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Differing Roles of Inflammation and Antigen in T Cell Proliferation and Memory Generation[1]

Dirk H. Busch,[2] Kristen M. Kerksiek, and Eric G. Pamer[3]

Recent studies have demonstrated that viral and bacterial infections can induce dramatic in vivo expansion of Ag-specific T lymphocytes. Although presentation of Ag is critical for activation of naive T cells, it is less clear how dependent subsequent in vivo T cell proliferation and memory generation are upon Ag. We investigated T cell expansion and memory generation in mice infected alternately with strains of *Listeria monocytogenes* that contained or lacked an immunodominant, MHC class I-restricted T cell epitope. We found substantial differences in the responses of effector and memory T cells to inflammatory stimuli. Although effector T cells undergo in vivo expansion in response to bacterial infection in the absence of Ag, memory T cells show no evidence for such bystander activation. However, Ag-independent expansion of effector T cells does not result in increased memory T cell frequencies, indicating that Ag presentation is critical for effective memory T cell generation. Early reinfection of mice with *L. monocytogenes* before the maximal primary T cell response induces typical memory expansion, suggesting that the capacity for a memory T cell response exists within the primary effector population. Our findings demonstrate that T cell effector proliferation and memory generation are temporally overlapping processes with differing requirements for Ag. *The Journal of Immunology*, 2000, 164: 4063–4070.

The innate and adaptive immune systems collaborate in mammalian defense against invasive pathogens, controlling primary infections and rapidly eliminating pathogens upon subsequent exposures. Protective immunity, which is mediated by Ag-specific memory B and T lymphocytes, is rapidly activated after re-exposure to the pathogen and greatly limits the extent and pathology of infection. The in vivo processes that generate and maintain protective immunity remain mysterious (1), and fundamental questions regarding T cell memory remain unanswered. When are memory T cells generated during the course of primary infection? Are memory T cells generated during the primary immune response, or do they develop during the T cell contraction phase after clearance of the infection? Are in vivo T cell expansion and T cell memory generation tightly linked, or can these processes be dissociated? What is the role of Ag during in vivo expansion of T cell populations and the generation of T cell memory?

$CD8^+$ CTL often play a major role in immunity to viral and intracellular bacterial infections (2). CTL recognize pathogen-derived peptide epitopes presented by MHC class I molecules on the surface of infected cells (3, 4), resulting in cytolytic destruction of the infected cell (5) and, potentially, direct killing of the pathogen (6). The generation and maintenance of specific $CD8^+$ memory T cells is crucial for the development of effective protective immunity against many intracellular pathogens (2, 7). The similarity of memory and effector TCR repertoires suggests that memory T cells derive from effectors by a stochastic rather than selective mechanism (8–12). Other studies also suggest that $CD8^+$ memory T cells are posteffector T cells, although this has not been conclusively demonstrated (1, 13). A recent study using an approach to "mark" genes that have been transiently induced indicates that memory T cells have a history of expressing genes associated with effector T cells. However, only a subset of effector T cells was maintained as memory T cells, and these were detectable during primary infection (14). These findings suggest that memory T cells represent a subset of the effector T cell population that is generated during the acute, primary immune response. The mechanisms that promote the development and expansion of early memory T cells and the factors that result in increased sensitivity and rapidity of memory T cell activation (15, 16) remain unknown.

The role of Ag during in vivo T cell expansion and memory T cell generation is poorly understood. Although Ag is required for T cell priming, long-term persistence of Ag-specific $CD8^+$ memory T cell populations is Ag-independent (17). Indeed, Ag-independent stimulation of memory T cell populations has been discussed as an important mechanisms for the maintenance of T cell memory (18). Recent studies demonstrate that memory T cells divide in the periphery in the absence of MHC molecules (19, 20), providing further evidence for Ag-independent proliferation of Ag-specific memory T cells. Ag-independent "bystander" activation of T lymphocytes has been proposed in the context of viral infections that induce massive populations of activated T lymphocytes, and the ability of type 1 IFN to play a role in this process has been demonstrated (21). However, more recent studies have demonstrated that most activated T cells after viral infection are Ag-specific and that bystander activation accounts for few activated T cells (22, 23). After in vivo T cell priming, Ag-specific T cell populations expand, plateau, and then contract into long-term memory populations. The size of memory T cell populations correlates with the T cell burst size (12, 24), suggesting that the processes that drive in vivo T cell expansion also influence memory T

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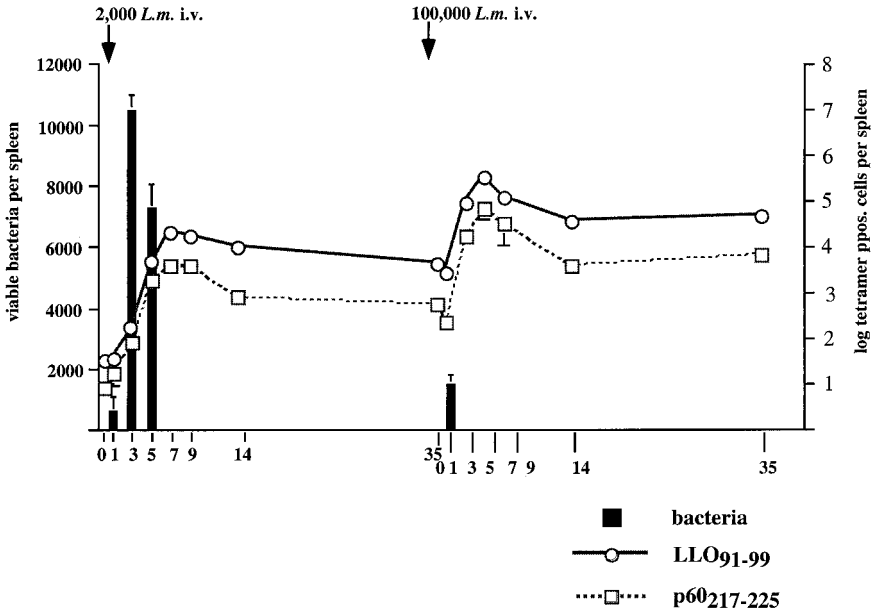

