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Initial T cell frequency dictates memory CD8$^+$ T cell lineage commitment

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Memory T cells can be divided into central memory T cell (T_{CM} cell) and effector memory T cell (T_{EM} cell) subsets based on homing characteristics and effector functions. Whether T_{EM} and T_{CM} cells represent interconnected or distinct lineages is unclear, although the present paradigm suggests that T_{EM} and T_{CM} cells follow a linear differentiation pathway from naive T cells to effector T cells to T_{EM} cells to T_{CM} cells. We show here that naive T cell precursor frequency profoundly influenced the pathway along which CD8$^+$ memory T cells developed. At low precursor frequency, those T_{EM} cells generated represented a stable cell lineage that failed to further differentiate into T_{CM} cells. These findings do not adhere to the present dogma regarding memory T cell generation and provide a means for identifying factors controlling memory T cell lineage commitment.

Based on homing characteristics and effector functions, at least two types of memory T cells have been described in CD4$^+$ and CD8$^+$ T cell populations. The original descriptions of central and effector memory T cells suggested that central memory T cells (T_{CM} cells) reside in lymphoid organs and express CCR7 and CD62L, whereas effector memory T cells (T_{EM} cells) reside mainly in nonlymphoid tissues, do not express CCR7 or CD62L and have immediate effector functions[1–3]. This raised the question of how T_{CM} cells and T_{EM} cells are generated and whether each is the product of interdependent or separate lineages.

Three models of differentiation have been proposed, with the first being that T_{CM} cells provide a continual source of T_{EM} cells. This model is based on the findings that memory CCR7$^+$ T cells in short-term *in vitro* culture can lose expression of this chemokine receptor and in the process become functionally competent[1,4]. Analysis of the T cell receptor (TCR) repertoire of human blood memory CD8$^+$ T cells has suggested an additional possibility in which T_{CM} and T_{EM} cells represent mostly separate lineages[5]. In contrast, an alternative model has indicated that over time T_{EM} cells convert to T_{CM} cells[6]. This conclusion was derived from analysis of TCR-transgenic CD8$^+$ memory T cells specific for lymphocytic choriomeningitis virus (LCMV) glycoprotein 33 (gp33) that had been separated by virtue of CD62L expression. In this system, adoptively transferred CD62L$^-$ memory T cells convert to CD62L$^+$ memory cells, however, the reverse does not occur. This discordance between models of memory CD8$^+$ T cell lineage development may be due in part to differences in experimental systems or could reflect a fundamental difference between human and mouse CD8$^+$ memory T cells.

To address those discrepancies, we have compared here the generation of memory cell lineages in adoptively transferred TCR-transgenic CD8$^+$ T cells versus endogenous CD8$^+$ T cells responding to the same infection *in vivo*. Our results indicated that commitment to a particular memory cell lineage was governed by the initial naive T cell precursor frequency. Moreover, the differentiative and proliferative capacity of memory cell subsets was dependent on the amount of initial clonal competition.

RESULTS

T_{EM} cells revert to T_{CM} cells in adoptive transfer systems

We first tested whether the conversion of T_{EM} cells to T_{CM} cells occurred when adoptively transferred naive TCR-transgenic T cells were the responding population. For this, we adoptively transferred naive LCMV-specific P14 cells into naive mice, which we then infected with the Armstrong strain of LCMV[7]. The LCMV-specific CD8$^+$ T cell response is characterized by an expansion phase, peaking on days 7–8, followed by a contraction phase between days 8 and 30; subsequently, a stable pool of P14 TCR–transgenic memory cells specific for an H-2D^b-restricted epitope of the LCMV glycoprotein, consisting of amino acids 33–41 (KAVYNFATM), is formed[8,9]. About 60% of the resulting P14 memory cells were CD62L$^+$ (**Fig. 1**). We sorted LCMV-specific memory CD8$^+$ T cells into T_{CM} and T_{EM} subsets on the basis of CD62L expression 40 d later and adoptively transferred the cells into naive recipients. At 34 d after transfer of sorted P14 T_{EM} cells (CD62L^{lo}) and T_{CM} cells (CD62L^{hi}) into separate hosts, a portion (48%) of T_{EM} cells had converted to T_{CM} cells (**Fig. 1a**), whereas transferred CD62L^{hi} cells retained CD62L expression. We obtained similar results when we sorted P14 cells and transferred them 117 d after infection (data not shown). To determine whether this phenomenon was unique to P14 cells and LCMV infection, we transferred

