H2-M3-Restricted Memory T Cells: Persistence and Activation Without Expansion

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H2-M3-Restricted Memory T Cells: Persistence and Activation Without Expansion

Kristen M. Kerksiek, Alexander Ploss, Ingrid Leiner, Dirk H. Busch, and Eric G. Pamer

H2-M3-restricted T cells respond more rapidly to primary *Listeria monocytogenes* infection than conventional MHC class I-restricted T cells. Reinfection with *L. monocytogenes*, while inducing explosive proliferation of H2-Kd-restricted T cells, does not stimulate significant expansion of H2-M3-restricted CTL. These disparate responses to reinfection are apparent within 5 days of primary *L. monocytogenes* infection. However, H2-M3-restricted memory T cells are generated, and are indistinguishable from classically restricted T cells in terms of cell surface memory markers and longevity. Early responses of H2-M3- and H2-Kd-restricted memory T cells to reinfection are similar, with increases in size and expression of activation markers. Interestingly, priming of H2-M3-restricted T cells with an *L. monocytogenes*-derived N-formyl peptide plus anti-CD40 generates memory T cells that expand upon re-exposure to Ag during *L. monocytogenes* infection. Our data indicate that disparate H2-M3- and MHC class Ia-restricted memory T cell responses reflect intrinsic differences between these T cell populations. Although distinct proliferative programs appear to be hardwired in these populations during primary *L. monocytogenes* infection, under different inflammatory circumstances M3-restricted T cell populations can maintain the ability to expand upon re-exposure to Ag. *The Journal of Immunology*, 2003, 170: 1862–1869.

Antigen-specific T cell responses following primary and secondary infections are quantitatively and qualitatively distinct (1–3). The frequency of pathogen-specific T cells after infection is higher than in naive individuals, a factor that probably contributes to the larger magnitude of memory responses. Qualitative differences between B cell responses to primary and secondary infection are well established (1, 4), and although the picture is somewhat less clear for T cells, there are similarities (1–3). T cells undergo in vivo selection with expansion of higher affinity clones upon re-exposure to Ag (5–8), and memory T cells require lower concentrations of Ag (9) and less costimulation (10) than naive T cells for activation. Memory T cells proliferate earlier (11–13), divide faster (11), and acquire effector functions (13–15) more rapidly than naive T cells.

However, distinguishing between naive, effector, and memory T cells is an inexact science. Naive T cells are often distinguished from Ag-experienced cells by cell surface expression of activation markers; naive cells are CD44<sup>low</sup>/CD62L<sup>high</sup> and become CD44<sup>high</sup>/CD62L<sup>low</sup> upon activation. However, CD62L is often re-expressed on memory cells, especially in the CD8 lineage, and expression of CD44 is not always consistent among mouse strains (16), complicating the use of these markers. The RB isoform of CD45 has been used to distinguish naive (CD45RB<sup>high</sup>) from memory (CD45RB<sup>low</sup>) cells, but it is not a reliable marker (2). Distinguishing memory from effector T cells can be even more difficult. Although CD25 (IL-2R<sup>α</sup>) and CD69 are expressed on recently activated effector T cells, their expression is rapidly down-regulated during the terminal effector phase of the T cell response. The IL-2Rβ-chain (CD122), which is a component of the IL-2 and IL-15 receptors, is expressed on some memory T cells and binds IL-15, a crucial cytokine for memory CD8<sup>+</sup> T cell maintenance (17, 18). In addition, expression of Ly-6C is low and heterogeneous on effector cells and high on memory T cells, providing a convenient marker to distinguish between these two populations (9, 19–21). Expression of these cell surface markers has been largely characterized on adoptively transferred transgenic T cells in C57BL/6 mice, and their utility for the analysis of complex memory T cell populations in infectious disease models and in other mouse strains has yet to be determined.

