Secondary memory CD8+ T cells are more protective but slower to acquire a central–memory phenotype

Ali Jabbari[1] and John T. Harty[1,2]

[1]Interdisciplinary Graduate Program in Immunology and [2]Department of Microbiology, University of Iowa, Iowa City, IA 52242

The formation of memory CD8 T cells is an important goal of vaccination. However, although widespread use of booster immunizations in humans generates secondary and tertiary CD8 T cell memory, experimental data are limited to primary CD8 T cell memory. Here, we show that, compared with primary memory CD8 T cells, secondary memory CD8 T cells exhibit substantially delayed conversion to a central–memory phenotype, as determined by CD62L expression and interleukin (IL)-2 production. This delayed conversion to a central–memory phenotype correlates with reduced basal proliferation and responsiveness to IL-15, although in vitro coculture with a high concentration of IL-15 is capable of inducing proliferation and CD62L upregulation. Functionally, secondary memory CD8 T cells are more protective in vivo on a per cell basis, and this may be explained by sustained lytic ability. Additionally, secondary memory CD8 T cells are more permissive than primary memory CD8 T cells for new T cell priming in lymph nodes, possibly suggesting a mechanism of replacement for memory T cells. Thus, primary and secondary memory CD8 T cells are functionally distinct, and the number of encounters with antigen influences memory CD8 T cell function.

CORRESPONDENCE
John T. Harty:
john-harty@uiowa.edu

Abbreviations used: LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; T_{CM}, central–memory T cell; T_{EM}, effector–memory T cell; tg, trangenic.

By virtue of their abilities to persist, undergo substantial secondary expansion in response to reinfection, and rapidly elaborate antimicrobial effector mechanisms, memory T cells provide enhanced resistance to infection with intracellular pathogens (1). Thus, generation of memory T cell responses is an important goal of vaccination, and much attention has been devoted to understanding the properties of memory CD8 T cells in experimental models. However, despite intense investigation, no single property or phenotypic marker has been revealed that will unequivocally identify a memory CD8 T cell. Furthermore, although most human vaccines involve multiple booster immunizations to generate secondary or tertiary memory populations (2), the vast majority of experimental studies to date have been performed on primary memory CD8 T cell populations. Thus, it is unknown if primary and secondary memory CD8 T cell populations are endowed with similar characteristics and whether these two populations are able to provide the same degree of protective immunity.

In response to infection of a naive host, pathogen-specific CD8 T cells expand in number, differentiate into effector cells that migrate throughout the body, and contribute to pathogen clearance (3–5). This expansion phase, which generally lasts for 7–10 d after acute infection, is followed by a programmed contraction phase in which ~90–95% of effector T cells are eliminated by apoptosis (6). The remaining antigen-specific CD8 T cells form the initial primary memory pool, which can remain stable in number for the life of the host (7–10). Interestingly, recent studies reveal substantial changes in the phenotype and function of memory CD8 T cell populations with time after infection (11). Specifically, early memory is composed predominantly of antigen-specific CD8 T cells that display an effector–memory phenotype. These cells are readily able to circulate through the blood and enter peripheral tissues and spleen but are excluded from lymph nodes because they lack important adhesion (CD62L) or chemokine receptor (CCR7) molecules (12, 13). In contrast, the majority of late memory CD8 T cells display a so-called central–memory phenotype and have regained surface expression of the molecules required to enter lymph nodes as well as the ability to produce

IL-2, in addition to IFN-γ, after antigen stimulation (14, 15). Although an early study was consistent with effector–memory T cells (T_{EM}) and central–memory T cells (T_{CM}) having different proliferative and protective functions (14), this has been recently questioned (16). In addition, recent studies from our laboratory suggest that the rate at which CD8 T cells acquire memory characteristics is not fixed, but rather controlled by inflammatory signals received by T cells during the initial stages of priming (17, 18). Thus, primary memory CD8 T cells generated by different infections or vaccination regimens may progress at different rates in their acquisition of specific memory characteristics.

In contrast, much less is known about the phenotype and function of secondary memory CD8 T cells. In general, boosting an immune host with higher doses of infection or heterologous antigen delivery vectors induces secondary expansion in the numbers of primary memory CD8 T cells, resulting in increased numbers of secondary memory CD8 T cells (2, 19). Whether these secondary memory CD8 T cells remain stable in number or progress from T_{EM} to T_{CM} has not been determined. Importantly, analysis of secondary memory T cells generated by reinfection or secondary immunization of a previously immune animal is complicated by the likelihood that naive precursors, either remaining unrecruited cells or newly generated precursors, will become activated and undergo a normal primary response to generate new primary memory T cells (20). Thus, boosting previously immunized mice may, in fact, engender mixtures of primary and secondary responses. To overcome this limitation, we (6, 20) and others (21, 22) have used adoptive transfer of relatively small numbers of allelically marked primary memory CD8 T cells into naive hosts followed by infection to generate primary and secondary CD8 T cell responses in the same animal that can readily be distinguished. Using this approach, we previously documented that the contraction phase of secondary CD8 T cell responses is delayed compared with the primary response after both viral and bacterial infection (6, 20). Similar results were obtained using viral infection and a TCR-transgenic (tg) T cell adoptive transfer model (21). In the current study, we used similar approaches to compare the phenotype and function of primary and secondary memory populations after infection. Compared with primary memory populations, secondary memory CD8 T cell populations exhibit a substantial delay in reacquisition of CD62L expression and the ability to produce IL-2, both important characteristics of T_{CM}. Despite this, secondary memory CD8 T cell populations were able to expand as well as primary memory CD8 T cell populations in response to antigen and, remarkably, were able to provide enhanced protective immunity against pathogen challenge. Furthermore, the inability of secondary memory CD8 T cells to traffic to the lymph nodes correlated with their ability to permit a new naive T cell response to be initiated. Thus, secondary memory CD8 T cells have properties that are functionally distinct from primary memory CD8 T cells, and the number of encounters with antigen influences memory CD8 T cell function.

RESULTS

Delayed CD62L expression by secondary memory cells

The primary CD8 T cell response to infection consists of expansion and differentiation to effector cells, followed by rapid contraction to stable numbers of memory cells. Importantly, recent evidence demonstrates that early primary memory CD8 T cells are $CD62L^{lo}$ (consistent with an effector–memory phenotype), whereas at later time points, the population of primary memory CD8 T cells are primarily $CD62L^{hi}$ (consistent with a central–memory phenotype) (11). This pattern of differentiation may be important in both immune function and vaccination, because, in at least some circumstances, T_{CM} provide superior protection to T_{EM} (14). To determine if secondary CD8 T cell responses exhibit the same pattern of differentiation, we initially infected BALB/c mice (Thy1.2) with lymphocytic choriomeningitis virus (LCMV) to elicit primary memory CD8 T cells specific for the dominant nucleoprotein (NP)118 epitope (23). Approximately 2×10^4 primary NP118-specific memory CD8 T cells, obtained 129 d after infection, were then transferred into naive Thy1.1 BALB/c mice. This experimental design allowed us to track both primary and secondary CD8 T cell responses in the same host, thus minimizing a potential source of environmental variability. The recipient mice were then infected with LCMV, and NP118-specific primary ($Thy1.2^{neg}$) and secondary ($Thy1.2^{pos}$) CD8 T cell responses were identified by NP118/L^d tetramer staining (Fig. 1 A). As previously noted (20), under these conditions both primary and secondary CD8 T cell responses exhibit vigorous expansion after LCMV infection (Fig. 1 B). As expected, the majority of primary NP118-specific effector CD8 T cells were $CD62L^{lo}$ at day 8 after LCMV infection, whereas the majority of primary memory CD8 T cells at day 72 were $CD62L^{hi}$ (Fig. 1 C), indicating a normal progression to T_{CM}. Similarly, NP118-specific secondary effector CD8 T cells in the same mice were also $CD62L^{lo}$ at day 8 after infection. In sharp contrast to primary memory CD8 T cells, however, secondary memory CD8 T cells remained primarily $CD62L^{lo}$ at day 72 (Fig. 1 C). Delayed acquisition of CD62L by secondary memory CD8 T cells was also observed with the same adoptive transfer protocol followed by challenge infection with a recombinant *Listeria monocytogenes* strain (LM-NPs [24]) expressing the NP118 epitope as a secreted fusion protein (Fig. 1 D), indicating that delayed acquisition of CD62L by secondary memory CD8 T cells also occurs after bacterial infection. Finally, we observed similar delayed acquisition of CD62L by secondary memory CD8 T cells specific for the ova257 epitope in previously immunized B6 mice, compared with naive B6 mice, after challenge with *L. monocytogenes* expressing the ova257 epitope (LM-OVA [25, 26]) (Fig. 1 E), indicating that this observation is not limited to BALB/c mice, the NP118-specific CD8 T cell response, or adoptive transfer models. Thus, compared with primary memory CD8 T cells, secondary memory CD8 T cells do not rapidly reacquire expression of surface CD62L, one of the major characteristics of T_{CM}.