FIGURE 1. Synchronous in vivo T cell kinetics of H2-K^d-restricted T cell populations during primary and recall infection with *L. monocytogenes*. A group of age-matched female BALB/c mice was infected with 2*000 L. monocytogenes* (*L.m.*) 10403s i.v. (day 0). At the indicated times after infection, the number of viable bacteria in the spleen was determined as indicated in *Materials and Methods*. The size of $LLO_{91–99}$- and $p60_{217–225}$-specific T cell population was determined by flow cytometry using H2-K^d tetramers. Absolute numbers of Ag-specific T cells were calculated on the basis of their frequency and the total number of splenocytes. A subgroup of mice received a second i.v. injection of 100,000 *L. monocytogenes* 5 wk after primary infection. Mean values and SD for two mice per time point are plotted. Filled bars, viable bacteria per spleen; ○, $LLO_{91–99}$-specific T cells per spleen; □, $p60_{217–225}$-specific T cells per spleen.

cell development. The relative importance of pathogen-derived Ag presentation as opposed to infection-induced inflammation in determining in vivo T cell expansion kinetics and memory generation is unknown. Although some studies indicate that Ag-independent expansion may be an important feature of in vivo T cell responses (25), direct evidence for this is lacking.

We have used murine infection with *Listeria monocytogenes* to study pathogen-specific T cell responses in vivo. Mice infected i.v. with a sublethal dose of this Gram-positive, intracellular bacterium rapidly clear the pathogen and develop long-lasting immunity, which is predominantly mediated by CD8$^+$ T cells (26, 27). Four different *Listeria* epitopes that are presented by the H2-K^d MHC class Ia molecule have been identified (12, 28, 29), and the kinetics of epitope generation and presentation (28) and the in vivo kinetics of the T cell response have been measured (10, 25, 30). The immunodominant epitopes $LLO_{91–99}$ (listeriolysin O)[4] (derived from the secreted virulence factor listeriolysin (31)) and $p60_{217–225}$ (derived from the mureine hydrolase p60 (32)) elicit large epitope-specific T cell populations. MHC-tetramer reagents and mutant *L. monocytogenes* strains, which are fully virulent yet lack distinct immunodominant epitopes, have been exploited to analyze in vivo T cell responses to this bacterial infection (25, 33).

In this study, we investigated the roles of Ag presentation and the inflammatory response induced by bacterial infection in promoting the in vivo expansion of Ag-specific T cell populations and in the generation of T cell memory. We find that effector T lymphocyte populations that are still responding to primary *L. monocytogenes* infection can be induced to undergo prolonged in vivo division by inflammatory stimuli in the presence but also in the absence of cognate Ag. However, in contrast to stimulation with additional Ag, expansion that is induced by inflammation alone does not result in the enhancement of memory T cell populations. Remarkably, the duration of T cell expansion elicited by reinfection during the primary immune response is identical with a typical memory response, suggesting that the ability to mount a memory T cell response is already established before the completion of the primary immune response. In concordance with work in other infectious systems, we do not find evidence for Ag-independent bystander activation of memory T cell populations. These studies demonstrate that in vivo expansion of Ag-specific effector T cell populations can be promoted by multiple factors but that the presence of Ag is critical for the generation of memory T cells.

[4] Abbreviations used in this paper: LLO, listeriolysin O; FSC, forward light scatter.

Materials and Methods

Mice and bacteria

BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Wild-type *L. monocytogenes* 10403s (obtained from Daniel Portnoy, University of California, Berkeley, CA) and the *L. monocytogenes* LLO_{92ser} mutant strain (mutation of the tyrosine in position 92 of listeriolysin to a serine (33)) were grown in brain heart infusion broth. For primary infection, 2000 *L. monocytogenes* were injected into the tail veins of 8- to 10-wk-old mice. Reimmunization was performed by i.v. injection of 100,000 *L. monocytogenes* 5 wk after primary infection. For the early rechallenge experiments, naive mice were first infected with 2000 *L. monocytogenes* before a second injection with 1×10^6 bacteria 6 days later. Bacterial numbers per infected organ were determined by plating out serial dilution of homogenate on brain heart infusion broth agarose as descibed (34).

Generation and purification of H2-K^d tetramers

Tetrameric H2-K^d/$LLO_{91–99}$ and H2-K^d/$p60_{217–225}$ complexes were generated as recently described (10, 35). Briefly, recombinant H2-K^d heavy chain and β_2-microglobulin were expressed as insoluble inclusion bodies in *Escherichia coli* and were further purified. The H2-K^d heavy chain molecule was mutated to remove the transmembrane and cytosolic domain and to add a specific biotinylation site at the C terminus (36). Purified proteins were refolded in vitro in the presence of high concentrations of synthetic peptide (Research Genetics, Huntsville, AL) to form stable and soluble MHC/peptide complexes (37). Complexes were purified by gel filtration over a Superdex 200 HR column (Pharmacia, Piscataway, NJ) and specifically biotinylated in vitro by adding the enzyme BirA (Avidity), d-biotin, and ATP. After further purification, biotinylated MHC/peptide complexes were multimerized with streptavidin-PE (SA-PE; Molecular Probes, Eugene, OR). Tetrameric complexes were purified by gel filtration and stored at 2–5 mg/ml at 4°C in PBS (pH 8.0) containing 0.02% sodium azide, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 0.5 mM EDTA. Throughout the presented studies, the reagents were frequently tested on *Listeria*-specific T cell lines to document staining capacity and signal intensity.