The rapid and dramatic expansion of H2-Kd (MHC class Ia)-restricted T cell populations in response to recall infection with *Listeria monocytogenes* is typical of immunological memory (22). In contrast, expansion of CTL populations restricted by the H2-M3 MHC class Ib molecule, which presents *L. monocytogenes*-derived N-formyl methionine peptides, is neither faster nor larger following recall *Listeria* infection than after primary infection (23). In this study, we show that the frequencies of H2-M3-IMIGWII(A) and H2-Kd/listeriolysin O (LLO)<sub>91–95</sub>, tetramer-positive memory cells are comparable following primary *L. monocytogenes* infection. To explain these disparate responses to reinfection, we tested two hypotheses: first, that inflammation and/or Ag presentation following reinfection do not provide adequate stimuli to drive H2-M3-restricted T cell expansion; and, second, that H2-M3- and H2-Kd-restricted T cells differ intrinsically in their responses to Ag re-exposure. We show that H2-M3-restricted T memory cells are generated, maintained, and do not differ from H2-Kd-restricted...
memory cells on the basis of cell surface phenotypic markers. Although H2-M3-restricted populations up-regulate expression of activation markers, they do not expand following reinfection with *L. monocytogenes*. Enhancing in vivo Ag exposure and inflammation did not enhance the expansion of H2-M3-restricted memory cells, arguing against the first hypothesis. Our results support the second hypothesis, suggesting that differences in memory T cell expansion are intrinsic to H2-M3- and H2-K\(^d\)-restricted T cell populations and reflect either differences in the naive T cell populations recognizing H2-M3 or H2-K\(^d\) or differences in the priming of T cells by these two MHC class I molecules.

**Materials and Methods**

**Mice, bacteria, and inoculations**

Female CB6F\(_1\) and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 M\(^{-}\)transgenic mice were generated by injection of fertilized oocytes with a 20-kDa fragment of the genomic H2-K\(^d\) gene, which was kindly provided by Dr. F. Lemmonier (Pasteur Institute, Paris, France). *L. monocytogenes* strain 10403s was obtained from D. Portnoy (University of California, Berkeley, CA) and grown in brain heart infusion broth at 37°C, shaking, until growth was exponential (OD\(_{600}\) = 0.05–0.1). Titters were calculated based on the following ratio: OD\(_{600}\) of 0.1 = 2 \(\times\) 10\(^5\) *L. monocytogenes*. All infections were performed i.v. by tail vein injection in a volume of 200 \(\mu\)l of PBS. Doses of 2 \(\times\) 10\(^6\) or 1 \(\times\) 10\(^5\) *L. monocytogenes* were used for inoculation of mice for primary and recall infections, respectively; any variations in dose are noted in the figures.

For peptide immunization, syngeneic (CB6F\(_1\)) splenocytes were harvested as described below, irradiated (1000 rad), incubated with 2 \(\times\) 10\(^{–6}\) M LLO\(_{99-99}\) and fMIGWII peptides for 2 h at 37°C, and washed twice. Approximately 3 \(\times\) 10\(^9\) peptide-coated splenocytes and 100 \(\mu\)g of anti-CD40 mAb (clone FGRK45) were injected i.v. in a volume of 200 \(\mu\)l of PBS. For concurrent live infection and anti-CD40 mAb treatment, mice received 10\(^5\) enriched cells (per well in a 96-well plate) was performed as above, or differences were used. Following the blocking step, cells were stained on ice for 1 h with PE-conjugated tetramers, anti-CD8α-allophycocyanin (clone 53-6-7; BD Pharmingen), and one of the following FITC-conjugated Abs specific for murine surface molecules (all from BD Pharmingen): CD62L (clone MEL-14), CD44 (clone IM7), CD45RB (clone 16A), CD69 (clone H1.2F3), CD25 (IL-2Rα, clone 7D4), CD122 (IL-2Rβ, clone TM-β1), and Ly-6C (clone AL-21). Either propidium iodide (PI) or ethidium monoazide bromide (EMA; Molecular Probes) was added to stain dead cells (specified in the figures). When PI was used, splenocytes were resuspended in SB after the final wash, transferred to chilled FACS tubes, and kept on ice at 4°C (unfixed) until acquisition. Just before acquisition, an equal volume of 8 \(\mu\)g/ml PI (in SB, final 4 \(\mu\)g/ml) was added to the cells. When EMA was used, a concentration of 1.25 \(\mu\)g/ml (in SB; stock 2.5 \(\mu\)g/ml in dimethylformamide) was added to the buffer during the staining of the cells. For the last 10 min of the (1-h) incubation, the cells were exposed to light to cross-link DNA-bound EMA; unbound EMA was washed away in the three subsequent washes. After the final wash, the splenocytes were fixed as above.