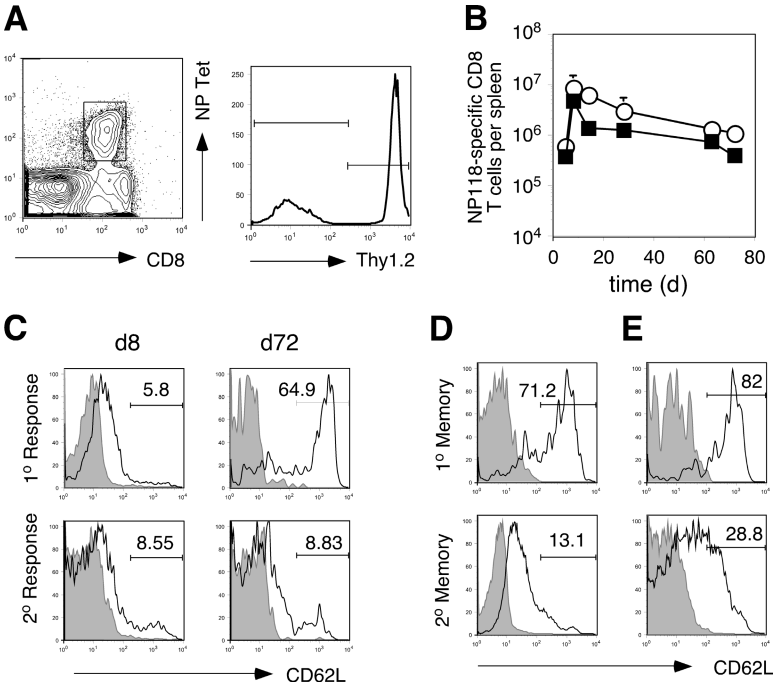

Figure 1. Reduced CD62L expression of secondary compared with primary memory CD8 T cells in the same host. Approximately 2×10^4 NP118-specific CD8 T cells from a LCMV-immune BALB/c, Thy1.2 mouse (day 129 after infection) were adoptively transferred into naive, Thy1.1 BALB/c mice. 1 d later, recipient mice were infected i.p. with LCMV-Armstrong. At various time points after infection, the number of NP118-specific CD8 T cells undergoing either a primary or secondary response was assessed in the spleen by MHC tetramers (left) and differential Thy1 expression (right). (A) Analysis of a representative mouse at day 8 after infection. Right panel gated on NP118/Ld tetramer$^+$ cells. (B) Total number of NP118-specific CD8 T cells in the spleen undergoing either a primary (■) or secondary (○) response over time. Data are mean ± SD for three mice/time point. (C) CD62L expression of NP118-specific cells undergoing either primary (top) or secondary (bottom) responses at day 8 (left) or day 72 (right) after infection. Open histograms, CD62L; shaded histograms, isotype control. (D) Adoptive transfer recipients were infected with 10^7 *actA*$^-$ LM-NPs, and CD62L expression on primary (top) and secondary (bottom) NP118-specific memory CD8$^+$ T cells was determined 39 d later. Shown are representative histograms of NP118/Ld tetramer gated cells. (E) Naive or LM-OVA–immune B6 mice were infected with 1.2×10^7 *actA*$^-$ LM-OVA, and CD62L expression on primary (top) and secondary (bottom) ova257-specific memory CD8$^+$ T cells was determined 39 d later. Shown are representative histograms of ova257-specific gated cells. 1°, primary; 2°, secondary.

Secondary memory cells eventually acquire T_{CM} characteristics

To eliminate the potential that precursor frequency (27) or changes in TCR utilization could influence the results, and to develop a model where we could purify memory T cells without ligating the TCR, we next analyzed CD62L expression in primary and secondary memory Thy1.1 OT-I TCR-tg CD8 T cells (specific for ova257 [28]). CD8-enriched naive OT-I T cells (2×10^4) were transferred into naive B6 (Thy1.2) mice, and primary OT-I T cell responses were generated by infection with LM-OVA. 5×10^4 Thy1.1-purified primary memory OT-I T cells (obtained >100 d after stimulation) or similarly purified naive OT-I T cells were then transferred into separate groups of naive B6 mice followed by infection with LM-OVA 1 d later to stimulate secondary and primary OT-I T cell responses, respectively. Both populations of OT-I T cells underwent substantial expansion after infection and generated memory populations (Fig. 2 A). As seen with nontransgenic T cells, both populations of OT-I T cells initially down-regulated CD62L at day 7, but by day 63 only the OT-I T cells in the primary, but not secondary, memory group had substantial CD62L expression (Fig. 2 B). As observed with endogenous responses, primary OT-I T cell responses exhibited progressive and relatively rapid reacquisition of CD62L with time after infection, reaching >75% positive by day 63 (Fig. 2 C). CD62L expression by OT-I T cell populations undergoing a secondary response also appeared to increase with time but at a much slower rate than observed in the primary response (Fig. 2 C), with <20% of secondary memory OT-I T cells expressing CD62L at day 63. The disparity in CD62L expression between primary and secondary memory was also observed in blood, bone marrow, and lungs, and the trafficking of secondary memory CD8 T cells to lymph nodes was substantially impaired (unpublished data). Secondary memory CD8 T cells also exhibited a substantial delay in acquiring the ability to produce IL-2 after in vitro antigen stimulation, another potentially important characteristic of T_{CM} (11) (Fig. 2 D). Additional studies revealed that the CD62L expression and capacity to produce IL-2 by tertiary memory OT-I CD8 T cells were similarly, if not more, delayed relative to secondary memory T cells (unpublished data).

