes*-OVA infection (Fig. 2E, experimental design).

Naive B6 mice contain 100–200 naive OVA$_{257}$-specific CD8 T cell precursors (32, 36). Not all of the transferred cells survive in the recipient upon adoptive transfer, and the percentage of the cells that survive has been referred to as “take.” Thus, even if take is as low as 3–4% of transferred memory numbers (most studies suggest that take is $\sim 10\%$) (20, 33, 37), equal if not higher numbers of memory CD8 T cells should be present in vivo before infection in this experimental design (Fig. 2E). Importantly, the endogenous naive CD8 T cells still expanded in numbers more than transferred polyclonal primary memory CD8 T cells (Fig. 2E, 2G).

Taken together, these data suggest that under various infections and priming conditions the magnitude of expansion of the re-
sponding naive cells is greater than the magnitude of expansion of the responding primary memory CD8 T cells and indicate that when analyzed on a per-cell basis naive CD8 T cells possess a higher proliferative potential than do memory CD8 T cells.

Differences in trafficking after adoptive transfer do not explain the higher magnitude of expansion of naive CD8 T cells

The results thus far have indicated that the expansion potential of naive CD8 T cells is greater than memory CD8 T cells when analyzed on a per cell basis under various priming conditions. A possible explanation for this is that naive and primary memory CD8 T cells could traffic to different areas after adoptive transfer, which might influence their priming. To test this idea, equal numbers of naive and primary memory OT-I cells (3 × 10^5 each) were co-transferred and the ratio of naive to primary memory OT-I cells in various organs 24 h after adoptive transfer was determined (Fig. 4A, experimental design. As in the experiments before, the memory OT-I cells were obtained from donor mice >200 d after primary infection, and phenotypic analysis showed that most of them were CD127, CD62L, CD27, and CD122 positive (Fig. 4B), which are characteristic of central or late memory (lateM) CD8 T cells (3, 11). Thus, these cells should be capable of vigorous secondary expansion and trafficking to secondary lymphoid organs where priming occurs (2, 33). Fig. 4C shows the ratio of naive and primary memory OT-I cells used in the adoptive transfer mix.

Examining the ratios of naive to primary memory OT-I cells in various tissues after adoptive transfer revealed a higher number of primary memory cells in all organs examined except for the inguinal lymph nodes (Fig. 4D). However, higher numbers of memory cells were detected in the spleen compared with naive cells (Fig. 4E). Importantly, the spleen is the major secondary lymphoid organ where priming occurs after L. monocytogenes infection (38). Thus, these data suggest that the differences in proliferation capacity between naive and memory CD8 T cells are likely not explained by differences in their localization at the time of priming.

Differences in tissue localization at the peak of the response do not explain the differences in expansion potential of naive and memory CD8 T cells

It is also possible that primary effectors are overrepresented in the circulation and that overall ratios between primary and secondary effectors differ in various organs of the mice. To address this question, equal numbers (1 × 10^5 each) of naive and primary memory OT-I cells were cotransferred into naive mice prior to Att L. monocytogenes-ova infection (Fig. 4F). Consistent with our previous experiments, we found a higher number of primary effectors in PBL at day 7 postinfection (Fig. 4F, 4G). Importantly, in each organ examined primary effectors were found in higher numbers than secondary effector CD8 T cells (Fig. 4F, 4G). Interestingly, even after the transfer of 20-fold lower numbers of both naive and memory CD8 T cells (50 cells transfer) the expansion potential of the naive T cell population surpasses that of primary memory CD8 T cells (Fig. 4F, 4G). Taken together, these results suggest that primary effectors are not overrepresented in circulation and that naive CD8 T cells possess a higher proliferative potential than do memory CD8 T cells when analyzed on a per-cell basis and in multiple organs of the mice.

Sustained proliferation of primary rather than increased death of secondary effectors leads to the higher magnitude of expansion of the naive CD8 T cells

The differences in the expansion capacity of naive and primary memory CD8 T cells after infection or immunization could be explained by differences in proliferation and/or survival of these populations after Ag stimulation. To test these possibilities, naive or primary memory OT-I CD8 T cells were transferred into separate groups of naive B6 mice before Att L. monocytogenes-ova infection (Fig. 5). As described before, differences in kinetics of CD8 T cell expansion were observed for primary and secondary CD8 T cell responses (Fig. 5A). Importantly, even after transfer into separate mice, a statistically significant increase in numbers (from day 6 to 7 p.i.) were observed only in the PBL of mice that contained primary CD8 T cells (Fig. 5A).