All data were acquired using a FACS Calibur flow cytometer and analyzed using CellQuest software (BD Biosciences, Mountain View, CA).

**Results**

**Activation and memory markers on H2-M3- and H2-K\(^d\)-restricted T cells are similar after primary Listeria infection**

Immunological memory is generated after infection with most pathogens, resulting in faster and more effective immune responses when the same pathogen is re-encountered (1). In mice reinfected with *L. monocytogenes*, H2-K\(^d\)-restricted, Listeria-specific T cells respond more quickly and reach much higher frequencies than seen during primary infection (6, 22–24). In contrast, H2-M3-restricted T cell populations undergo only minimal expansion following a second exposure to *Listeria* (23, 26). To investigate the differences between MHC class Ia (H2-K\(^d\))- and class Ib (H2-M3)-restricted memory responses, we performed phenotypic analyses of these populations following primary and secondary *L. monocytogenes* infection.

H2-M3/fMIGWII and H2-K\(^d\)/LLO\(_{99-99}\) tetramer-positive cells expressed similar activation and memory markers during primary and secondary *L. monocytogenes* infection. Seven days following primary infection, M3/fMIGWII and K\(^d\)/LLO\(_{99-99}\) tetramers stained ~4% and 2.3% of CD8\(^+\) cells, respectively (Fig. 1, left columns). Both populations were CD62L\(^{low}\), CD44\(^{high}\), CD45R\(^{high}\), CD69\(^{low}\), and CD25\(^{low}\) 7 days postinfection and throughout the primary response (days 5, 7, 9, 11, 14, 17, and 21 postinfection). Differences in activation marker expression between the H2-K\(^d\)- and H2-M3-restricted populations were detected only for CD122 (IL-2Rβ) and Ly-6C. These differences most likely reflect the distinct kinetics of the T cell populations; on day 5 postinfection (not shown), H2-M3-restricted T cells resembled H2-K\(^d\)-restricted T cells that are present 7 days postinfection (Fig. 1), with higher levels of CD122 and more heterogeneous expression of Ly-6C. By day 9 postinfection, LLO\(_{99-99}\)-specific cells were CD122\(^{low}\) and more uniformly high for Ly-6C (not shown).

Four weeks following primary *L. monocytogenes* infection, fMIGWII- and LLO\(_{99-99}\)-specific T cell populations constituted ~0.65% and 0.40% of CD8\(^+\) cells, respectively. Both populations had become more heterogeneous in expression of CD62L (Fig. 1, right columns), confirming our previous finding that some LLO\(_{99-99}\)-specific T cells re-express CD62L over time (22). Some heterogeneity in the expression of CD44, and particularly CD45RB, also developed 4 wk postinfection, although the majority of epitope-specific cells retained high levels of these markers. Some studies have indicated that memory cells are CD45RB\(^{low}\), in our system, only a very small percentage of
cells would fit that criterion. Finally, in contrast to the memory-like cells that undergo homeostatic expansion in "empty" mice and maintain uniformly high levels of CD122 (17, 20, 21), neither fMIGWII- nor LLO91–99-specific T cells expressed high levels of CD122 in the weeks following primary infection. No clear differences between the H2-M3- and H2-Kd-restricted T cell populations were detected, supporting the notion that H2-M3-restricted memory cells are generated and maintained following primary infection with *L. monocytogenes*.