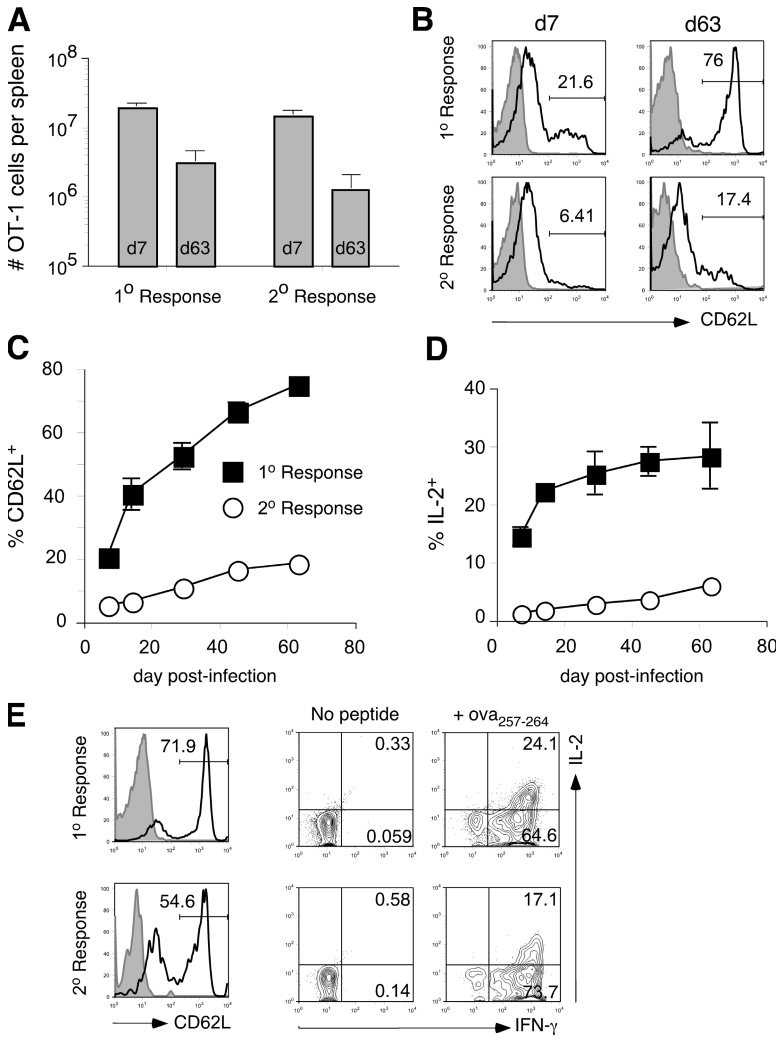

Figure 2. OT-I CD8 T cells undergoing a secondary response do not adopt central–memory-like characteristics until very late time points after infection. 10^4-purified naive or primary memory (day 126 after infection) OT-I/Thy1.1 cells were adoptively transferred into separate groups of naive, Thy1.2 B6 mice, and recipient mice were subsequently infected with 1.4×10^7 *actA*$^-$ LM-OVA to initiate primary and secondary responses, respectively. (A) Enumeration of OT-I cells (CD8$^+$/Thy1.1$^+$) in the spleen at days 7 and 63 after infection. Data are mean ± SD of three mice/group. (B) CD62L expression of OT-I cells in the spleen undergoing either primary (top) or secondary responses (bottom) at days 7 (left) or 63 (right) after infection. Representative histograms are gated on CD8$^+$/Thy1.1$^+$ splenocytes. Open histograms, CD62L; shaded histograms, isotype control. (C) CD62L expression by OT-I cells undergoing either primary (■) or secondary (○) responses at various time points after infection. Data are mean ± SD of three mice/time point. (D) IL-2 production by primary and secondary memory OT-I cells detected by ova257-stimulated intracellular cytokine staining at various days after infection. Data are mean ± SD of three mice/time point. (E) Representative CD62L expression (left) and IL-2 production (middle and right) at 227 d after infection on primary (top) and secondary (bottom) memory OT-I T cells. 1°, primary; 2°, secondary.

However, the data suggest that CD62L expression and the capacity to produce IL-2 were slowly increasing in secondary memory CD8 T cells with time. Consistent with this notion, ~50% of secondary memory OT-I T cells at day 227 after infection expressed CD62L and 17% produced IL-2 in response to antigen (Fig. 2 E). Although this represents ~60% of the level of expression of these molecules at day 227 in the primary memory CD8 T cell population, the population of secondary memory OT-I T cells at day 227 still had not attained the CD62L expression or IL-2 production capacity displayed by day 30 after infection in the primary OT-I T cell response. Together, these results demonstrate that secondary memory CD8 T cells acquire T_{CM} characteristics with a substantial delay compared with primary memory T cells.

It is possible that the precursors of the secondary memory CD8 T cells were derived solely from CD62L^{lo} primary memory CD8 T cells (27). To address this possibility, we transferred purified CD62L^{hi} cells from mice containing primary memory or naive OT-I CD8 T cells (Fig. 3 A) into

naive B6 mice. 1 d later, these mice were infected with LM-OVA, and we analyzed OT-I cells at days 7 and 44 after infection (Fig. 3 B). Similar to the previous results we obtained with mixed T_{EM}/T_{CM} primary memory, we found that $CD62L^{hi}$ primary memory CD8 T cells gave rise to secondary memory CD8 T cells that exhibited a delayed conversion to a $CD62L^{hi}$, T_{CM}-like phenotype (Fig. 3 C). Therefore, the delayed acquisition of CD62L by secondary memory CD8 T cells is not a consequence of selective activation of T_{EM} primary memory CD8 T cells.

Forced antigen-independent proliferation of secondary memory CD8 T cells effects adoption of a central–memory phenotype

One of the hallmarks of primary memory CD8 T cells, thought to be important in maintaining stable memory levels, is their ability to undergo proliferative renewal (basal proliferation) in the absence of cognate antigen, or even MHC class I (29, 30). It has been suggested that primary T_{EM} ($CD62L^{lo}$) are able to convert directly to T_{CM} ($CD62L^{hi}$) without proliferation (14); however, this finding has recently been questioned (27). Thus, basal proliferation may facilitate, or even be required for, conversion of memory CD8 T cell populations from T_{EM} to primarily T_{CM}. To address this, we asked if secondary memory CD8 T cells undergo similar basal proliferation as primary memory CD8 T cells. BrdU treatment to identify dividing cells was initiated at day 69 after the last LM-OVA infection, in mice containing either primary or secondary memory OT-1 T cells. A substantial fraction of primary memory OT-I T cells, both $CD62L^{hi}$ and $CD62L^{lo}$, incorporated BrdU during the 8-d pulsing period (Fig. 4 A). In striking contrast, few secondary memory OT-I T cells, whether $CD62L^{hi}$ or $CD62L^{lo}$, incorporated BrdU (Fig. 4 A), indicating that secondary memory CD8 T cells have a substantially reduced rate of basal proliferation compared with primary memory CD8 T cells. These data additionally indicate that secondary memory CD8 T cells are not identical to primary T_{EM} ($CD62L^{lo}$), because primary T_{EM} underwent basal proliferation.

Proliferative renewal of primary memory CD8 T cells requires IL-15 (31–33), but not expression of the high-affinity IL-15 receptor on the T cells themselves, where expression of CD122 and the common γ chain of the IL-2 receptor are sufficient (34). To determine if modulation of the IL-15 response could account for the reduced basal proliferation of secondary memory cells, we compared CD122 expression and in vitro proliferation in response to IL-15 of purified primary and secondary memory OT-I cells. Secondary memory CD8 T cells have reduced levels of CD122 on their surface compared with primary memory CD8 T cells analyzed at the same time point after infection (Fig. 4 B) and also display a reduced ability to proliferate in vitro in response to IL-15 (Fig. 4 C); the cell division in vitro supports the potential functional relevance of the difference in the levels of CD122 expression. Our preliminary evidence suggests that IL-15Rα expression is similar on both primary and secondary memory OT-I cells (unpublished data), suggesting that these results are not a consequence of differential capacities to transpresent IL-15 (35) in vitro. These data suggest that the reduced basal proliferation in secondary memory CD8 T cells could potentially result from decreased responsiveness of these cells to IL-15.

As mentioned previously, reduced basal proliferation could account for the delayed acquisition of T_{CM} characteristics in secondary memory CD8 T cells. As high dose IL-15 induced proliferation of secondary memory CD8 T cells (Fig. 4 C), we asked whether secondary memory CD8 T cells that had undergone IL-15–mediated proliferation exhibited an increased conversion to a central–memory phenotype. Purified secondary memory OT-I T cells cocultured in the presence of a high concentration of IL-15 divided, and successive generations of daughter cells exhibited increased proportions of cells expressing high levels of CD62L (Fig. 4 E); in the absence of IL-15, the fraction of $CD62L^{hi}$ secondary memory cells did not change from that observed directly ex vivo, before coculture (Fig. 4 D; unpublished data). Additionally, secondary memory CD8 T cells undergo

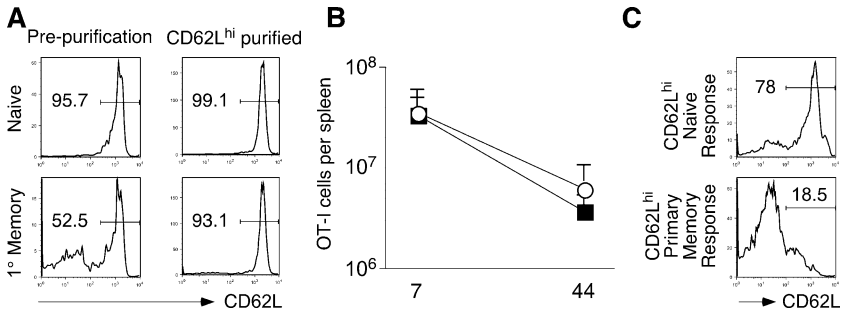

Figure 3. T_{CM} primary memory CD8 T cells exhibit delayed CD62L reacquisition after infection. Primary memory OT-I CD8 T cells were generated in vivo as described in Fig. 2. (A) $CD62L^{hi}$ cells were purified from splenocytes of mice containing primary memory OT-I cells as well as from naive OT-I mice. (B) Approximately 5×10^4 $CD62L^{hi}$-purified naive or primary memory CD8 T cells were then adoptively transferred into naive B6 mice, and these mice were infected 1 d later with *actA*$^-$ LM-OVA. At days 7 and 44 after infection, the numbers of OT-I cells were assessed. (C) At day 44 after infection, the CD62L expression of cells that had undergone a primary (top) or secondary (bottom) response was assessed. 1°, primary.

