ticipate in the autoimmune process. Potentially, neuronal OPN secretion could modulate inflammation and demyelination and could influence the clinical severity of the disease. Consistent with this idea, a role for neurons in the pathophysiology of MS and EAE has recently been described (*21*, *22*), and neurons are known to be capable of cytokine production (*35*, *36*). OPN inhibits cell lysis (*6*), and thus, neuronal OPN might even protect the axon from degeneration during autoimmune demyelination.

CD44 is a known ligand of OPN, mediating a decrease of IL-10 production (*10*). As shown here, $OPN^{-/-}$ mice produced elevated IL-10 during the course of EAE. We recently demonstrated that antibodies against CD44 prevented EAE (*37*), suggesting that the proinflammatory effect of OPN in MS and EAE might be mediated by CD44. The binding of OPN to its integrin fibronectin receptor $\alpha_V \beta_3$ through the arginine-glycine-aspartate tripeptide motif may also perpetuate T_H1 inflammation (*10*). In active MS lesions, the α_V subunit of this receptor is overexpressed in macrophages and endothelial cells, and the β_3 subunit is expressed on endothelial cell luminal surfaces (*23*). By means of its tripeptide-binding motif, OPN inhibits inducible nitric oxide synthetase (iNOs) (*38*), which is known to participate in autoimmune demyelination (*1*). Thus, in conclusion, OPN is situated at a number of checkpoints that would allow diverse activities in the course of autoimmune-mediated demyelination.

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Priming of Memory But Not Effector CD8 T Cells by a Killed Bacterial Vaccine

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Killed or inactivated vaccines targeting intracellular bacterial and protozoal pathogens are notoriously ineffective at generating protective immunity. For example, vaccination with heat-killed *Listeria monocytogenes* (HKLM) is not protective, although infection with live *L. monocytogenes* induces long-lived, CD8 T cell–mediated immunity. We demonstrate that HKLM immunization primes memory CD8 T lymphocyte populations that, although substantial in size, are ineffective at providing protection from subsequent *L. monocytogenes* infection. In contrast to live infection, which elicits large numbers of effector CD8 T cells, HKLM immunization primes T lymphocytes that do not acquire effector functions. Our studies show that it is possible to dissociate T cell–dependent protective immunity from memory T cell expansion, and that generation of effector T cells may be necessary for long-term protective immunity.

CD8 T lymphocytes mediate immunity to a broad range of viral, bacterial, and protozoal pathogens (*1*), and increasing evidence suggests that effector T cells primed during infection evolve into long-lived memory cells (*2*–*4*). Memory T cells can be subdivided into two categories on the basis of activation markers, homing receptor expression, and effector function (*5*). Central memory T cells, which express high levels of the chemokine

receptor CCR7 and the adhesion molecule CD62L and do not express effector functions, may differentiate into effector memory T cells, which express low levels of CCR7 and CD62L but produce cytokines (*6*). Whether these memory T cell subsets differ in their capacity to mediate protective immunity is unknown. Furthermore, the stimuli that generate central versus ef-