Recall infection with *L. monocytogenes* activates H2-M3-restricted T cells

To further characterize the disparity between H2-M3- and H2-Kd-restricted memory T cells, we measured cell size and intensity of CD8α staining, as an indication of T cell activation, at early time points following reinfection. Changes in both parameters were very similar in the two populations (Fig. 2). Both M3/fMIGWIIA and Kd/LLO91–99 tetramer-positive (and CD62Llow) T cell populations increase in size on day 3 postchallenge and return to a small size (equivalent to the tetramer-negative, CD62Lhigh population) by day 7. H2-M3-restricted CTL also appear to be activated on the basis of CD8 expression levels; the kinetics of CD8 up-regulation and subsequent down-regulation following secondary *Listeria* infection are nearly identical with those detected for the LLO91–99 peptide-specific population (Fig. 2).

M3/fMIGWII tetramer-positive T cells also express early activation markers following challenge with *L. monocytogenes* (Fig. 3). One day following reinfection, fMIGWII- and LLO91–99-specific T cell populations constitute ~0.5 and 0.3% of total CD8+ T cells, respectively, and significant percentages of both populations express CD69 (>60%) and, particularly for M3/fMIGWII-positive cells, CD25 (20–45%). During primary infection, populations of epitope-specific CTL expressing CD69 and CD25 are not detectable (data not shown) because of the low frequencies at early time points. Three days following reinfection, levels of CD122 are dramatically up-regulated, with 65% of fMIGWII- and nearly 80% of LLO91–99-specific CTL expressing the marker. However, CD122 levels decrease by day 5 postinfection and remain low at subsequent time points (data not shown). Expression of these activation markers is shown on the right- and y-axes. Dot plots are gated on live CD8+ cells. Percentages in the upper right corners represent the percentage of activation/memory marker-positive cells among tetramer-staining cells. Three mice were analyzed each day, and data are shown for one representative animal.
markers is not detected on all tetramer-positive cells, which may reflect heterogeneity within the populations or, given the low frequency of memory T cells, the inadvertent inclusion of nonspecifically stained cells. Nevertheless, by size parameters, modulation of CD8 levels, and expression of early activation markers, the activation states of H2-M3- and H2-Kd-restricted CTL are indistinguishable at early time points following reinfection.

Extended exposure to L. monocytogenes during secondary infection does not increase the expansion of H2-M3-restricted T cells

We next considered the possibility that the amount of N-formyl peptide present during secondary infection, although sufficient to induce activation, is not enough to result in dramatic proliferation of H2-M3-restricted T cells. Although immune mice are typically challenged with doses of L. monocytogenes 50-fold larger than the primary inoculum, bacteria are cleared much more quickly (27). To extend secondary infection and increase the duration of in vivo Ag presentation, immune mice were repeatedly infected with high doses of L. monocytogenes (Fig. 4). Infection with 1 \( \times 10^9 \) L. monocytogenes for 2 (Fig. 4, middle column) or 3 (right column) consecutive days did not increase expansion of H2-M3/fMIGWII or H2-Kd/LLO91-99 tetramer-positive cells; in fact, frequencies of epitope-specific cells were remarkably similar to expansion detected following a typical challenge with 1 \( \times 10^5 \) L. monocytogenes (Refs. 22 and 23, and data not shown). The size of the spleens in mice reinfected with one, two, or three doses of L. monocytogenes was similar (2.8, 2.7, and 2.5 \( \times 10^8 \) splenocytes, respectively), and viable bacteria were detected at the time of spleen harvest in most repeatedly infected mice (data not shown).