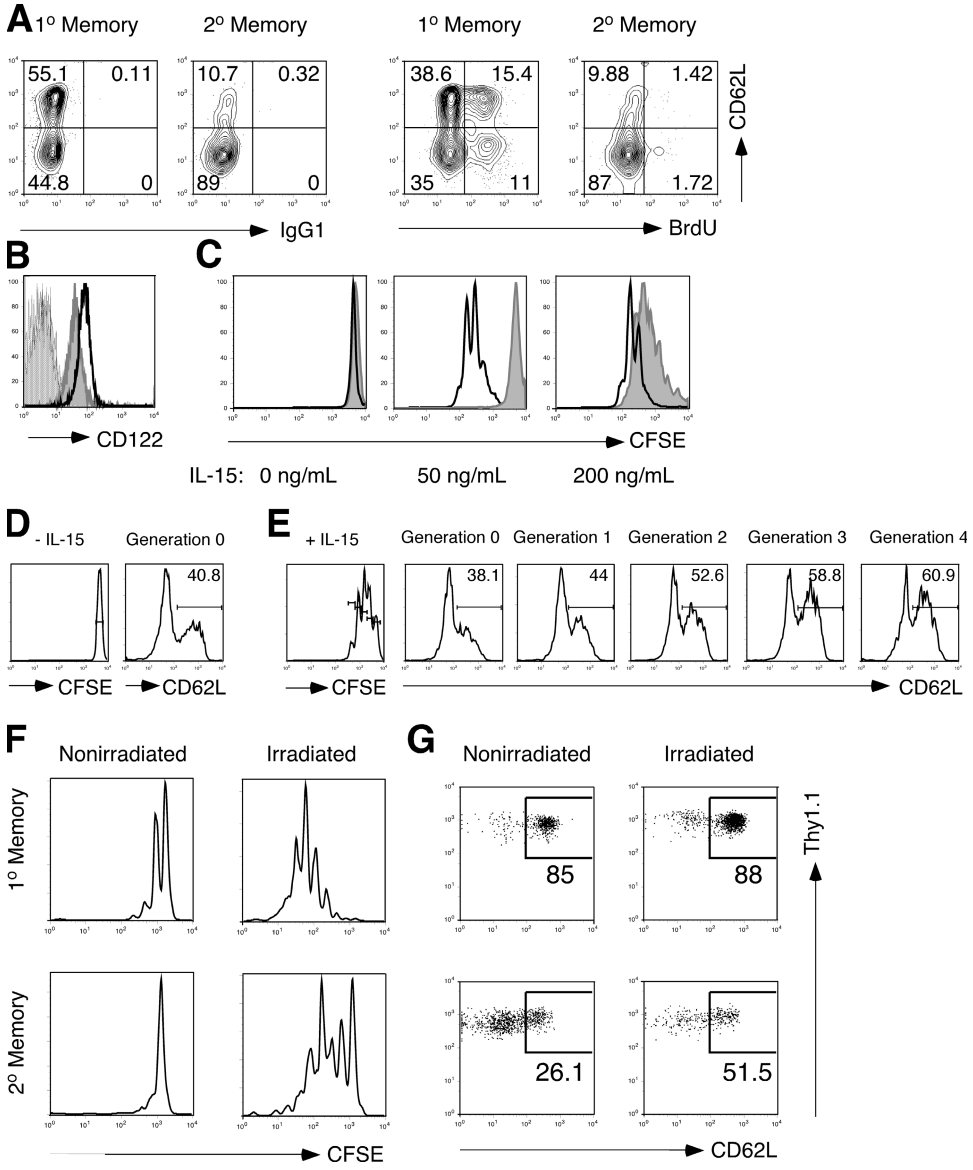

Figure 4. Reduced IL-15R expression and reduced responsiveness to IL-15 correlate with decreased basal proliferation and delayed central–memory phenotype. Primary and secondary memory OT-I CD8 T cells were generated in vivo as described in Fig. 2. (A) Starting at day 69 after infection, mice received BrdU for the following 8 d. Representative profiles of primary and secondary memory OT-I cells for expression of surface CD62L and intracellular isotype control (IgG1, left two panels) or BrdU (right two panels). Contour plots are gated on $CD8^{+}Thy1.1^{+}$ cells. (B) Representative expression at 63 d after infection on primary memory OT-I cells of CD122 (open histogram), isotype control (vertical line-fill), or secondary memory OT-I cells (CD122, shaded histogram), or isotype control (diagonal line-fill). (C) Purified primary or secondary memory OT-I cells at 63 d after infection were labeled with CFSE and cocultured in the presence of 0, 50, or 200 ng/ml IL-15. After 3 d, dilution of CFSE as a measure of proliferation was assessed by flow cytometry. Open histograms, primary memory OT-I cells; shaded histograms, secondary memory OT-I cells. (D and E) Purified secondary memory OT-I cells at 75 d after infection were labeled with CFSE and cocultured for 3 d in the absence (D) or in the presence (E) of 200 ng/ml IL-15. Cells were subsequently stained for CD62L expression. Left-most panels, CFSE profile of cultured primary or secondary memory OT-I cells; right panels, CD62L profiles of CFSE peaks as gated in top panels. (F and G) CFSE-labeled Thy1.1-purified primary or secondary memory OT-I cells were adoptively transferred into nonirradiated B6 mice or B6 mice sublethally irradiated 24 h prior. 14 d later, spleens of mice were harvested and assessed for CFSE dilution (F) or CD62L expression (G). 1°, primary; 2°, secondary.

homeostatic proliferation in a sublethally irradiated host (Fig. 4 F), albeit at a reduced rate compared with primary memory CD8 T cells. These proliferating secondary memory CD8 T cells exhibited increased CD62L expression relative to cells transferred into a nonirradiated host, which did not divide (Fig. 4 G). These data highlight one potential mechanism to account for the slow conversion of secondary memory CD8 T cells to a central–memory phenotype: reduced

basal proliferation, most likely as a result of reduced responsiveness to IL-15.

Secondary memory T cells potently expand and provide enhanced protection against *L. monocytogenes*

In some (14) but not all cases (36), primary T_{CM} ($CD62L^{hi}$) are superior in mediating protective immunity to primary T_{EM} ($CD62L^{lo}$). Our studies suggested that secondary memory CD8 T cells exhibit reduced proliferative capacity in several different circumstances relative to primary memory CD8 T cells. To address whether secondary memory CD8 T cells have a reduced capacity to proliferate in response to infection, we transferred 5×10^5–purified primary or secondary memory Thy1.1 OT-I T cells (both at d 66 after LM-OVA infection) into naive B6 mice and determined the number of OT-I T cells in the spleen at days 0, 3, and 5 after LM-OVA challenge (Fig. 5 A). No substantial differences in seeding of the spleen were observed in mice that received primary or secondary memory cells, and this approach generated a number of memory cells ($\sim 5 \times 10^4$/spleen) in recipient mice that is less than that achieved in the endogenous response to LM-OVA (37). Importantly, we also observed no differences in the antigen-driven proliferation of OT-I T cells between the groups of mice; in each case, expansion in the number of OT-I T cells was >1,000-fold by day 5 after challenge infection. These data indicate that, despite the reduced basal proliferation, secondary memory CD8 T cells are able to undergo a vigorous proliferative response to infection.

Given their ability to potently expand in number in response to infection, we next compared the protective capacity of primary and secondary memory CD8 T cells against bacterial infection. Naive mice or mice that had received 5×10^6 primary or secondary memory OT-I T cells were challenged with a high dose of virulent LM-OVA, and the number of bacteria were determined at day 3 after challenge (Fig. 5 B). Mice containing primary memory OT-I T cells reduced the bacterial load ~20-fold in their spleens compared with mice without memory T cells, indicating protection by the primary memory CD8 T cells. Strikingly, mice that received the same number of secondary memory OT-I T cells had 500-fold fewer bacteria than the mice without memory T cells. These data suggest that, despite, or perhaps because of, their slow progression to a central–memory phenotype, secondary memory CD8 T cells are more potent in providing protective immunity than primary memory CD8 T cells. This enhanced protection is most likely not mechanistically explained by an enhanced capacity to expand in response to infection. Additionally, the functional avidity of secondary memory OT-I cells, as measured by IFN-γ production in response to titrated amounts of peptide (38, 39), was no different than that of primary memory CD8 T cells (unpublished data), indicating that differences in sensitivity to antigen also was not sufficient to explain the observed differential protective capacities.

Enhanced lytic ability by secondary memory cells

A major effector mechanism of memory CD8 T cells in resistance to infection is cytolysis, whereby vectorial degranulation of preformed vesicles containing perforin and granzymes results in lysis of infected cells (5). Studies from our own lab demonstrate that perforin-dependent cytolysis is the major effector mechanism of CD8 T cells in resistance to *L. monocytogenes* infection (39). To address the mechanism for enhanced protection by secondary memory CD8 T cells, we performed in vivo cytolytic assays. Our results indicate that secondary memory CD8 T cells have increased lytic capacity early after target cell transfer when compared with an equal number of primary memory CD8 T cells (Fig. 6, A and B). These results are consistent with the hypothesis that increased cytolytic potential accounts for the increased protective capacity conferred by secondary memory CD8 T cells.