![Figure 5](image-url)
Examining the kinetics of proliferation revealed that nearly all primary and secondary effector OT-I cells incorporated BrdU from days 4 to 5. However, from days 6 to 7 in the PBL and days 5 to 6 and 6 to 7 in the spleen, a significantly higher percentage of primary effector OT-I cells incorporated BrdU than secondary effector OT-I cells (Fig. 5B, 5C). Thus, these data suggest that primary effector CD8 T cells exhibit sustained and prolonged proliferation compared with secondary CD8 T cells.

In addition to the BrdU proliferation assay, caspase-3/7 staining (as an indication of cell death) was performed on primary and secondary OT-I cells found in the spleens at days 6 and 7 after Att L. monocytogenes-OVA infection. Similar percentages of primary and secondary CD8 T cells were positive for caspase-3/7 at days 6 and 7 p.i. (Fig. 5D, 5E), suggesting that the rate of death for both primary and secondary CD8 T cell responses was similar. Therefore, these results indicate that differences in proliferation but not the rate of cell death can explain the observed differences in naive and memory CD8 T cell expansion p.i.

Population dynamics of naive and memory CD8 T cell responses after Ag stimulation in vivo

Previously, it has been shown that in contrast to the vigorous and relatively short contraction (death) phase of primary CD8 T cell responses, secondary CD8 T cells die at a substantially protracted rate (7, 18–21). Importantly, it is not known if and when contraction of secondary CD8 T cells is resolved and how the prolonged contraction influences the numbers of secondary memory CD8 T cells. In other words, is the potential for generating a stable memory CD8 T cell population different for responding naive and primary memory CD8 T cells? To address this issue, low numbers of naive (1 × 10^3 per mouse) OT-I cells were cotransferred with increasing numbers of primary memory CD8 T cells (naive/memory CD8 T cell ratios of 1:1, 1:10, and 1:100) before infection with Att L. monocytogenes-OVA (Fig. 6A, experimental design). Detailed longitudinal kinetic analysis of primary and secondary CD8 T cell responses (shown as frequencies of OT-I cells in the PBL) is presented in Fig. 6B.

Plotting the ratio of the primary to secondary CD8 T cell responses at various time points p.i. revealed a dynamic pattern of regulation (Fig. 6C). As shown above, during the expansion phase primary OT-I cells were present in higher numbers than secondary OT-I cells. During the contraction phase, secondary OT-I cells were present in higher numbers than primary OT-I cells, consistent with the prolonged contraction of secondary CD8 T cell responses. However, due to the continual decline in secondary CD8 T cell numbers, primary memory CD8 T cells were more prevalent than secondary memory OT-I cells when analyzed late p.i. (Fig. 6C). Importantly, the changes in population dynamics of primary and secondary CD8 T cell responses were not influenced by the number of memory CD8 T cells present at the time of infection (Fig. 6C).

The changes in dynamics of primary and secondary CD8 T cell responses p.i. could be a consequence of examining the representation of primary and secondary memory populations in PBL and may not be representative of other tissues in the host. To address this issue, the percentages of primary and secondary

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**FIGURE 6.** Longitudinal analysis of primary and secondary CD8 T cell responses in vivo. A, Experimental design. Naive Thy1.1/1.1 OT-I cells (1 × 10^3) were mixed with primary memory Thy1.1/1.2 OT-I cells (day 200+ after primary VacV-OVA infection) at the indicated ratios and injected into naive B6 Thy1.2/1.2 recipients. Mice were challenged 24 h later with Att L. monocytogenes-OVA (5 × 10^6 CFU/recipient; i.v.). B, Kinetic analysis of OT-I in PBL at the indicated days p.i. or immunization. Data are presented as the mean percentage of primary or secondary OT-I cells in PBL ± SEM for four or five mice/group. C, Kinetic analysis of primary and secondary OT-I CD8 T cell responses presented as the ratio of primary to secondary OT-I cells in PBL (mean ± SEM for four or five mice/group). The dashed line indicates the starting ratio of naive and memory OT-I cells transferred before infection. The data are representative of at least three similar and independent experiments.
Memory CD8 T cells were determined in secondary lymphoid organs and tertiary tissues at a memory time point (day 227 p.i.). The data clearly showed that in all organs examined a higher percentage of primary memory OT-I cells was detected compared with secondary memory OT-I cells (Fig. 7A, 7B).