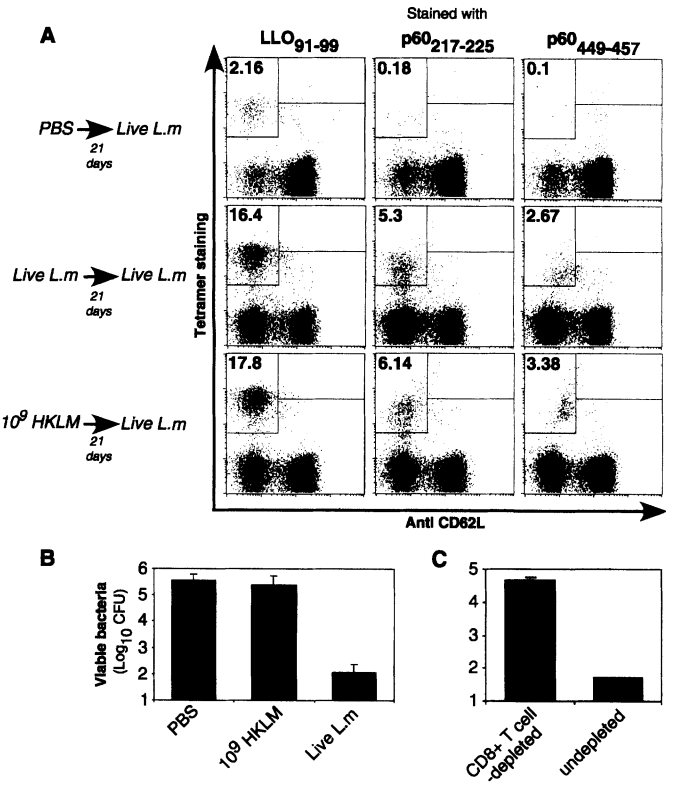

Fig. 1. HKLM immunization primes antigen-specific memory CD8$^+$ T cell responses. (**A**) CB6 mice (6 to 8 weeks old) were immunized intravenously with PBS, 10^4 live *L. monocytogenes*, or two consecutive daily doses of 10^9 HKLM (*13*). Mice were infected 21 days later with 10^4 (naïve and HKLM-immunized mice) or 10^5 (immune mice) live *L. monocytogenes*. Splenocytes, 7 days after infection in PBS-treated mice and 5 days after infection in HKLM and live *L. monocytogenes*–immunized mice, were stained for CD8α and CD62L and with H2-K^d tetramers complexed with three *L. monocytogenes*–derived epitopes (LLO_{91-99}, $p60_{217-225}$, and $p60_{449-457}$). Dot plots are gated on live CD8 T lymphocytes and show CD62L and H2-K^d tetramer staining. The percentage of activated tetramer-positive CD8 T cells is shown in the upper left quadrant of each panel. (**B**) CB6 mice were immunized with two intravenous doses of 10^9 HKLM, 10^4 live *L. monocytogenes*, or PBS and were challenged 21 days later with 10^4 live *L. monocytogenes*. Protective immunity was measured by dissociating spleens 72 hours after infection and quantifying viable bacteria. Mean numbers of colony-forming units (CFU) from six mice per group are shown (error bars, SD). (**C**) BALB/c mice were immunized with 2000 live *L. monocytogenes* and, 4 weeks later, left untreated or depleted of CD8 T cells by intravenous administration of three consecutive daily doses of 100 μg of CD8α-specific mAb (anti Lyt 2, 53-6.72, American Type Culture Collection) before rechallenge with 10^5 live *L. monocytogenes*. Mice received an additional dose of CD8 mAb 3 days after infection. CD8 T cell depletion was greater than 90%. Mean numbers of bacteria in spleens (three mice per group) 72 hours after infection are shown (error bars, SD).