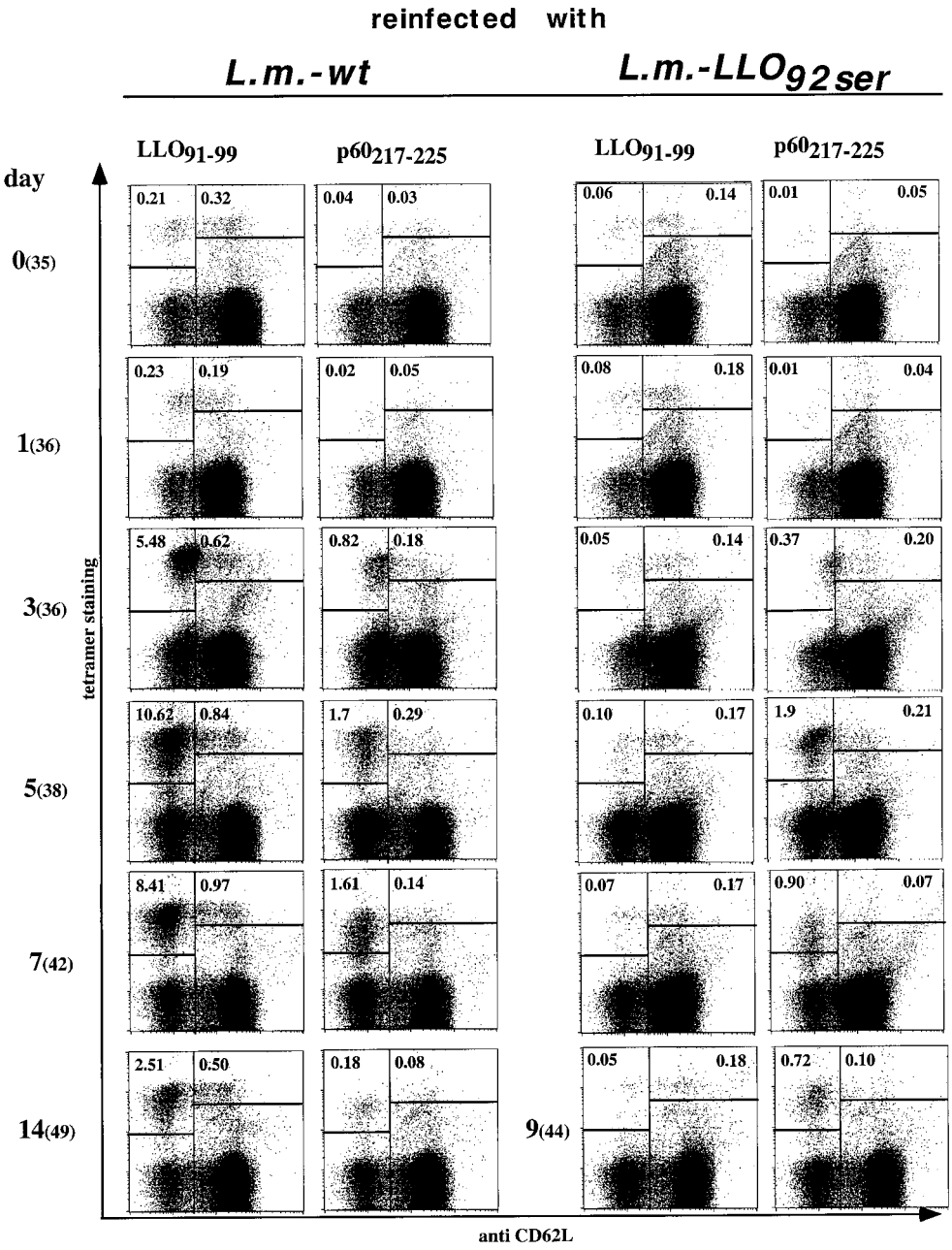

FIGURE 2. Lack of bystander activation of *L. monocytogenes*-specific memory T cells. BALB/c mice were reinfected 5 wk after primary infection with either 100,000 wild-type *L. monocytogenes* (*L.m.-wt*) or with 100,000 *L. monocytogenes* lacking the dominant $LLO_{91–99}$ epitope (*L.m.-LLO*$_{92ser}$). This strain expresses normal amounts of the $p60_{217–225}$ epitope. At the indicated time points during the recall response, $LLO_{91–99}$- and $p60_{217–225}$-specific T cell populations were analyzed by flow cytometry using H2-K^d tetramers. Dot plots of CD8α-gated cells are shown with tetramer staining (PE-conjugated) on the *y*-axis and CD62L staining (FITC-conjugated) on the *x*-axis. Some dot plots show cell populations on the diagonal with intermediate intensity, most likely representing enriched dead cells due to the positive separation procedure (also see *Materials and Methods*).

Preparation of splenocytes

Spleens were removed at various time points after primary infection or reinfection with *L. monocytogenes*. Splenocytes were harvested by dissociation through a wire mesh and lysis of erythrocytes with ammonium chloride and were subsequently resuspended in $RP10^+$, which consists of RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% FCS, L-glutamine, HEPES (pH 7.5), 2-ME, penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml). For the recall experiments using wild-type and LLO_{92ser} *L. monocytogenes*, splenocytes were positively enriched for $CD8^+$ T cells using magnetically activated cell sorting (MACS), anti-CD8α microbeads (clone 53-6.7), and type LS columns (all from Miltenyi, Bergisch Gladbach, Germany). Because this procedure also enriches for dead cells in the preparations (see Fig. 2), we modified our method in subsequent experiments by negatively enriching $CD8^+$ T cells by depleting splenocytes with anti-CD4 (GK1.4) and anti-MHC class II (TIB120) mAbs and anti-rat IgG microbeads (Miltenyi).