The in vivo lytic capacities were measured over the course of 2 h, consistent with the rapid perforin-granzyme–mediated killing mechanism (40). We have already shown that this killing pathway is the predominant mechanism used to eradicate virulent *L. monocytogenes* (39). To address the mechanism(s) underlying the increased lytic capacity of secondary memory CD8 T cells, we examined the kinetics of degranulation (surface exposure of CD107a after in vitro peptide stimulation [41]) of primary and secondary memory CD8 T cells. We observed no difference in the kinetic or

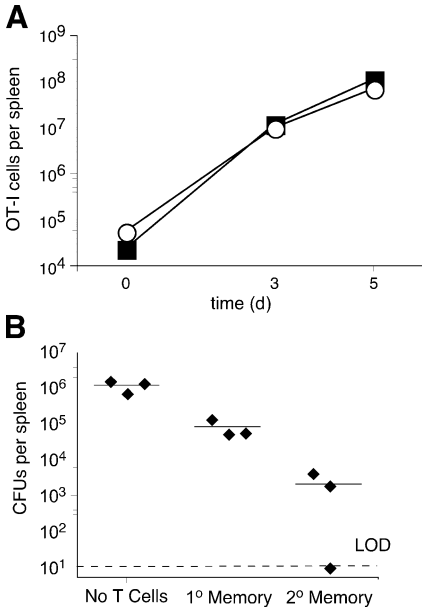

Figure 5. Robust expansion and enhanced protection in response to infection by secondary memory CD8 T cells. (A) 5×10^5 purified primary or secondary memory CD8 T cells (day 66 after infection) were adoptively transferred into separate groups of naive, Thy1.2 B6 mice. 1 d later, mice were infected with 1.5×10^7 *actA*$^-$ LM-OVA. Shown are total numbers of OT-I cells/spleen at days 0, 3, and 5 after infection of recipients of primary (■) or secondary (○) memory OT-I cells. Data represent mean ± SD from three mice/group/time point. (B) Approximately 5×10^5 primary or secondary memory OT-I cells were adoptively transferred to naive, B6 recipients. 1 d later, mice were infected with 2.7×10^5 virulent LM-OVA. The number of bacteria in the spleen was determined 3 d after challenge infection. LOD, limit of detection. 1°, primary; 2°, secondary.

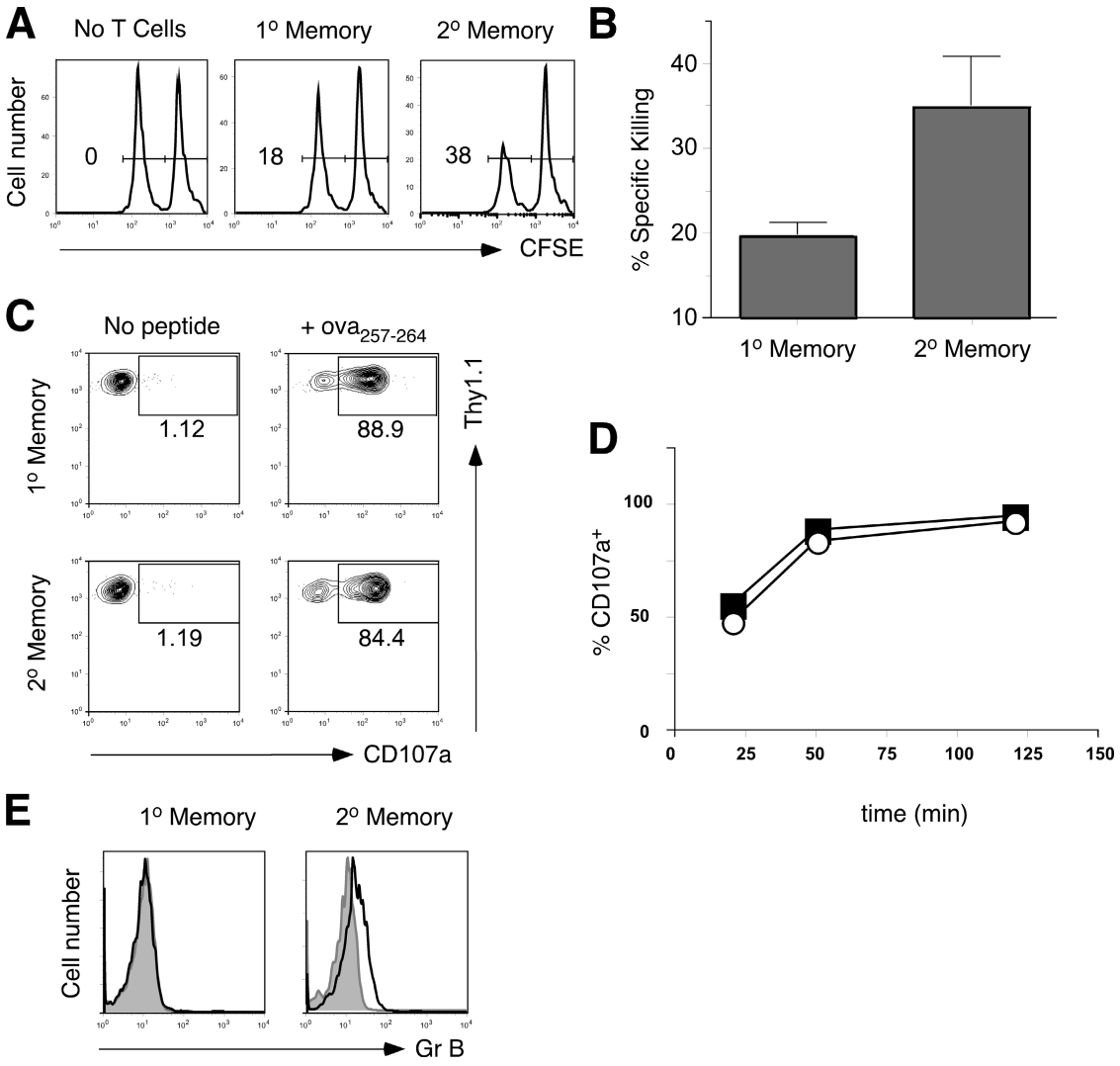

Figure 6. Secondary memory CD8 T cells exhibit enhanced in vivo killing capacity, equivalent degranulation kinetics, and increased expression of granzyme B compared with primary memory cells. (A) Approximately 1.5 × 10^6 primary (middle) or secondary memory (right) OT-I cells were adoptively transferred into naive B6 recipients. 1 d later, recipient mice and a group of naive B6 mice (left) were injected with target cells consisting of 2 × 10^6 $CFSE^{hi}$-labeled B6 splenocytes (no peptide) and 2 × 10^6 $CFSE^{lo}$-labeled B6 splenocytes (coated with ova257 peptide). Representative histograms of CFSE-labeled cells recovered from each group at 2 h after target cell injection. Number in histograms is percentage of specific killing. (B) Cumulative data from three mice per group for the in vivo cytolysis assay. (C) Primary and secondary memory OT-I cells were assessed for degranulation (cell surface presence of CD107a) in the absence (left) or presence (right) of ova257 peptide at 50 min after stimulation. Contour plots are gated on $CD8^+Thy1.1^+$ cells. (D) Percentage of primary (■) or secondary (○) memory OT-I T cells expressing surface CD107a at various time points after peptide stimulation. (E) Intracellular stain of primary (left) and secondary (right) memory OT-I cells obtained at 63 d after infection for granzyme B. Histograms are gated on $CD8^+Thy1.1^+$ cells. Open histograms, granzyme B; shaded histograms, isotype control. GrB, granzyme B. 1°, primary; 2°, secondary.

magnitude of degranulation between these populations (Fig. 6, C and D), indicating that differences in the rates of degranulation are not sufficient to explain the observed increased lytic capacities. Detectable intracellular granzyme B differentiates highly lytic effector CD8 T cells, from primary memory cells, either T_{EM} or T_{CM}, that generally express much reduced granzyme B and display decreased rapid ex vivo lytic function (41). Consistent with this explanation for enhanced killing by secondary memory CD8 T cells, we detected substantial granzyme B expression in secondary but not primary memory CD8 T cells (Fig. 6 E). These data demonstrate that secondary memory CD8 T cells remain more poised for rapid cytolysis relative to primary memory CD8 T cells, most likely caused by constitutive expression of the relevant lytic molecules. Additionally, secondary memory CD8 T cells are functionally distinct from T_{EM} primary memory CD8 T cells, as $CD62L^{lo}$-enriched (Fig. 7 A) secondary memory CD8 T cells provide enhanced protection