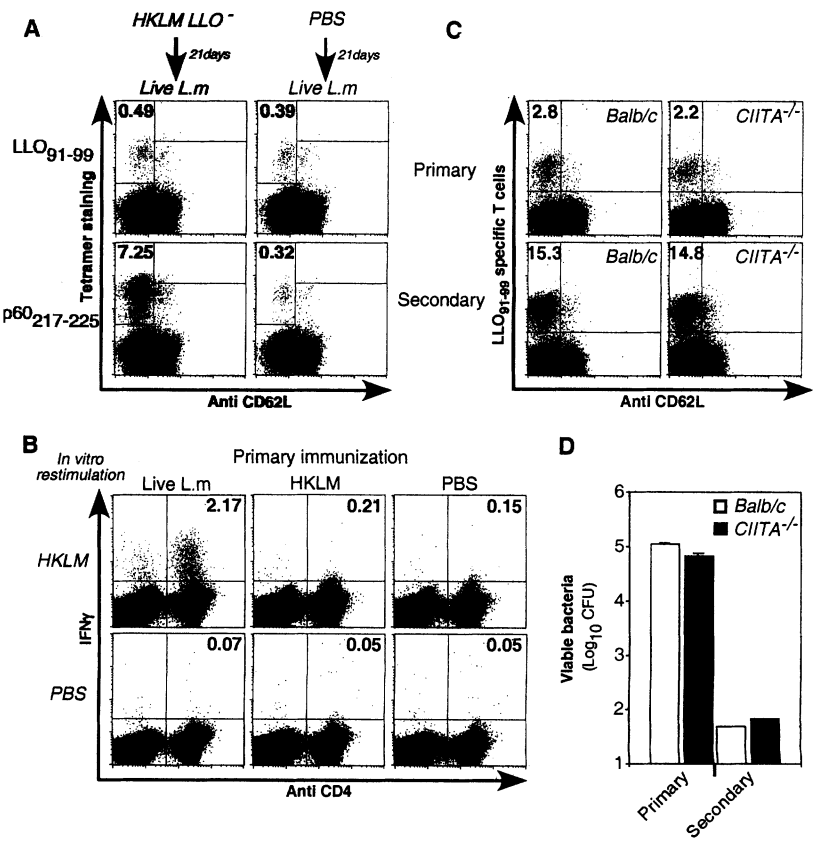

Fig. 2. HKLM directly primes antigen-specific CD8$^+$ T cells, but not *L. monocytogenes*–specific CD4$^+$ T cells. (**A**) CB6 mice (three per group) were injected intravenously with PBS or HKLM derived from a mutant *L. monocytogenes* strain lacking the LLO_{91-99} epitope (see text) and were rechallenged 21 days later with live *L. monocytogenes*. The frequencies of CD8 T cells specific for LLO_{91-99} and $p60_{217-225}$ were determined by tetramer staining 5 days after infection. (**B**) Mice were immunized with live *L. monocytogenes*, HKLM, or PBS; 6 days later, splenocytes were restimulated in vitro with HKLM (top row) or PBS (bottom row) in the presence of brefeldin A (BFA) for 6 hours. IFN-γ production was measured by intracellular cytokine staining using manufacturer's protocols (Cytofix/Cytoperm, Pharmingen). Dot plots show CD4 and IFN-γ staining; percentages of CD4 T cells producing IFN-γ are shown. (**C**) BALB/c or CIITA$^{-/-}$ mice (8 to 10 weeks old) were infected intravenously with live *L. monocytogenes* and analyzed 7 days later for the frequency of LLO_{91-99}–specific T cells by tetramer staining (top row). Mice that had received live *L. monocytogenes* 21 days earlier were rechallenged with 10^5 live bacteria, and the LLO_{91-99}–specific CD8 T cell response was measured 5 days later. (**D**) The number of live bacteria in the spleens of mice 72 hours after primary or secondary infection was determined as described in Fig. 1. Means of two mice per group are shown (error bars, SD). In (A) and (C), the percentage of activated, tetramer-positive CD8 cells is shown in the upper left quadrant of each panel.

fector memory T cells remain undefined.

Listeria monocytogenes is a Gram-positive, facultative intracellular bacterium that causes severe disease in immunocompromised patients (*7*). Studies in a mouse model of listeriosis have demonstrated that CD8 T cells mediate protective immunity after immunization with live bacteria (*8–10*). HKLM immunization, on the other hand, does not induce protective immunity (*11*). Infection of mice with live *L. monocytogenes* induces antigen-specific CD8 T cell responses, with peak frequencies 7 to 9 days after infection of naïve mice and 5 days after reinfection of immune mice (*12*). To determine whether HKLM immunization primes *L. monocytogenes*–specific CD8 T cells, we immunized CB6 (C57BL/6 × BALB/c F_1) mice with live *L. monocytogenes* or HKLM (*13*) and, 21 days later, intravenously infected these mice with live *L. monocytogenes*. The magnitude of the *L. monocytogenes*–specific CD8 T cell response was measured by H2-K^d tetramer staining of splenocytes (Fig. 1A). CD8 T cells specific for the immunodominant listeriolysin epitope LLO_{91-99} were detectable 7 days after infection of naïve mice (Fig. 1A, upper left panel), whereas responses to the subdominant $p60_{217-225}$ and $p60_{449-457}$ epitopes were smaller. As expected, reinfection of mice immunized with live *L. monocytogenes* induced markedly larger CD8 T cell memory responses (Fig. 1A, middle row), and these mice were resistant to reinfection (Fig. 1B). In contrast, HKLM immunization was not protective (Fig. 1B). Surprisingly, HKLM-immunized mice mounted memory CD8 T cell responses that were indistinguishable in size from those detected in immune mice (Fig. 1A, bottom row). Mice immunized with HKLM derived from an avirulent strain of *L. monocytogenes* lacking LLO also primed CD8 T cell responses to p60 (*14*), indicating that residual LLO associated with HKLM is not allowing access to the cytosol of APCs.