Tetramer staining and flow cytometry

Epitope-specific T cell populations were detected with PE-conjugated, tetrameric MHC/peptide complexes and concurrently stained for other surface molecules using directly conjugated mAbs as described previously (10, 25, 30). Briefly, after blocking with unconjugated streptavidin (0.5 mg/ml; Molecular Probes) and Fc-block (PharMingen, San Diego, CA), $\sim 5 \times 10^5$ $CD8^+$-enriched cells were incubated in FACS staining buffer (PBS (pH 7.45), 0.5% BSA, and 0.02% sodium azide) for 1 h on ice in the presence of saturating concentrations of tetramer reagents (0.25–0.5 mg/ml) and the various mAbs. Subsequently, cells were washed three times in staining buffer and then fixed in 1% paraformaldehyde/PBS (pH 7.45).

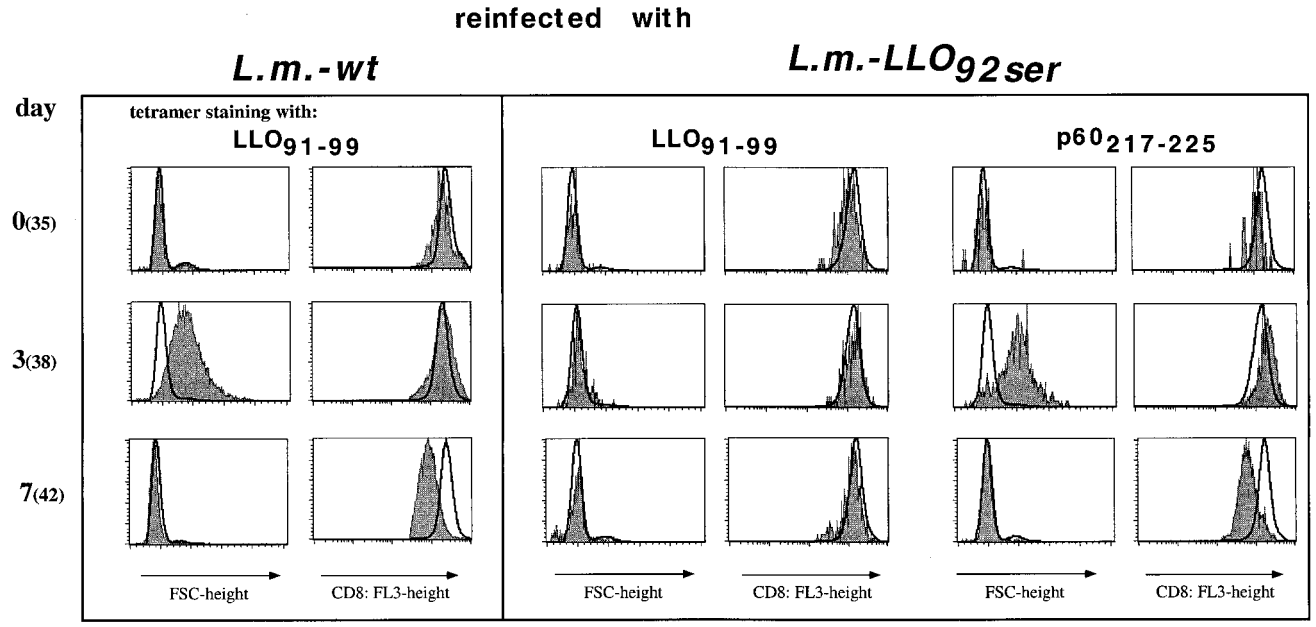

FIGURE 3. Bacterial infection in the absence of Ag does not result in down-regulation of CD8α expression or blasting of *L. monocytogenes*-specific memory T cells. BALB/c mice were reinfected 5 wk after primary infection with either 100,000 wild-type *L. monocytogenes* (*L.m.-wt*) or 100,000 *L. monocytogenes* lacking $LLO_{91–99}$ (*L.m.-LLO$_{92ser}$*), as described in the legend to Fig. 2. At the indicated time points during the recall infection, $LLO_{91–99}$- and $p60_{217–225}$-specific T cell populations were identified using H2-K^d tetramers. Tetramer-positive cell populations were gated, and their FSC and CD8α (CyChrome-conjugated) surface expression profiles are shown (filled histograms). As controls, FSC and CD8α profiles of CD62L^{high} and tetramer-negative populations are shown (black line). Time points after reinfection are indicated (days after primary infection in parentheses).

Flow cytometry was performed using a FACSCalibur, collecting 200,000 CD8$^+$ events; data were further analyzed with CellQuest software (Becton Dickinson, Mountain View, CA). The following mAbs were used (all obtained from PharMingen): CyChrome-conjugated anti-CD8α (clone 53-6.7) and FITC-conjugated anti-CD62L (clone MEL-14).

Results

Synchronized in vivo kinetics of Ag-specific T cell populations

Several epitopes that are recognized by CD8$^+$ CTL during infection of mice with the intracellular bacterium *L. monocytogenes* have been identified. Four *Listeria* epitopes ($LLO_{91–99}$, $p60_{217–225}$, $p60_{449–457}$, and $mpl_{84–92}$) are recognized in the context of the H2-K^d MHC class Ia molecule. Two of these epitopes, $LLO_{91–99}$ and $p60_{217–225}$, induce relatively large, immunodominant T cell responses (12, 25). As shown in Fig. 1, infection of naive BALB/c mice with *L. monocytogenes* results in an initial increase of the number of bacteria in the spleen until day 3, when a rapid, T cell-mediated clearance phase begins. Similar kinetics for bacterial growth and clearance are seen in the livers of infected mice. Unlike many viral infections, which cause prolonged or chronic infections, *L. monocytogenes* is completely cleared from the infected mouse, usually within 6–7 days after primary infection. Reinfection with much higher bacterial doses results in very limited infections with viable bacteria detectable only on the first day after bacterial inoculation. Using MHC tetramers complexed with *L. monocytogenes* epitopes, we found that the expansion kinetics of H2-K^d-restricted *Listeria*-specific T cell populations specific for different peptides are similar during primary and recall *L. monocytogenes* infections, peaking 7–8 days after primary infection and 5 days after recall infection (Fig. 1). The synchronous in vivo expansion of T cells specific for the different *L. monocytogenes* epitopes is surprising because these peptides are present in vastly different amounts and have dramatically different stabilities.