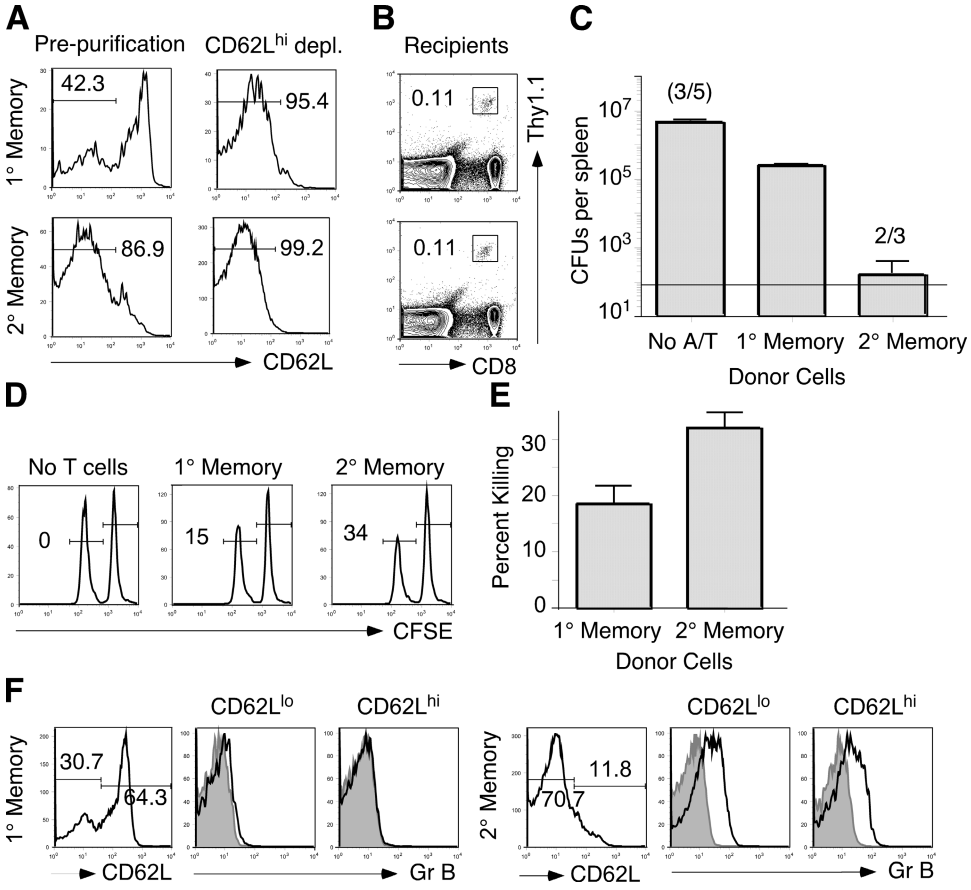

Figure 7. CD62L^{lo} secondary memory CD8 T cells offer greater protection and exhibit more efficient cytolysis than CD62L^{lo}, primary T_{EM}. Primary and secondary memory OT-I/Thy1.1 CD8 T cells were generated in vivo as described in Fig. 2. Splenocytes were harvested and depleted of CD62L^{hi}-expressing cells. (A) CD62L expression on OT-I cells pre and after purification. (B) Approximately 9.2×10^5 CD62L^{lo} primary or secondary memory OT-I/Thy1.1 CD8 T cells were adoptively transferred into naive, Thy1.2-expressing B6 mice. 1 d later, spleens of recipient mice were harvested and stained for CD8 and Thy1.1. (C) 1 d after transfer, recipient mice were infected with 1.27×10^6 virulent LM-OVA. 3 d after infection, the bacterial burden in the spleens of three to five infected mice per group was assessed as in Fig. 6. Two out of five naive mice died. One out of three recipients of secondary memory OT-I cells did not have detectable levels of LM-OVA. Line, limit of detection. (D) Recipients of CD62L^{lo} primary or secondary memory CD8 T cells were injected with target cells consisting of 1.25×10^6 CFSE^{hi}-labeled B6 splenocytes (no peptide) and 1.25×10^6 CFSE^{lo}-labeled B6 splenocytes (coated with ova257 peptide). Representative histograms of CFSE-labeled cells recovered from each group at 2 h after target cell injection. Number in histograms is percentage of specific killing. (E) Cumulative data from three mice per group for the in vivo cytolysis assay. (F and G) Unpurified primary (F) and secondary (G) memory OT-I cells were harvested from spleens and stained for CD62L and intracellular granzyme B directly ex vivo. Left panels are gated on OT-I cells, middle panels are gated on CD62L^{lo} OT-I cells, and right panels are gated on CD62L^{hi} OT-I cells. 1°, primary; 2°, secondary.

(Fig. 7, B and C), exhibit greater cytolytic capacity (Fig. 7, D and E), and express higher levels of granzyme B (Fig. 7 F) than their CD62L^{lo}-enriched, T_{EM} primary memory counterparts.

Secondary memory T cells allow priming of naive precursors

Relatively slow progression to T_{CM} and the protracted contraction phase in secondary CD8 T cell response (6, 20, 21) may have evolved to maintain higher levels of protective immunity in areas with recurring pathogen exposure. In addition, the relatively slow acquisition of CD62L, and thus the reduced ability to enter lymph nodes could facilitate the priming of new naive CD8 T cells by eliminating the competition that would occur with CD62L-positive primary memory CD8 T cells (20, 42). In this way, multiple exposures to the same pathogen would result in priming of replacement memory T cells. To examine the trafficking of these populations, we used a novel homing assay, where CFSE-labeled primary memory Thy1.1 OT-I T cells were mixed with unlabeled primary or secondary memory Thy1.1 OT-I T cells to achieve a known input population (Fig. 8 A). These mixed populations were then injected into naive B6 (Thy1.2) mice, where the ratio of labeled to unlabeled OT-I T cells recovered in various tissues serves as a normalized homing index to primary memory OT-I T cells. We found that the ratios of labeled to unlabeled primary memory OT-I T cells recovered from various tissues did not change from the input ratios (Fig. 8, B and C), demonstrating that the CFSE labeling procedure had no adverse effect on the trafficking of cells to tissues.