Memory CD8 T cell populations are maintained by a process of slow basal turnover, and it has been shown that primary memory CD8 T cells undergo higher rates of basal proliferation than secondary memory CD8 T cells (19, 39). We used BrdU incorporation to determine whether differences in basal proliferation might explain the shift in primary and secondary memory CD8 T cell numbers. A significantly higher percentage of primary memory OT-I cells incorporated BrdU in the PBL and spleen when compared with secondary memory CD8 T cells (Fig. 7C, 7D). Because basal proliferation of both primary and secondary memory CD8 T cell populations was measured in the same mice, the differences observed were not related to environmental factors, but rather represent inherent differences between these memory populations.

Taken together, these results reveal the differences in population dynamics of primary and secondary CD8 T cell responses and suggest that one naive CD8 T cell does have a greater memory generation potential than one primary memory CD8 T cell.

**FIGURE 7.** Tissue distribution and homeostatic proliferation of primary and secondary memory CD8 T cells analyzed in the same host. A. The percentage of primary or secondary OT-I per indicated organ on day 227 after Att L. monocytogenes-OVA infection. Data are presented as mean ± SEM for three mice/organ. B. The ratio of primary to secondary OT-I in the indicated organs at day 227 after Att L. monocytogenes-OVA infection. C. Representative dot plots of BrdU staining of primary or secondary OT-I cells in the spleen or PBL on day 216 p.i. (the ratio of naive to primary memory OT-I cells used for adoptive transfer was 1:10). Numbers inside the plots indicate the percentage of OT-I cells positive for BrdU. D. The percentage of BrdU-positive primary or secondary OT-I cells in spleen or PBL at day 216 after Att L. monocytogenes-OVA infection. Dots represent individual mice, solid lines indicate the mean, and the dashed line indicates the starting ratio of naive and memory OT-I cells transferred before infection. *p = 0.01–0.05, **p < 0.01.

**Higher proliferation potential but indistinguishable kinetics of secondary CD8 T cell responses generated from late versus early memory CD8 T cells**

Memory CD8 T cells undergo phenotypic and functional changes throughout time p.i. (1, 34, 40–42). Consequently, the quality of memory CD8 T cells used to examine the population dynamics of naive and primary memory CD8 T cells could affect the interpretation of our results. For each of the experiments that we have discussed so far, we have used memory CD8 T cells that were at least 200 d after primary infection. It has been shown that most primary memory CD8 T cells at this time postinfection have a lateM phenotype (from CD62L expression) and function (ability to “vigorously” expand to Ag challenge) (29, 33). To test the extent to which the observed changes in population dynamics of primary and secondary CD8 T cell responses were influenced by the quality of primary memory CD8 T cells, an additional set of adoptive cotransfer experiments was performed (Fig. 8). Primary memory OT-I cell populations generated 33 d (earlyM) or 255 d after VacV-OVA infection (lateM) were cotransferred at different ratios into naive B6 recipient mice before infection with Att L. monocytogenes-OVA or Vir L. monocytogenes-OVA infections (Fig. 8A, experimental design). Phenotypic analysis of both...
memory OT-I populations revealed that day 33 cells express an earlyM phenotype in contrast to the lateM phenotype observed at day 255 (from high frequencies of cells expressing CD62L and CD27 markers) (Fig. 8B).

The longitudinal kinetic analysis of secondary CD8 T cell responses generated either from earlyM or lateM OT-I donors revealed that proliferation capacity of lateM CD8 T cells is higher than that of earlyM cells. Interestingly, despite the initial differences in the ability to expand in numbers, the ratio of earlyM and lateM cells remained relatively constant up to 5 mo p.i., indicating that whereas lateM cells were able to undergo a higher magnitude of expansion than earlyM CD8 T cells (29, 33), the overall kinetics of the secondary CD8 T cell responses were nearly identical (Fig. 8C, 8D). The same results were obtained in all groups of mice, suggesting that intrinsic differences in proliferative expansion and overall kinetics of the secondary CD8 T cell responses are not influenced by the numbers of cells transferred or the virulence of the pathogen used for the infection (Fig. 8C, 8D).