To rule out the possibility that H2-M3-restricted T cells fail to expand in response to challenge because of overstimulation and subsequent deletion of epitope-specific cells, immune mice were also reinfected with lower doses of L. monocytogenes. Following challenge with 1 \( \times 10^5 \) L. monocytogenes, LLO91-99-specific T cells expanded to somewhat lower frequencies than those detected after challenge with higher doses. However, no expansion of H2-M3-restricted T cells was detected (data not shown).

H2-M3-restricted T cells do not undergo enhanced expansion if re-exposed to L. monocytogenes during the primary immune response

H2-M3-restricted T cell populations in Listeria-immune mice become activated, but do not expand dramatically if mice are reinfected 4–7 wk after the primary infection (Ref. 23; Figs. 2 and 3). To determine whether H2-M3-restricted CTL expand if re-exposed to L. monocytogenes earlier following initial inoculation, immune mice were boosted with 1 \( \times 10^6 \) L. monocytogenes 6 days after primary infection. LLO91-99-specific T cells underwent further expansion under these conditions; at the peak of expansion, the LLO91-99-specific CTL population was ~5-fold larger than the peak response in control mice (Ref. 28; Fig. 5A). The kinetics of the additional expansion, with a peak 5 days after the boost, suggest that LLO91-99 peptide-specific cells with the potential to undergo memory expansion are present within the primary effector population (22, 23). In contrast to the enhanced expansion of H2-Kd-restricted T cells, there was little additional expansion of fMIGWII-specific T cells following boosting (Fig. 5A). The slight increase in the frequency of fMIGWII-specific T cells is not a consistent finding and was not detected in a similar, independent experiment (D. H. Busch and E. G. Pamer, unpublished results).

We were concerned that the differences between H2-M3- and H2-Kd-restricted CTL expansion in response to the day-6 boost might reflect the distinct expansion kinetics of these T cell populations following primary L. monocytogenes infection; on day 6 after primary infection, H2-M3-restricted CTL are beginning the contraction phase, while H2-Kd-restricted T cells are in the midst of vigorous expansion (23). To address this issue, mice were boosted 4 days after primary infection (Fig. 5B) because, on day 4 after primary infection, both H2-M3- and H2-Kd-restricted T cell populations are expanding. The results of this experiment also

![FIGURE 3.](image)

**FIGURE 3.** Up-regulation of early activation markers on H2-M3-restricted T cells following L. monocytogenes reinfection. Four weeks following primary L. monocytogenes infection, CB6/F1 mice were reinfected with 1 \( \times 10^9 \) bacteria. Splenocytes were harvested 3 (left columns) and 5 (right columns) days postchallenge and stained as described in Fig. 1. Dot plots are gated on live CD8+ T cells, with staining for activation/memory markers (listed to the right) on the x-axis and tetramer staining on the y-axis. The tetramer used (H2-M3/fMIGWII or H2-Kd/LLO91-99) is specified above each column. The percentage of tetramer-staining cells positive for each activation marker is shown. Three mice were analyzed per day, and representative data for one individual are shown.

![FIGURE 4.](image)

**FIGURE 4.** Repeated exposure to L. monocytogenes does not enhance expansion of H2-M3/fMIGWII tetramer-positive memory cells during recall infection. Female CB6/F1 mice were infected with 2000 L. monocytogenes and, 15 wk later, reinfected with 1 \( \times 10^8 \) Listeria one, two, or three times at daily intervals (left to right, as indicated above the dot plots). Splenocytes harvested 5 days after the first reinoculation were MACS-enriched for CD8+ T cells and stained with anti-CD45e and anti-CD26L mAb as well as MHC class I tetramers (listed to the left). Dot plots are gated on CD8+ T cells, with CD62L on the x-axis and tetramer staining on the y-axis. The tetramer used (H2-M3/fMIGWII or H2-Kd/LLO91-99) is specified above each column. The percentage of tetramer-staining cells positive for each activation marker is shown. Three mice were analyzed per day, and representative data for one individual are shown.