Absence of bystander activation of Ag-specific memory T cell populations

Synchrony of in vivo T cell responses suggests that factors other than Ag determine the in vivo kinetics of T cell expansion and entry into the memory compartment. To investigate the influence of inflammatory factors produced during bacterial infection on the duration of in vivo T cell expansion, we infected mice with a fully virulent strain of *L. monocytogenes* that lacks the immunodominant $LLO_{91–99}$ epitope (33). We first determined the effect of infection with *L. monocytogenes* lacking $LLO_{91–99}$ on established memory T cell populations. The in vivo role and influence of inflammatory stimuli on memory T cells remains controversial (21, 38). As shown in Fig. 2, recall infection with wild-type *L. monocytogenes* results in rapid and massive expansion of $LLO_{91–99}$-specific T cell populations, accounting for 10–20% of CD8$^+$ T cells, and an increase in the frequency of $p60_{217–225}$-specific T cells, accounting for ~2% of CD8$^+$ T cells. Although recall infection with *L. monocytogenes LLO$_{92ser}$* induces normal activation and expansion of $p60_{217–225}$-specific T cell populations, $LLO_{91–99}$-specific T cells do not increase in number and do not undergo phenotypic changes characteristic of activation. Thus, the ratio of CD62L^{high} to CD62L^{low} on $LLO_{91–99}$-specific T cells remains constant (Fig. 2) and there are no detectable changes on forward light scatter (FSC) profiles (Fig. 3) or on the level of surface CD8 expression (Fig. 3). Taken together, these data indicate that bystander activation of $LLO_{91–99}$-specific memory T cells is not detectable during recall responses to *L. monocytogenes* infection.

Expansion of in vivo-activated T cell populations in the absence and presence of Ag

Although inflammation induced by bacterial infection does not activate resting memory T cells, we wanted to determine whether bacterial infection in the absence of Ag can promote the in vivo