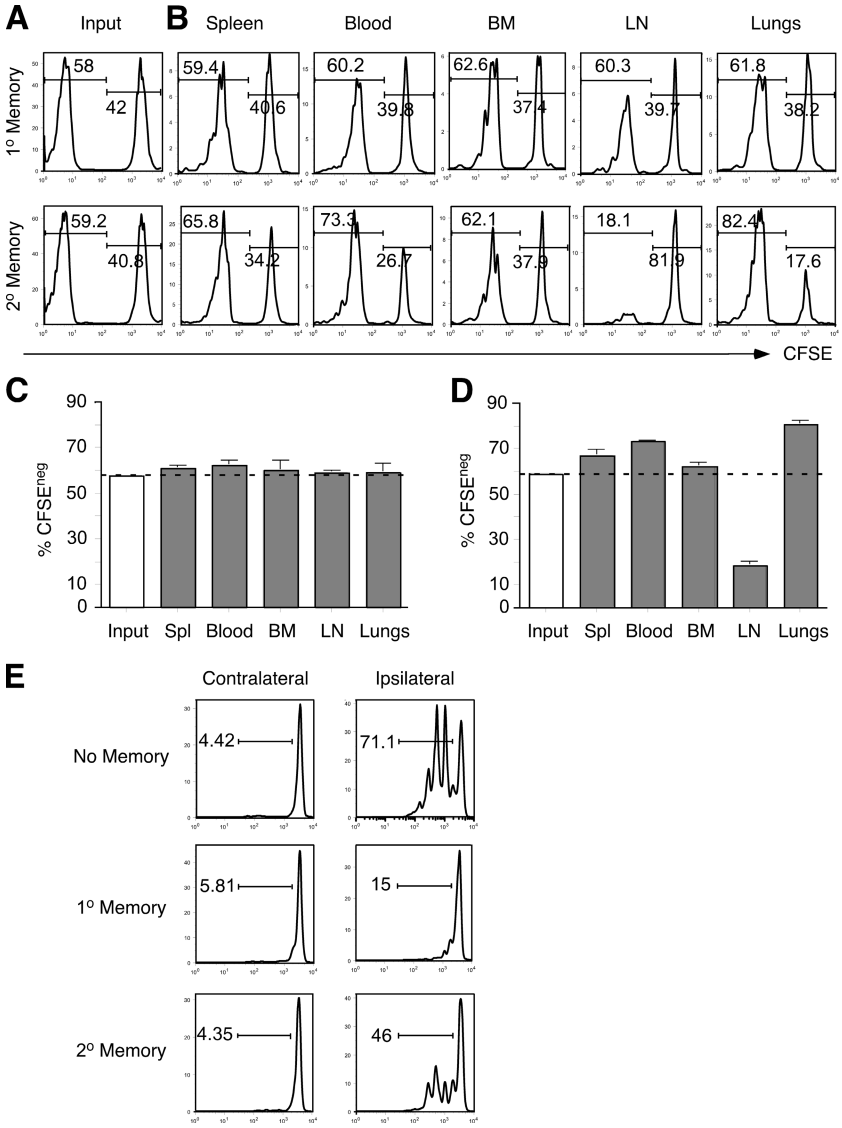

Figure 8. Decreased capacity of secondary memory CD8 T cells to traffic to lymph nodes permits enhanced priming of naive CD8 T cells. (A) Primary memory OT-I cells generated in Thy1.2 B6 mice were labeled with CFSE and mixed with unlabeled primary (top) or secondary (bottom) memory OT-I cells (Thy1.1$^+$). Histograms of the CFSE profiles are gated on CD8$^+$Thy1.1$^+$ cells. Approximately 3×10^6 memory OT-I cells in these mixed populations were adoptively transferred into naive Thy1.2 B6 mice. (B) Representative histograms obtained 3 d after transfer of blood, spleens, bone marrow (BM), lymph nodes, and lungs were assessed for presence of unlabeled primary (top) or secondary (bottom) memory OT-I cells relative to CFSE-labeled primary memory OT-I cells. Representative histograms, gated on CD8$^+$Thy1.1$^+$ cells, are shown. (C) Percentage of unlabeled primary and (D) secondary memory OT-I cells (mean ± SD of three mice/group) in various tissues compared with input. (E) Primary and secondary memory OT-I T cells were generated in Thy1.2 B6 mice. At day 69 after infection, naive mice (top) or with preexisting primary (middle) or secondary (bottom) OT-I memory received 10^6 naive, CFSE-labeled OT-I cells. 1 d later, mice were injected with $\sim 1.2 \times 10^7$ *actA*$^-$ LM-OVA s.c. in the lower right flank. Proliferation of the CFSE-labeled naive OT-I cells was assessed in inguinal lymph nodes contralateral (left) and ipsilateral (right) to the injection site at 2 d after infection. Representative histograms, gated on CD8$^+$Thy1.1$^+$CFSE$^+$OT-I cells, are shown. Numbers are the percentages of CFSE-labeled cells that had undergone cell division. 1°, primary; 2°, secondary.

In contrast, we observed modest increases in the homing of secondary memory cells to the spleen and blood with a more noticeable increase in homing to the lung (Fig. 8, B and D). However, we saw a substantial decrease in homing of secondary memory cells to the lymph nodes, consistent with the reduced expression of CD62L in the population.

The inability of secondary memory CD8 T cells to home to lymph nodes has the potential to eliminate competition for priming of naive T cell responses that has been observed with primary memory CD8 T cells (20, 42). Alternatively, enhanced protection by secondary memory CD8 T cells could result in faster clearance of infection and a reduction of new

T cell responses. To differentiate between these possibilities, we generated primary and secondary OT-I memory mice and, 69 d after infection, transferred 10^6 CFSE-labeled, purified naive OT-I T cells into each group, as well as into naive mice. 1 d later, all groups were challenged by subcutaneous LM-OVA infection at a single site on the right flank. Priming of the naive OT-I T cells, as indicated by CFSE dilution, was assessed in the draining ipsilateral as well as contralateral inguinal lymph nodes at day 2 after infection (Fig. 8 E). LM-OVA infection resulted in substantial proliferation of the CFSE-labeled naive OT-I T cells in mice that did not contain memory OT-I T cells. As previously described (20, 42), the predominantly $CD62L^{hi}$ primary memory OT-I T cell population efficiently competed for priming with the naive OT-I T cells, substantially reducing the fraction of OT-I T cells that had diluted CFSE. In contrast, despite their enhanced ability to control LM infection, secondary memory OT-I T cells only minimally inhibited the response from naive OT-I T cells. These data suggest that the relatively slow acquisition of CD62L by secondary memory CD8 T cells may occur, in part, to facilitate replacement of secondary memory CD8 T cells with newly primed memory CD8 T cells while maintaining an environment of enhanced protective immunity.

DISCUSSION

Acute infections have the potential to generate high levels of protective CD8 T cell memory that can persist for the life of the organism (1, 8). Although an extensive body of literature describing CD8 T cell memory has been amassed, the vast majority of these studies have focused on primary memory cells that arise after a single infection or vaccination. Importantly, in the case of vaccination, a single immunization may not be sufficient to generate an adequate level of immunity for protection from infection, and a temporally separated booster immunization may be required (2). The characteristics of secondary responses, specifically the extent to which secondary CD8 T memory differs from primary CD8 T memory is, therefore, critical for the optimization of vaccination protocols.

Here we have shown, in infection models that differ with respect to mouse strain, pathogen, antigen, and host environment, that secondary CD8 T cell responses result in memory populations that have unique qualities that distinguish them from primary memory CD8 T cell populations. As defined by CD62L expression and IL-2 production, primary CD8 T cell responses after acute infection pass relatively rapidly through an effector–memory phase and stabilize as a predominantly T_{CM} population. In contrast, secondary memory CD8 T cells appear to be severely delayed in this progression. Furthermore, secondary memory CD8 T cells, besides sharing a similarly low CD62L expression level, are clearly not the same as primary T_{EM}; secondary memory CD8 T cells display reduced basal proliferation, a higher level of expression of cytolytic molecules, exhibit more potent cytolytic capabilities, and are more protective than primary T_{EM}.

The IL-15–driven basal proliferation important for the long-term maintenance of primary memory CD8 T cells is substantially reduced in secondary memory CD8 T cells, which may account for the delayed acquisition of T_{CM} characteristics. This correlation between the rate of cell division and the acquisition of a central–memory phenotype for memory CD8 T cells may be explained by several possibilities. One possibility is that cell division is a necessary step before memory cells can reexpress CD62L. However, studies by others (14) do not support this hypothesis. Another possibility that may explain the correlation between cell division and CD62L acquisition is that T_{CM} may accumulate faster than T_{EM}, resulting in an increase in the representation of T_{EM} over time. Proponents of this hypothesis often cite data that indicate that T_{CM} undergo a faster rate of basal proliferation than T_{EM} (14). However, it is necessary that the death rate of this population is also known to invoke preferential accumulation; it is possible for a dividing population to decrease in total numbers if its death rate is greater than that which can be replenished by cell division. Resolution of this issue will likely require progress in understanding the death rate of memory T cell populations.

We found that secondary memory CD8 T cells are not only equally able to undergo expansion in response to antigen, but are better at immediate cytolysis and protecting hosts against challenge by a virulent pathogen compared with primary memory CD8 T cells of the same specificity. Additionally, their decreased representation in the lymph nodes and therefore increased representation in the peripheral organs may be a mechanism to maintain highly lytic memory CD8 T cells for longer periods at potential sites of pathogen entry. In this way, the host may tailor memory CD8 T cell migration to circumstances in which infection with the same pathogens are a recurring event.

Immediately after an infection, the preexisting naive CD8 T cell population becomes activated by receiving antigenic and costimulatory signals in the secondary lymphoid organs and will eventually differentiate into a memory CD8 T cell population (43, 44). Throughout these responses, new naive CD8 T cells of the same specificity will be populating the periphery, either through thymic or extrathymic development (45). Also, it may be possible that, during an initial infection, not all of the preexisting naive CD8 T cells will have been recruited to respond (46). Therefore, it is likely that most anamnestic CD8 T cell responses include both primary and secondary responses (20). Here, we show that trafficking into lymph nodes by secondary memory CD8 T cells is severely decreased compared with primary memory CD8 T cells, which more rapidly adopt a $CD62L^{hi}$ central–memory phenotype. Because primary memory CD8 T cells are present in lymph nodes, they are able to suppress a new naive CD8 T cell response from occurring by, most simply, competing for presented antigen on dendritic cells (42). Because secondary memory CD8 T cells, on the other hand, are excluded from entering the lymph nodes until very late time points after infection, they are more permissive for the initiation of a new naive CD8 T cell response. Their exclusion from the lymph nodes, therefore, increases the potential for

the generation of a higher number of replacement memory cells in future exposures to the same pathogen.