Although CD8 T cells can provide protective immunity to *L. monocytogenes* infection, CD4 T cells and antibodies have also been implicated in immunity (*10, 15*). To measure their contribution to protective immunity, we depleted CD8 T cells from immune mice and challenged them with *L. monocytogenes*. Depleted animals were markedly more susceptible than control immune mice to *L. monocytogenes* infection (Fig. 1C), demonstrating the essential role that CD8 T cells play in protective immunity. Depletion of CD8 T cells with a monoclonal antibody (mAb) spe-

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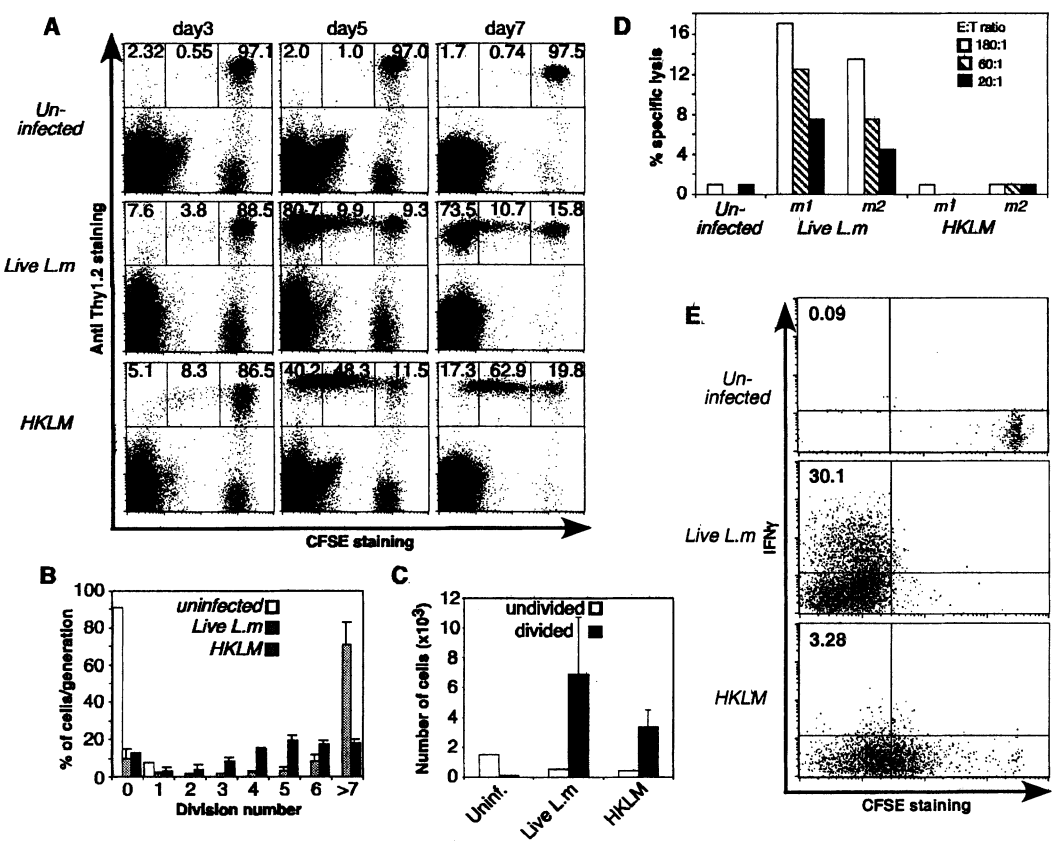