**2.** \( 1 \times 10^6 \) Lm: 1 dose, 2 doses, 3 doses

**H2-Kd/LLO91-99**

<table>
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<th>CD6L</th>
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<td>53.0</td>
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**H2-M3/fMIGWII**

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**anti-CD62L**

**TABLE 4.**
show that LLO91-specific T cells undergo additional expansion while fMIGWII-specific T cells are unaffected. Thus, the different capacities of H2-M3- and H2-K4-restricted T cells to respond to an Ag re-exposure are already apparent during the primary expansion phase of the T cell populations.

Peptide immunization primes fMIGWII and LLO91–99 peptide-specific T cells that can respond to subsequent infection with L. monocytogenes

The challenge inoculum of L. monocytogenes, whether administered 4 days or 7 wk after the primary infection, is cleared very rapidly (data not shown). To determine whether Ag-experienced, H2-M3-restricted CTL are capable of expansion under primary infection conditions, we primed epitope-specific T cells by immunization of mice with peptide-coated splenocytes and anti-CD40 mAb. Immunization with fMIGWII- and LLO91–99-coated splenocytes primed peptide-specific T cell responses restricted by H2-M3 and H2-K4, respectively (Fig. 6). Subsequent L. monocytogenes infection of peptide-immunized mice resulted in impressive expansion of M3/fMIGWII and K4/LLO91–99 tetramer-positive T cells (Fig. 6). The frequency of LLO91–99-specific T cells 5 days after reinfection was slightly lower in mice that were peptide-immunized than in mice previously immunized with L. monocytogenes (Fig. 6B, left panels). However, the absolute numbers of LLO91–99-specific T cells that expanded under these two conditions were similar (Fig. 6A). The recall frequencies in this experiment are low compared with previous studies (23) because the challenge dose was only 5000 bacteria, because peptide-immunized mice were not expected to have high-level protective immunity. Remarkably, the fMIGWII-specific T cell population expanded to a similar extent as LLO91–99-specific T cells in peptide-primed mice (Fig. 6, B, lower right panel, and A). As expected, the H2-M3 memory response in mice previously immunized with live L. monocytogenes was small (Fig. 6A) and stained with lower intensity with the tetramers (data not shown). It is interesting that LLO91–99-specific T cells did not expand dramatically in L. monocytogenes–primed mice reimmunized with peptide-coated splenocytes. It is possible that LLO-coated APCs are rapidly eliminated by memory T cells in these immune mice.

To determine whether anti-CD40 stimulation during primary infection with live bacteria alters the generation of subsequent H2-M3-restricted memory responses, we treated mice with three doses of 100 μg of FGK45 anti-CD40 Ab, 48 and 24 h before and at the time of primary L. monocytogenes infection. Fig. 6C demonstrates that anti-CD40 treatment in the context of a live infection did not enhance the H2-M3-restricted memory T cell response.

The increased frequencies of fMIGWII- and LLO91–99-specific T cells in peptide-immunized mice (compared with naive mice) indicates that the T cells are Ag experienced. However, the T cell memory responses in mice primed with peptide and live L. monocytogenes are not identical. For example, LLO91–99 and fMIGWII-specific memory T cells in mice immunized with live L. monocytogenes are large, as measured by forward scatter (FSC), 3 days postchallenge and become small by the fifth day (Figs. 2, and 6B, top histograms). In contrast, memory T cells in peptide-immunized mice remain large on the fifth day after live bacterial challenge, suggesting that these T cells are still actively proliferating (Fig. 6B, bottom histograms). Although we interpret these differences in T cell size as a reflection of different kinetics of bacterial clearance and inflammation in the two experimental groups, it is also possible that they result from disparities in the priming of CD8+ T cells by live infection vs peptide immunization.