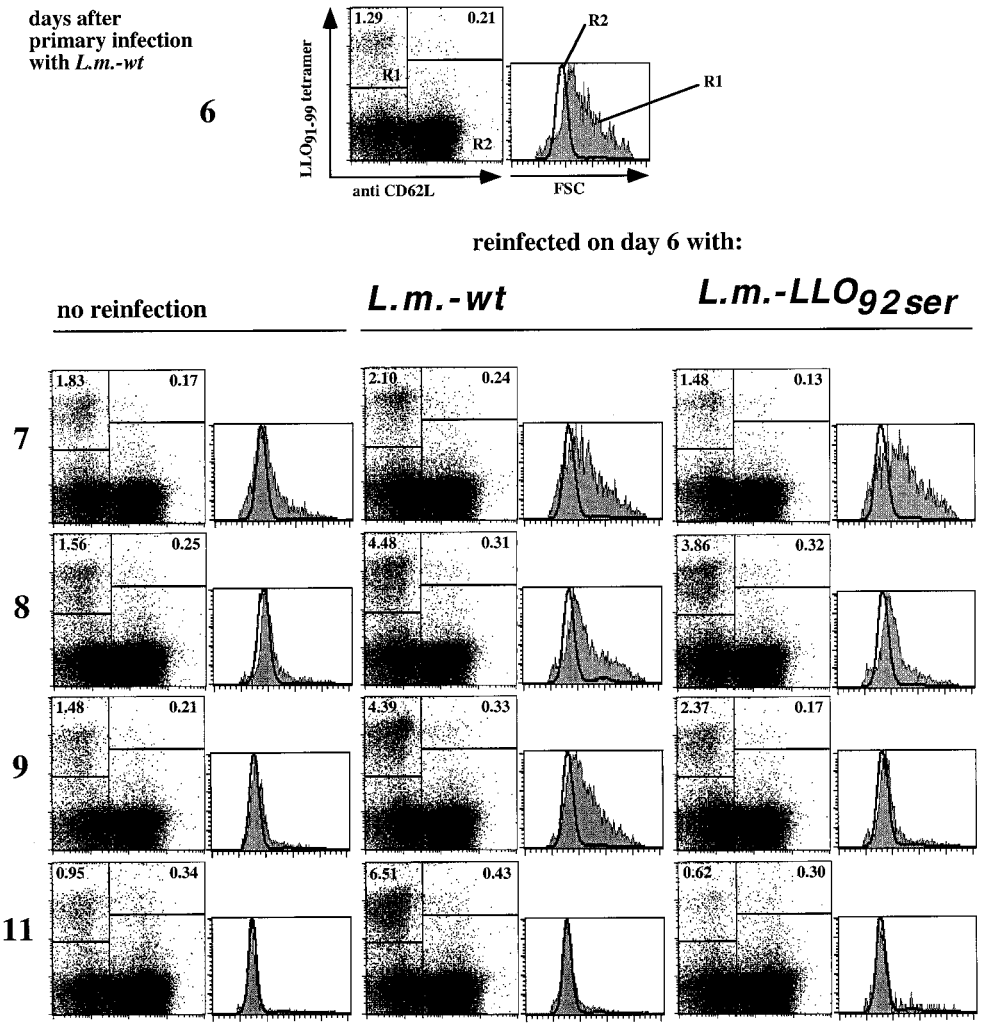

FIGURE 4. Bacterial infection in the presence or absence of Ag induces proliferation of *L. monocytogenes*-specific effector T cells. BALB/c mice were infected with a sublethal dose of 2000 wild-type *L. monocytogenes*. Six days after inoculation, the infected mice were split into three groups; the first group received no further treatment (no reinfection), the second was reinfected with 1×10^6 wild-type *L. monocytogenes* (*L.m.-wt*), and the third group was reinfected with 1×10^6 *L. monocytogenes* lacking LLO_{91-99} (*L.m.-LLO_{92ser}*). At the indicated time points during the course of reinfection, LLO_{91-99}-specific T cell populations were identified and analyzed by H2-K^d tetramer staining (two mice for each group were analyzed per time point, as described in *Materials and Methods*). Representative dot plots with tetramer staining on the *y*-axis and CD62L staining on the *x*-axis are shown (gated on CD8α-positive cells). Gates R1 (tetramer-positive, $CD62L^{low}$ cells) and R2 (tetramer-negative, $CD62L^{high}$ cells) indicate the regions that were chosen for the FSC analysis shown on the right side of the corresponding dot plot.

expansion of activated effector T cells. Therefore, we reinfected mice 6 days after primary infection with wild-type *L. monocytogenes* with a high dose of either wild-type bacteria or *L. monocytogenes* LLO_{92ser}. Without reinfection, the expansion of the LLO_{91-99}-specific T cell populations terminates 7–8 days after bacterial inoculation, which is also reflected by a rapid loss of blasting cells as measured by FSC. However, in mice challenged with *L. monocytogenes* LLO_{92ser}, LLO_{91-99}-specific T cells continued to increase in frequency for several days (Figs. 4 and 5). As determined by FSC profiles, Ag-specific T cells continued to blast on the seventh day after primary infection (Fig. 4), resulting in an increase in the frequency (Fig. 4) and absolute number (Fig. 5) of

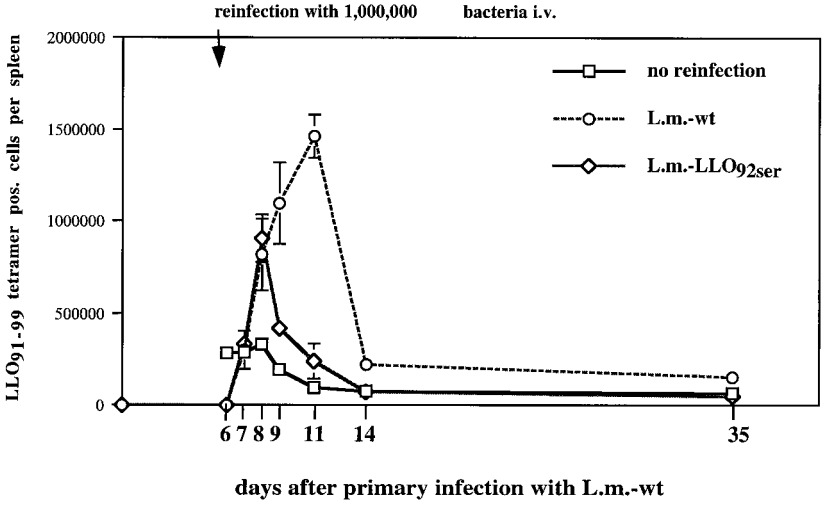

FIGURE 5. Enhanced T cell expansion only results in increased memory T cell numbers in the presence of additional Ag. BALB/c mice were reinfected with wild-type *L. monocytogenes* (*L.m.-wt*) or *L. monocytogenes* lacking LLO_{91-99} (*L.m.-LLO_{92ser}*) before completion of the primary response, as described in the legend to Fig. 4. LLO_{91-99}-specific T cell populations were indentified by H2-K^d tetramer staining, and their absolute numbers were calculated and plotted. The time points after infection are indicated on the *x*-axis. □, no reinfection during the primary response; ○, reinfection with wild-type *L. monocytogenes*; ◇, reinfection with the *L. monocytogenes* lacking LLO_{91-99}.