Fig. 3. HKLM-primed $CD8^+$ T cells undergo limited primary expansion and do not acquire effector functions. (**A**) TCR-transgenic mice specific for $p60_{217-225}$ were generated with the TCRα and β chain genes from CTL clone L9.6 and backcrossed to the BALB/c (Thy1.2) background (*19*). L9.6 TCR-transgenic splenocytes (2.2×10^7) were labeled with 5 μM CFSE and transferred into BALB/c Thy1.1 mice. One day later, mice were intravenously injected with PBS, live *L. monocytogenes*, or HKLM. Splenocytes were recovered 3, 5, and 7 days after immunization and stained with antibodies specific for CD8α and Thy1.2. CFSE staining intensity and staining for Thy1.2, which identifies transferred T cells, are plotted for live CD8 T cells. Percentages of $Thy1.2^+$ cells that are CFSE low, intermediate, and high are shown in each plot. (**B**) Percentages of cells that have divided from 0 to >7 times (as determined by the intensity of CFSE fluorescence) are plotted for uninfected, live *L. monocytogenes*–infected, and HKLM-immunized mice on the fifth day after inoculation. (**C**) Absolute numbers of transferred $Thy1.2^+$ T cells that remained undivided or that proliferated in uninfected, live *L. monocytogenes*–infected, and HKLM-immunized mice were determined on the fifth day after inoculation. (**D**) For determination of the cytolytic activity of transferred L9.6 TCR-transgenic T cells, CD8 T cells were enriched with MACS anti-CD8α beads (Ly-2, Miltenyi Biotech) from mice immunized 7 days previously with PBS, live *L. monocytogenes*, or HKLM. Direct cytolytic activity of these CD8 T cells was measured in a 6-hour ^{51}Cr release assay using $p60_{217S-225}$–pulsed P815 target cells. Percent specific lysis is plotted for three different effector-to-target ratios for an uninfected control and two live and HKLM-immunized mice. (**E**) $p60_{217-225}$ TCR-transgenic spleen cells (Thy1.2; 2.2×10^6) were labeled with CFSE and transferred into Thy1.1 BALB/c recipient mice. One day later, mice were injected intravenously with PBS (upper panel), live *L. monocytogenes* (middle panel), or HKLM (lower panel). Splenocytes were taken 5 days later and stimulated in vitro for 6 hours with 10 μM $p60_{217S-225}$ in the presence of BFA. IFN-γ synthesis by the specific T cells was determined by intracellular cytokine staining of $Thy1.2^+$ CD8 T cells. The percentage of IFN-γ–producing, $CFSE^{lo}$ CD8 T cells is indicated in the upper left quadrant of each plot.

cific for CD8β yielded similar results (*16*).

The robust CD8 T cell memory responses in HKLM-immunized mice could have resulted from direct priming of antigen-specific CD8 T cells or, indirectly, through priming of *L. monocytogenes*–specific CD4 T cells that would act to accelerate naïve CD8 T cell responses upon challenge with live bacteria. To distinguish between these two mechanisms, we immunized mice with HKLM lacking the immunodominant LLO_{91-99} CD8 T cell epitope ($HKLM^{LLO-}$). This strain of *L. monocytogenes* was generated by point mutation of an essential anchor residue of LLO_{91-99} and expresses functional LLO (*17*).

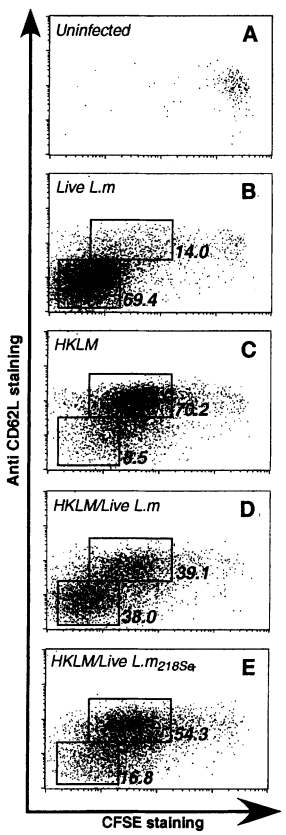

Fig. 4. Concurrent live infection with *L. monocytogenes* does not alter the phenotype of HKLM-primed CD8 T cells. L9.6 TCR-transgenic splenocytes (Thy1.1; 10^6) were labeled with CFSE and transferred into Thy1.2 mice. One day later, mice were either left uninfected (**A**), infected with live wild-type *L. monocytogenes* (**B**), or immunized with HKLM alone (**C**) or together with live wild-type *L. monocytogenes* (**D**). One group of mice was immunized with HKLM and concurrently infected with live *L. monocytogenes* 218Ser (**E**), which contains a point mutation in p60, eliminating the $p60_{217-225}$ epitope. After 4.5 days, splenocytes were stained with mAbs specific for CD8, Thy1.1, and CD62L. Dot plots show CFSE and CD62L staining of gated Thy1.1 CD8 T cells. Each plot shows results for a single animal and is representative of two mice per experimental group. The percentage of transferred CD8 T cells in the $CD62L^{hi}$ and $CD62L^{lo}$ quadrants is shown.