Discussion

H2-M3-restricted T cells are early participants in the adaptive immune response to primary L. monocytogenes infection, reaching peak numbers before and frequently expanding to greater frequencies than H2-K4-restricted populations (23, 26). However, in response to secondary infection, which induces dramatic expansion of MHC class Ia (H2-K4)-restricted CTL, only minimal expansion of H2-M3-restricted T cell populations is detected. In this report, we demonstrate that M3-restricted memory T cells are generated and maintained; the residual populations of H2-M3- and H2-K4-restricted T cells detected weeks after primary infection do not...
differ in their expression of activation and memory markers. Furthermore, secondary infection activates H2-M3-restricted T cells. Although the extent of recall expansion is small, H2-M3-restricted T cells peak in frequency 5 days postinfection and undergo similar size shifts and CD8 modulation as their class Ia-restricted counterparts. Thus, H2-M3-restricted memory T cell responses share similarities with MHC class Ia-restricted responses but, notably, do not undergo dramatic expansion upon secondary challenge with L. monocytogenes.

We first hypothesized that secondary infection with L. monocytogenes might not provide an adequate stimulus for H2-M3-restricted memory T cells to undergo expansion. In this scenario, H2-M3- and H2-Kd4-restricted memory T cells are not inherently different, but secondary L. monocytogenes infection selectively supports expansion of H2-Kd4-restricted T cells, perhaps reflecting better Ag presentation. Primary and secondary L. monocytogenes infections are remarkably different; immune mice rapidly clear viable bacteria in 1–3 days, despite a 50-fold greater dose than that used for primary infection, which requires 5–7 days for clearance (27). Differences between the peptides presented by H2-M3 and H2-Kd4 might provide an explanation for the lesser expansion of H2-M3-restricted memory T cells. Short N-formyl peptides are shed by bacteria and probably do not require processing before presentation by H2-M3 (29, 30). In contrast, peptides presented by H2-Kd4 derive from secreted proteins (31) that must be degraded to generate the nonamer peptide epitopes. It is possible that the requirement for processing extends the duration of peptide presentation by H2-Kd4 beyond clearance of the pathogen, while the duration of N-formyl peptide presentation is restricted to the period of infection with viable bacteria.

Arguing against the role of Ag presentation in the disparate memory responses is our finding that H2-M3-restricted T cells become activated during a secondary Listeria infection. H2-M3-restricted memory T cells up-regulate CD69, CD25, and CD122 early following challenge (Fig. 3), and changes in FSC and CD8 levels exhibited by IMIGWII-specific T cells are indistinguishable from those detected for LLO91.99-specific T cells (Fig. 2). Shifts in FSC and CD8 levels, at least for MHC class Ia-restricted CTL, require in vivo Ag presentation (28). Although Ag presentation is sufficient for activation of H2-M3-restricted T cells following L. monocytogenes challenge, it is possible that this exposure is not sufficient for maximal expansion. However, consecutive daily challenges with high doses of L. monocytogenes failed to increase the frequency of either H2-M3- or H2-Kd4-restricted memory T cell responses (Fig. 4), strongly suggesting that Ag is not a limiting factor in H2-M3-restricted T cell responses. These experiments argue against the hypothesis that secondary infection does not provide an adequate stimulus to drive expansion of H2-M3-restricted memory T cells.

Our second hypothesis was that inherent differences between H2-M3- and H2-Kd4-restricted memory T cell populations account for their disparate expansion kinetics in response to secondary L. monocytogenes infection. Because neither enhanced Ag presentation nor inflammation promote the expansion of H2-M3-restricted memory T cells, it is reasonable to suggest that H2-M3- and H2-Kd4-restricted memory T cells may have been programmed differently at the time of primary infection. Recent studies suggest that differences between H2-M3- and H2-Kd4-restricted T cells result from disparate forms of thymic selection (32). Our finding that Ag responsiveness of H2-Kd4 and H2-M3-restricted T cells already differs at early time points during primary infection supports the notion that these T cell populations are intrinsically different.