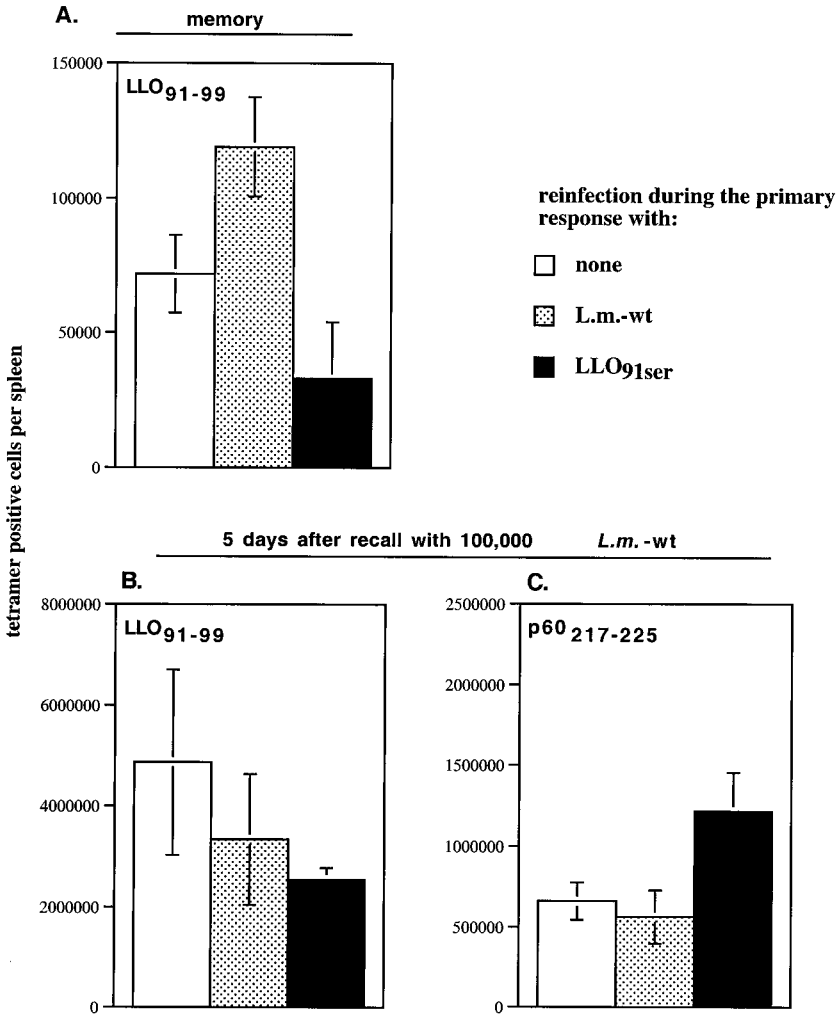

FIGURE 6. Differences in the kinetics of primary T cell responses do not significantly impact memory T cell responses. BALB/c mice were infected with wild-type *L. monocytogenes* and then divided into three groups and reinfected as described in Figs. 4 and 5. □, No reinfection during the primary response; ▨, reinfection with 1×10^6 wild-type *L. monocytogenes* (*L.m-wt*) on 6 days after primary infection; ■, reinfection on day 6 with 1×10^6 epitope knockout *L. monocytogenes* (LLO_{92ser}). $LLO_{91–99}$- and $p60_{217–225}$-specific T cell populations were identified by H2-K^d tetramer staining, and absolute numbers were calculated. Mean values of three to four mice per group and SD are plotted. *A*, Absolute numbers of $LLO_{91–99}$-specific memory T cell populations in the spleen 5 wk after primary infection. *B*, $LLO_{91–99}$-specific T cell populations in the spleen 5 days after recall infection with 100,000 wild-type *L. monocytogenes*. *C*, $p60_{217–225}$-specific T cell populations in spleens 5 days after recall infection, as in *B*.

$LLO_{91–99}$-specific T cells. This Ag-independent in vivo expansion of $LLO_{91–99}$-specific T cells is followed by a rapid contraction phase. Taken together, these data demonstrate that accentuated inflammatory responses during the terminal phases of the primary T cell response are capable of promoting further T cell expansion that does not depend upon the presence of Ag.

We also reinfected mice on the sixth day after primary *L. monocytogenes* infection with a large dose of *L. monocytogenes* expressing the $LLO_{91–99}$ epitope. In this setting, $LLO_{91–99}$-specific T cells also continue to expand, as measured with H2-K^d tetramers complexed with $LLO_{91–99}$. However, in contrast to reinfection with *L. monocytogenes* lacking $LLO_{91–99}$, expansion continues for an additional three days, resulting in a substantially larger effector T cell population. Interestingly, the kinetics of the accentuated $LLO_{91–99}$-specific T cell response elicited by reinfecting mice on the sixth day after primary infection approximate the previously described kinetics of *L. monocytogenes*-specific memory T cell responses (25).

Epitope presence and memory T cell generation

Reinfection of mice during the primary immune response with epitope-knockout and wild-type *L. monocytogenes* both induce the further expansion of $LLO_{91–99}$-specific T cell populations, but the consequences for memory T cell generation are quite different (Figs. 4 and 5). The initial expansion of $LLO_{91–99}$-specific T cells after reinfection with *L. monocytogenes* LLO_{92ser} is followed by a dramatic contraction phase, leaving low numbers of $LLO_{91–99}$-specific memory T cells. These data indicate that the correlation between the effector T cell burst magnitude and the size of memory T cell populations is not maintained when T cells are induced to undergo increased expansion in the absence of Ag. Reinfection with wild-type *L. monocytogenes* results in enhanced expansion, and the $LLO_{91–99}$-specific memory T cell frequency is substantially increased (Figs. 5 and 6*A*). Thus, Ag presentation appears to be essential for enhanced generation of memory T cells.

To further investigate the differences in memory T cell generation after Ag-dependent and -independent T cell expansion, we reinfected mice with wild-type *L. monocytogenes* that had been infected 5 wk previously with various sequential combinations of wild-type and $LLO_{91–99}$-deficient bacteria. As shown in Fig. 6*B*, mice previously immunized with wild-type *L. monocytogenes* alone develop a normal memory response upon reinfection; this response is manifested by massive expansion of $LLO_{91–99}$- and $p60_{217–225}$-specific T cells (also see Fig. 2). Similarly, *L. monocytogenes*-immunized mice that received a second dose of bacteria 6 days after primary infection also developed normal $LLO_{91–99}$- and $p60_{217–225}$-specific memory responses after reinfection 5 wk later. In contrast, mice that received a primary infection with wild-type *L. monocytogenes* and then a second dose of $LLO_{91–99}$-deficient *L. monocytogenes* 6 days later mounted a somewhat smaller $LLO_{91–99}$-specific response upon reinfection 5 wk later. This result further supports the conclusion that inflammation-driven effector T cell expansion does not increase memory T cell populations. Indeed, our results suggest that inflammation-driven expansion in the

absence of Ag may diminish the size or responsiveness of memory T cell populations.