If CD4 T cell responses accelerate CD8 T cell responses upon live challenge of HKLM-immunized mice, LLO_{91-99}–specific responses would be expected to still be enhanced in $HKLM^{LLO-}$-immunized mice. Infection of mice previously immunized with $HKLM^{LLO-}$ resulted in a primary-like CD8 T cell response to LLO_{91-99}, similar to that obtained upon primary infection of naïve mice with *L. monocytogenes* (Fig. 2A, top row), but a memory-like CD8 T cell response to $p60_{217-225}$ (Fig. 2A, bottom row). This experiment demonstrates that HKLM immunization primed CD8 T cell responses independently of CD4 T cell priming. Indeed, CD4 T cells from HKLM-immunized and naïve mice, in contrast to mice infected with live bacteria, do not respond to bacterial antigen (Fig. 2B), which suggests that HKLM immunization does not efficiently prime CD4 T cells. Furthermore, infection of HKLM-immunized mice did not result in memory CD4 T cell responses (*14*).

One explanation for a lack of protective immunity in HKLM-immunized mice may be that deficient CD4 T cell responses limit *L. monocytogenes*–specific CD8 T cell function. To determine whether CD4 T cells influence CD8 T cell responses to *L. monocytogenes* infection, we measured the LLO_{91-99}–specific CD8 T cell response in mice lacking the class II transactivator gene ($CIITA^{-/-}$). These mice have a 95% reduction in peripheral CD4 T cells and do not mount peripheral CD4 T cell responses because B cells, dendritic cells, and macrophages lack major histocompatibility complex class II molecules (*18*). The primary CD8 T cell response to *L. monocytogenes* infection was identical in CIITA-deficient and control mice (Fig. 2C). Similarly, reinfection of previously immunized $CIITA^{-/-}$ and control mice elicited indistinguishable memory LLO_{91-99}–specific CD8 T cell responses. CIITA-deficient mice cleared bacterial infection and developed similar protective immunity (Fig. 2D). Thus, CD4 T cell responses do not account for the difference in protective immunity after live and HKLM immunization.

To further characterize CD8 T cell priming by in vivo HKLM immunization, we generated a transgenic mouse line expressing T cells with specificity for $p60_{217-225}$ (*19*). Naïve, antigen-specific T cells from these mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and transferred into naïve recipient mice, allowing us to trace their fate after live infection or HKLM immunization. Donor CD8 T cells can be distinguished from recipient cells by a congenic Thy1 disparity. In the absence of infection, transferred $p60_{217-225}$–specific CD8 T cells remained CFSE positive (Fig. 3A, top row), indicating that they had not undergone in vivo divisions after transfer. In contrast, infection with live *L. monocytogenes* resulted in a large population of CFSE-low donor CD8 T cells (Fig. 3A, middle row). Immunization with HKLM also induced the expansion of $p60_{217-225}$–specific T cells, with the majority of transferred T cells entering division (Fig. 3A, bottom row). However, in contrast to priming by live infection, $p60_{217-225}$–specific T cells primed with HKLM underwent fewer divisions (Fig. 3B). The proportions of naïve $p60_{217-225}$–specific T cells that proliferated after infection with live bacteria or immunization with HKLM were not significantly different (Fig. 3C), which suggests that transferred T cells encounter similar levels of processed antigen. It is unlikely that decreased T cell proliferation reflects insufficient antigen presentation, because mice immunized with 10^8, 10^9, and 10^{10} HKLM yielded similar results. Furthermore, we and others have demonstrated that naïve CD8 T cells require only brief encounter with antigen to undergo prolonged division (*20*–*22*). Thus, it is unlikely that a shorter duration of in vivo antigen presentation after HKLM immunization accounts for decreased proliferation.

To determine whether *L. monocytogenes*–specific CD8 T cells activated by HKLM immunization express cytolytic activity, we transferred $p60_{217-225}$–specific T cells and infected recipients with live *L. monocytogenes* or immunized with HKLM. Seven days later, CD8 T cells were assayed for cytolytic activity. CD8 T cells from infected recipients were cytolytic, whereas those from HKLM-immunized recipients displayed no lytic activity (Fig. 3D). Because the ratios of antigen-specific T cells to target cells were similar, these results demonstrate that CD8 T cells induced to proliferate by HKLM do not acquire cytolytic activity. Similarly, the proportion of $p60_{217-225}$–specific T cells producing interferon-γ (IFN-γ) after HKLM immunization is reduced relative to live infection (Fig. 3E). These experiments show that HKLM immunization supports CD8 T cell expansion, but not differentiation into effector CD8 T cells.