In terms of immune function, is there a compelling reason to keep secondary memory CD8 T cells out of lymph nodes for extended periods? Secondary memory CD8 T cells are, by definition, the progeny of many rounds of division caused by the expansion phase of their initial response, their maintenance by basal proliferation as primary memory CD8 T cells, and the expansion phase of their secondary response. Although telomerase is thought to be activated in responding CD8 T cells (47, 48), potentially permitting many more divisions before senescence compared with other cells, even memory T cells will likely reach a limit of division. Thus, delayed acquisition of CD62L by secondary memory CD8 T cells may be a mechanism to allow for a new population of CD8 T cell memory to be generated from naive precursors while maintaining adequate or even enhanced protection from infection. These results have practical implications for vaccines that rely on multiple immunizations by defining an optimal window for the third boost, before the reacquisition of CD62L. This may result in both efficient boosting of secondary memory CD8 T cells as well as the most effective generation of new primary CD8 T cell responses. This approach to timing may ensure that the induced memory is long lasting and able to respond to multiple encounters with the specific pathogen.

In cases where the induction of humoral responses by vaccination is not sufficient to protect against pathogenic infection, the generation of CD8 T cell memory has substantial potential in the rational design of vaccines (49). Insufficient primary memory CD8 T cell responses may be boosted by secondary immunizations, which quantitatively enhance memory cell numbers and alter certain qualitative aspects of memory. Whether secondary memory CD8 T cells will be more protective, as shown in our studies with *L. monocytogenes* against all, or only a subset, of pathogens, is a critical issue for the design of the most potent vaccines. For example, a potent CD8 T cell response to a single immunization may be most effective against one subset of pathogens, whereas a low priming vaccination and robust booster immunization to generate secondary CD8 T cell memory may be more effective against another subset of pathogens. Comparison of primary and secondary memory CD8 T cells for protection against a variety of pathogens will be required to resolve this issue.

MATERIALS AND METHODS

Mice, *L. monocytogenes*, and LCMV. C57BL/6 (B6) and BALB/c mice were obtained from the National Cancer Institute, Frederick, MD. Thy1.1 BALB/c mice were provided by Dr. Richard Dutton (Trudeau Institute, Saranac Lake, NY). OT-I TCR-tg mice have been previously described (28). *L. monocytogenes* expressing the ovalbumin gene (LM-OVA) was obtained from Dr. Hao Shen (University of Pennsylvania, Philadelphia, PA) and Dr. Leo Lefrancois, (University of Connecticut, Farmington, CT). Mice were bred and maintained in our animal facilities at the University of Iowa. All animal protocols were approved by the University of Iowa Institutional Animal Care and Use Committee. Attenuated strains of LM (*actA*$^-$ LM-OVA [26] and *actA*$^-$ XFL303 [50]) were previously described. For infections, LM were grown and injected i.v. as described (51). The number of colony-forming units was confirmed by plating dilutions on selective media. LCMV-Armstrong (2×10^5 plaque-forming units) was injected i.p. as described (52).

Antibodies and reantigenents. Antibodies of the indicated specificities and with the appropriate combination of conjugated fluorophores were used in these studies: IFN-γ, Thy1.1, BrdU, CD107a, CD62L, IL-2, IgG2a, IgG1, CD8 (BD PharMingen), IgG2b, CD122, Thy1.2 (eBiosciences), and granzyme B (CalTantigen). Granzyme B stain of splenocytes was performed after treatment with Brefeldin A (BD PharMingen). CFSE (Molecular Probes) was used at 0.5 μM, unless indicated otherwise, to label cells. BrdU (BD PharMingen) was injected i.p. (2 mg) on the first day and was administered in the drinking water (0.8 mg/ml) for 8 d. Synthetic peptides $NP_{118-126}$ (NP118) and $ova_{257-264}$ (ova257) have been previously described (24, 28).

Intracellular cytokine staining. Intracellular cytokine staining was performed as previously described (53). In brief, splenocytes were cocultured with Brefeldin A in the presence or absence of specific peptide for 6 h. Cells were then washed, surface stained, and treated with Cytofix/Cytoperm (BD PharMingen) before staining for cytokines.

Generation of primary and secondary responses in the same mouse. We generated primary and secondary responses in the same mouse as previously described (20). In brief, splenocytes from a LCMV-immune BALB/c Thy1.2 mouse, containing ~1.5–2 × 10^4 NP118-specific CD8 T cells, were adoptively transferred i.v. into naive, Thy1.1 BALB/c mice. These mice were then infected i.p. with LCMV-Armstrong, and the subsequent responses were distinguished by differential Thy1 expression.

Generation of primary and secondary memory OT-I cells. OT-I/Thy1.1 splenocytes were CD8 enriched by negative selection (Milltenyi Biotech), and 2×10^4 cells were adoptively transferred into naive, Thy1.2 B6 mice. Recipient mice were infected i.v. with ~1×10^7 *actA*$^-$ LM-OVA. Over 100 d later, spleens were harvested and OT-I cells purified using anti–Thy1.1-PE and anti-PE magnetic beads. Approximately 5×10^4 purified primary memory OT-I cells were adoptively transferred into a new group of naive Thy1.2 B6 mice. At the same time, an equal number of Thy1.1-purified naive OT-I cells were adoptively transferred into a group of naive Thy1.2 B6 mice. Both groups of mice were infected with 10^7 *actA*$^-$ LM-OVA to generate secondary and primary OT-I responses. For primary and secondary memory OT-I studies, spleens were harvested around 65 d after infection, and memory OT-I cells were purified by anti–Thy1.1-PE antibodies and anti-PE magnetic beads. For experiments that dealt with purifying or depleting CD62L^{hi} cells, spleens containing primary or secondary memory OT-I cells were harvested and stained with anti–CD62L-PE and anti-PE magnetic beads before AutoMACS purification.

In vitro culture with IL-15. Purified OT-I memory T cell populations were CFSE labeled, and 10^5 of either cell type was cultured in vitro in a 96-well round bottom plate in the presence of 0, 50, or 200 ng/ml IL-15 (Peprotech). CFSE dilution was assessed by flow cytometry 3 d later.

Homeostatic proliferation in irradiated hosts. 10^5 CFSE-labeled, Thy1.1-purified primary or secondary memory OT-I cells were adoptively transferred into hosts irradiated (6.5 Gys) 24 h earlier. At the same time, 5×10^5 cells were transferred into nonirradiated B6 hosts. After 14 d, spleens were harvested and CFSE dilution was assessed by flow cytometry.

Degranulation assay. Splenocytes were cultured with monensin (BD PharMingen) and anti–CD107a-FITC in the presence or absence of 1 μM specific peptide. At different time points after infection, cells were washed and surface stained for the indicated markers.

In vivo cytolytic assay. The indicated number of primary and secondary memory OT-I cells were adoptively transferred into naive, Thy1.2 B6 mice. 1 d later, a mixture of 2×10^6 unpulsed splenocytes labeled with 0.5 μM

CFSE and 2×10^6 splenocytes pulsed with 1 μM specific peptide and labeled with 0.0625 μM CFSE was administered i.v. to the indicated groups as well as a control group that received no memory cells. 2 h later, spleens were harvested, and the percentages of $CFSE^+$ cells that were $CFSE^{hi}$ and $CFSE^{lo}$ was assessed flow cytometrically. The percent killing was calculated as: $100 - (100 \times [(\%\ CFSE^{lo}/\%\ CFSE^{hi})/(\%\ CFSE^{lo}$ in no memory cells group/% $CFSE^{hi}$ in no memory cells group)]).

In vivo protection. The indicated number of primary and secondary memory OT-I T cells were adoptively transferred into naive, Thy1.2 B6 mice. Mice were subsequently challenged with the indicated dose of virulent LM-OVA, and bacterial numbers were determined in spleen homogenates 3 d later as described (17).

Lymphocyte isolation. Other than blood, the organs and tissues were harvested after cardiac perfusion with PBS and heparin. Axillary and inguinal lymph nodes were pooled and mechanically disrupted using frosted glass slides. Bone marrow was aspirated from femurs and tibias. Spleens and lungs were forced through metal meshes. Where appropriate, RBCs were lysed with ACK buffer.

Ability to prime new naive response. Primary and secondary memory Thy1.2 OT-I T cells were generated as described earlier. Approximately 65 d after infection, CD8-enriched, CFSE-labeled naive OT-I/Thy1.1 cells were adoptively transferred into these mice. The following day, mice were injected s.c. with $\sim 1 \times 10^7$ *actA*$^-$ LM-OVA in the right lower flank. 2 d after infection, ipsilateral and contralateral inguinal lymph nodes were harvested, and CFSE dilution was monitored in $CD8^+$Thy1.1–positive cells by flow cytometry. Contralateral lymph nodes were pooled before analysis.

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