## Discussion

CD8 T cells mediate protection to infection due to their ability to employ effector functions and undergo Ag-driven clonal expansion. CD8 T cells respond to infection by the production of cytolytic molecules and cytokines, which results in destruction of infected cells and the recruitment of additional cells of the immune system.
to the site of infection. With regard to these processes, memory CD8 T cells are superior to naive CD8 T cells, as they are able to display their effector molecules faster (16, 30, 36). However, the precise mechanisms that allow memory CD8 T cells to kill and produce cytokines faster are not completely understood, and there is some evidence that memory CD8 T cells may exist in a “ready to respond” state through steady-state phosphorylation of proteins involved in signaling as well as chromatin remodeling around gene loci encoding for cytolytic molecules (43–48).

The capacity to undergo vigorous proliferation in response to infection is a critical component of protective primary and secondary CD8 T cell responses. It has been suggested that compared with naive CD8 T cells, memory CD8 T cells may be able to encounter Ag earlier (due to differences in trafficking and tissue distribution), begin to cycle faster, and proliferate to a higher extent after Ag encounter (1–6). However, interpretation of these observations has in some instances been complicated by the experimental systems employed. Additionally, recent studies have suggested that primary and repeatedly stimulated memory CD8 T cells differed substantially in their functional properties, including the ability to proliferate and redifferentiate into long-lived memory (19).

In this study, we show that naive CD8 T cells have a greater per-cell expansion potential than do primary memory CD8 T cells when analyzed in the same environment. The intrinsic ability of one naive cell to give rise to more effector CD8 T cells than one memory CD8 T cells is independent of type and dose of infection as well as the number of primary memory cells present in vivo. Additionally, the proliferation capacity of lateM (predominantly CD62Llow) is higher than capacity of earlyM CD8 T cells (predominantly CD62Lhigh) p.i. (Fig. 8 and Refs. 29, 33), confirming that differences in expansion of naive and memory CD8 T cells are not influenced by the quality of memory CD8 T cells used in our cotransfer studies. Therefore, and in line with the notion that multiple Ag encounters decrease the proliferation capacity of responding CD8 T cells (18, 19), even primary Ag stimulation leads to a decreased ability of Ag-specific CD8 T cells to respond to new Ag stimulation (Fig. 9, model).

However, the inflammatory milieu (signal 3) (31, 49, 50) encountered during Ag recognition influences the accumulation of primary and secondary CD8 T cells. The sensitivity to inflammatory cues might be greater for memory CD8 T cells since increasing inflammation during the initial priming decreases the differences in the magnitude of the expansion between primary and secondary CD8 T cell responses. Recently, we showed that inflammatory cytokines (signal 3) exert the greatest impact on proliferating CD8 T cells (26), suggesting the possibility that memory CD8 T cells, by their ability to enter cell cycle earlier than naive CD8 T cells, might be influenced by inflammation for a longer period of time or at the time when inflammation peaks in vivo (usually in the first few days after challenge). Although additional studies are needed to further investigate the differential susceptibility of naive and memory CD8 T cells to inflammatory stimuli, the data presented in this study suggest that the choice of booster pathogen and modulation of the inflammatory milieu during Ag restimulation might represent a viable approach to enhance the magnitude and composition of responding CD8 T cells.

By examining the population dynamics after Ag stimulation we also showed that memory generation potential is higher for responding naive than primary memory CD8 T cells. Initially, contraction of the secondary CD8 T cell response was delayed, leading to a greater representation of secondary than primary CD8 T cells. However, contraction over time was not reduced, but rather contraction of the secondary CD8 T cell response was protracted. As contraction of secondary CD8 T cell responses resolved, primary memory cells were actually present at greater numbers than secondary memory CD8 T cells in all organs analyzed. Again, the observed changes in population dynamics of primary and secondary CD8 T cell responses were not influenced by the quality of Ag stimulation (18, 19), even primary Ag stimulation leads to a decreased ability of Ag-specific CD8 T cells to respond to new Ag stimulation (Fig. 9, model).

In summary, the data presented in this study provide new insights into the functional properties of primary memory CD8 T cells that will help further delineate differences between naive and primary memory CD8 T cells. It also establishes the functional relationship between naive, primary, and multiple stimulated memory CD8 T cells that is clearly dependent on the Ag stimulation history, a finding that has to be taken into consideration in future vaccine development.

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**Disclosures**

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**References**