Consistent with their disparate effector functions, $p60_{217-225}$–specific CD8 T cells after live immunization became $CD62L^{lo}$, whereas HKLM-primed T cells remained $CD62L^{hi}$ (Fig. 4, B and C). To determine the possible impact of CD4 T cell help and/or inflammation on CD8 T cell priming, we coimmunized mice with HKLM and live bacteria. Concurrent immunization of mice with HKLM and live wild-type bacteria resulted in two distinct populations, one $CD62L^{hi}$ and the other $CD62L^{lo}$, suggesting that some T cells are primed by live infection while others follow the HKLM route (Fig. 4D). Concurrent immunization with wild-type HKLM and live bacteria lacking the $p60_{217-225}$ epitope primed T cells that underwent fewer divisions and remained $CD62L^{hi}$ (Fig. 4E), demonstrating that CD4 T cell responses and in-

flammation do not promote differentiation of HKLM-primed CD8 T cells. These results suggest that different antigen-presenting cells, perhaps in different sites, prime naïve CD8 T cells after HKLM and live infection.

Innate inflammatory responses to lipopolysaccharide enhance proliferation and survival of antigen-stimulated CD4 T cells (*23*), an effect that may result from the induction of antiapoptotic cellular factors (*24*). Although inflammatory responses to live infection and HKLM immunization differ, HKLM consists of a remarkable constellation of adjuvants: lipoteichoic acid, peptidoglycan, flagellin, lipoproteins, and bacterial DNA. Nevertheless, stimulation of CD40 during HKLM immunization augments the magnitude of *L. monocytogenes*–specific immune responses (*25*). It is possible that HKLM-derived molecules do not access relevant innate immune receptors, perhaps because killed and live bacteria are cleared by different cells. For example, HKLM uptake may be restricted to macrophages, whereas live infection may provide greater access of antigen to dendritic cells.

Recent studies have demonstrated that memory T cells reside predominantly in peripheral tissues (*26, 27*) and that central and effector memory cells differ with respect to trafficking (*6*). Previous studies have also shown that adoptively transferred CD8 T cells only limit bacterial growth if administered during the first day of infection (*28*). It is possible, therefore, that HKLM-primed memory CD8 T cells traffic and/or acquire effector functions too sluggishly to mediate protective immunity. Our findings suggest that differentiation of CD8 T cells into effector cells during primary immune responses has important consequences for the development of protective immunity. We believe this has important implications for pathogen- and cancer-specific vaccine development.

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19. VDJ regions of the T cell receptor (TCR) β and α chain genes were amplified with the polymerase chain reaction (PCR) from genomic DNA of cytolytic T lymphocyte (CTL) clone L9.6, which is specific for the *L. monocytogenes*–derived epitope $p60_{217\text{-}225}$ (*29*). The β chain VDJ region was amplified with Pfu polymerase (Stratagene) using the sense Vβ14 primer 5′-CGCTCGAGTGAACTATGCTGTACTCTCTCCTTGCC-3′ and the antisense Jβ2.4 primer 5′-TCTTATCGATACAGCCCCAGAACCCAAC-3′. The resulting fragment was digested with Xho I and Cla I and cloned into the TCR β cassette (*30*). The TCR α chain VJ region was amplified using the sense Vα4.2 primer 5′-CGCCCGGGCAGAAAATGAACCTTTGTCCTGAACTGGGG-3′ and the antisense Jα4 primer 5′-GCGGCGGCCGCGCTACCACCAAAGCCTGTGG-3′, which include Xma I and Not I restriction sites, respectively. This fragment was digested with Xma I and Not I and cloned into the TCR α cassette (*30*). Plasmids were screened by restriction mapping for presence of the complete TCR β or α gene. The TCR β gene insert was separated from the prokaryotic fragment of the vector by digestion with Kpn I and the TCR α gene insert by digestion with Sal I. These DNA fragments were purified and comicroinjected into fertilized oocytes of C57BL6 × SJL F_1 mice at the Yale transgenic mouse facility. Founders were identified by PCR screening of DNA purified from mouse tails, and were bred for 10 generations onto the BALB/cJ background.
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