Because both populations are similarly activated by repeat infection, it is possible that proliferation of H2-M3- and H2-Kd4-
restricted T cells in response to secondary *L. monocytogenes* infection is equivalent but that expanding H2-M3-restricted populations undergo increased apoptosis. Levels of Bcl-2 are higher in CD8+ memory T cells than in naive or effector cells (33). Bcl-2 is regulated by IL-7, and mice deficient in the IL-7R are unable to maintain CD8+ memory cells (34). Although our studies demonstrate that H2-M3-restricted T cells are generated and maintained, it is conceivable that deficiency or differential regulation of Bcl-2 or other antiapoptotic molecules in H2-M3-restricted memory cells results in decreased cell survival during the proliferative phase that follows reinfecion with *L. monocytogenes*.

Our results with peptide-immunized mice indicate that H2-M3-restricted T cells can, under some circumstances, undergo memory expansion. Immunization with peptide-coated splenocytes primes peptide-specific T cells (35, 36) but does not induce significant immunity to *L. monocytogenes* infection. Peptide immunization and live infection provide different stimuli to naive T cells, possibly resulting in distinctly programmed, H2-M3-restricted memory T cells. Alternatively, because bacterial clearance is delayed in H2-M3-restricted T cells following Ag-experienced H2-M3-restricted T cells are capable of an anergic T cells make a major contribution to the more rapid and efficient memory CD8+ T cell response.

Rapid expansion of H2-M3-restricted T cells following primary *L. monocytogenes* infection kinetically resembles a memory T cell response (23). One scenario, which was proposed by Lenz and Bevan (38), is that H2-M3-restricted T cells are preactivated by formyl peptides to enable its trafficking to the cell surface. This surge in CD8+ memory T cells, which expand dramatically in response to secondary *L. monocytogenes* infection in vivo; although MHC class Ia-restricted T cells in these mice do not expand significantly in response to reinfecion, depletion of CD8+ T cells, which are primarily H2-M3-restricted, results in decreased protection (26). We also have evidence that the potential of CD8+ T cell expansion is limited; Kd+LLO91-99 tetramer-positive cells, which expand dramatically in response to secondary *L. monocytogenes* infection, undergo minimal expansion following a third bacterial inoculation (D. H. Busch and E. G. Pamer, unpublished data). Thus, it is possible that murine CD8+ T cells have a limited ability to proliferate in vivo, and that H2-M3-restricted T cells may have a history of proliferation before the first infection with *L. monocytogenes*.

Another intriguing explanation for the minimal expansion of H2-M3-restricted T cells is related to the low surface expression of the MHC class Ia molecule in vivo. Because there are few endogenous N-formyl peptides to enable its trafficking to the cell surface, most H2-M3 is retained intracellularly in the unstimulated cell (39, 40). The results of investigations addressing requirement for MHC in the maintenance of memory T cells would suggest that low H2-M3 expression levels do not lead to a defect in memory cell maintenance (41–43). Our ability to detect M3-restricted memory T cells with MHC tetramers fits with these findings. However, a recent study found that, although allogenic MHC engagement was not necessary for memory CD4+ T cell survival, it was essential to maintain the full functional capabilities of the memory cells (44). Interestingly, much like H2-M3-restricted memory cells, memory cells with an MHC-deficient history increased in size upon re-infecion but were not able to expand. However, the MHC class Ia-deficient mice argues against a full functional defect in H2-M3-restricted memory cells (26).

Although many questions about H2-M3-restricted memory responses to *L. monocytogenes* infection remain, the data presented in this study provide evidence that M3-restricted memory T cells are generated, maintained, and activated in response to challenge with the bacterium. Determination of the mechanisms responsible for differential expansion of H2-M3- and H2-Kd-restricted T cell populations in response to secondary *L. monocytogenes* infection may provide important insights into the nature of immunological memory.

References