Discussion

Studies of in vivo T cell responses to various infectious pathogens have demonstrated that the size of resulting T cell populations can range from relatively small to massive. When different pathogens contain the same T cell epitope, the magnitude of the epitope-specific T cell response is substantially determined by the infectious context (39, 40). Although it is possible that the duration of in vivo Ag presentation may differ in these circumstances and may account for the disparate T cell responses, it is also possible that the inflammatory response triggered by different pathogens influences the duration of T cell proliferation. The role of Ag during in vivo T cell expansion and memory T cell generation is only partially understood. Our studies demonstrate that whereas activation and expansion of memory T cell populations is Ag-dependent, activated effector T cells can be induced to proliferate in vivo by inflammatory stimuli in the absence of additional Ag. Although such Ag-independent expansion does not result in increased memory T cell frequencies, T cell expansion that is driven by Ag does increase the size of the memory T cell population.

The development of new techniques to identify epitope-specific T cells directly ex vivo has permitted detailed analyses of normal T cell response dynamics (23, 30, 35, 41). As demonstrated here and in previous studies, the in vivo kinetics of T cell populations that differ in specificity are often synchronized (23, 25), a finding that is particularly striking during the recall infection with *L. monocytogenes.* Although the Ags for the four known H2-K^d-restricted *Listeria* epitopes are expressed by the bacterium in very different amounts and the epitopes are processed in infected cells with different kinetics and form MHC/peptide complexes with markedly different half-lives (28), the T cell kinetics are very similar. This finding suggests that factors other than epitope prevalence determine the in vivo kinetics of T cell expansion.

What effects do inflammatory stimuli have on epitope-specific T cell populations in the absence of Ag? To address this question, we used a mutant strain of *L. monocytogenes* that is fully virulent but does not produce the immundominant H2-K^d-restricted epitope $LLO_{91–99}$ (33). Using MHC-tetramer staining we demonstrate that $LLO_{91–99}$-specific memory T cells are not activated by *L. monocytogenes* infection if $LLO_{91–99}$ is not presented. $LLO_{91–99}$-specific memory T cells do not increase in number and do not undergo phenotypic changes during the course of reinfection if the specific epitope is not present. These findings confirm the results of other studies that showed that Ag-independent "bystander activation" of memory T cells is a rare event that is unlikely to be crucial for the maintenance of T cell memory (22, 23, 38).

In contrast to our findings with memory T cells, early reinfection with *L. monocytogenes* lacking $LLO_{91–99}$ promoted further in vivo expansion of effector T cells specific for $LLO_{91–99}$. This finding indicates that activated effector T cell populations respond to the in vivo inflammation induced by *L. monocytogenes* infection in the absence of additional Ag presentation. Our experiments do not identify the specific factors mediating this Ag-independent T cell expansion, but they demonstrate that inflammation induced by bacterial infection serves as a potent stimulus for effector T cell proliferation.

T cell activation is accompanied by transient surface expression of the high-affinity IL-2 and IL-15 receptors, promoting T cell proliferation upon exposure to these cytokines (42). From in vitro studies it is clear that IL-2 in the absence of Ag can promote limited effector T cell proliferation even in the absence of TCR-mediated signals (43). Thus, it is possible that early reinfection with *L. monocytogenes* induces the production of IL-2 and perhaps other growth-promoting cytokines, which drive further proliferation of effector T cells. Alternatively, it is possible that bacterial products such as lipoteichoic acids or peptidoglycan directly stimulate the growth of effector T cells in an Ag-independent fashion (44). Another possibility is that early reinfection provides anti-apoptotic stimuli that promote effector T cell survival (45).

It is interesting that reinfection of mice before resolution of a primary infection induces further expansion of Ag-specific T cells that kinetically approximates the typical memory response that is induced by reinfection months after the resolution of a primary infection. We have previously shown that memory T cells undergo dramatic in vivo expansion for 5 days after reinfection. Similarly, reinfection of mice 6 days after primary infection, which precedes the maximal in vivo primary T cell response by 1–2 days, results in 5 additional days of expansion. This observation suggests that the ability to mount a memory T cell response is established before the completion of the expansionary phase of the primary T cell response. This interpretation of our findings is supported by a recent study using a transgenic approach that detected potential memory T cells during the effector phase of infection (14).

The presence or absence of cognate epitope during in vivo restimulation of effector T cells has significant consequences for the development of memory T cell populations. The presentation of additional Ag increases the burst size of the effector T cell population, ultimately resulting in a substantially larger memory T cell population, as we have previously described for recall responses to *L. monocytogenes* infection (25). In contrast, early reinfection with *L. monocytogenes* LLO_{92ser}, the strain lacking the dominant $LLO_{91–99}$ epitope, results in continued expansion of $LLO_{91–99}$-specific T cells and then very rapid contraction and a lower frequency of $LLO_{91–99}$-specific memory T cells. Thus, effector T cell expansion that occurs in the absence of Ag does not enhance T cell memory, demonstrating that the T cell burst size does not always correlate with the size of the resulting memory population. This finding may have important implications for vaccine and adjuvant development.

It is likely that Ag and inflammatory factors collaboratively promote effector T cell proliferation and survival and that both play a role during productive in vivo T cell expansion. Our results suggest that while inflammation alone can promote in vivo T cell expansion, Ag presentation is required for the expansion of the memory compartment. On the basis of our results, we suggest that the capacity for a memory T cell response is "hard-wired" in the Ag-specific effector T cell population before the completion of the expansionary phase of the primary T cell response. Whether memory T cells constitute a distinct subpopulation of effector T cells remains unclear. It is possible that all effector T cells acquire a "memory program" that requires a TCR-mediated signal to be activated. If this is the case, then exposure of a large effector T cell population, such as the one present 6 days after primary infection, should result in a very large memory T cell response. Our results do not support this hypothesis because the "memory" response induced by reinfection during the primary T cell response is smaller than the response obtained 6 wk after the resolution of infection. Our results are more consistent with the idea that a distinct subset of the total effector T cell population is capable of further expansion upon re-exposure to Ag.

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