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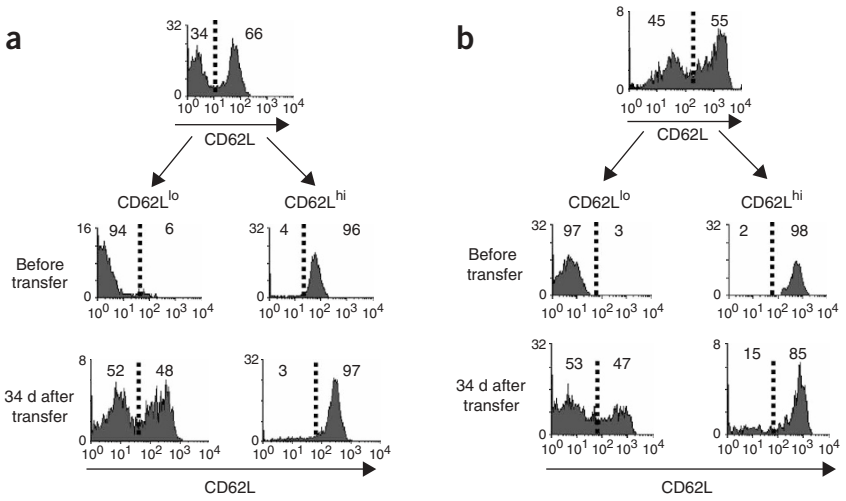

Figure 1 T_{EM} cells convert to T_{CM} cells when derived from adoptively transferred TCR-transgenic $CD8^+$ T cells. (**a**) Splenocytes from mice transgenic for the TCR specific for an H-$2D^b$-restricted epitope of the LCMV glycoprotein consisting of amino acids 33–41 (P14; 1×10^6 cells per mouse) were transferred into syngeneic (B6 × B6D2) F1 mice that were infected intraperitoneally 1 d later with LCMV Armstrong (2×10^5 PFU). At 40 d after infection, $CD62L^{lo}$ and $CD62L^{hi}$ memory T cells from the spleen were purified by flow cytometry and were adoptively transferred into separate naive mice. After 34 d, CD62L expression by splenic H-$2D^b$–gp33$^+CD8^+$ T cells of recipients was assessed. Data represent one of three mice per time point. (**b**) Splenocytes (1×10^5) from CD45.1 OT-I TCR–transgenic mice were transferred into CD45.2 B6 mice, which were infected intravenously 1 d later with VSV-OVA (1×10^6 PFU). At 56 d after infection, $CD62L^{hi}$ cells from the spleen and $CD62L^{lo}$ memory T cells from the spleen were purified by flow cytometry and were adoptively transferred into separate naive mice. Between 34 and 40 d, CD62L expression by splenic OVA-specific memory T cells of recipients was assessed. Data are from one representative mouse of three to four mice analyzed per time point from three individual experiments. Vertical axes, relative cell number; numbers to the right or left of the dashed vertical line (which represents negative control staining) indicate the percentage of cells in that area.

ovalbumin-specific OT-I $CD8^+$ T cells and infected the recipients with vesicular stomatitis virus expressing ovalbumin (VSV-OVA)[10]. Then, 56 d later we sorted OT-I memory $CD8^+$ T cells into T_{CM} and T_{EM} subsets on the basis of CD62L expression and adoptively transferred the cells into naive recipients (**Fig. 1b**). After 34 d, the transferred $CD62L^{hi}$ T_{CM} population remained $CD62L^{hi}$, whereas approximately half of the $CD62L^{lo}$ population had converted to $CD62L^{hi}$, confirming the ability of OT-I T_{EM} cells to acquire the phenotype of T_{CM} cells.

Stable T_{EM} cell generation from endogenous $CD8^+$ T cells

To determine whether the conversion of T_{EM} cells to T_{CM} cells was also characteristic of polyclonal populations of $CD8^+$ memory T cells, we generated memory cells by infecting naive C57BL/6 (B6) mice with LCMV. In this setting, an LCMV-specific $CD8^+$ memory cell population, consisting of 40% $CD62L^{lo}$ and 60% $CD62L^{hi}$ cells, developed in the spleen 111 d after infection (data not shown), at which time we sorted memory cells from spleen or liver into $CD62L^{lo}$ and $CD62L^{hi}$ populations and adoptively transferred them into naive mice. Unexpectedly, at 34 d after transfer, the gp33-specific T_{EM} ($CD62L^{lo}$) population remained uniformly $CD62L^{lo}$ in both the spleen and liver, indicating that the endogenous LCMV-specific T_{EM} cells did not give rise to T_{CM} cells (**Fig. 2a**). Similarly, $CD62L^{lo}$ $CD4^+$ T cells transferred from LCMV-immune mice into naive recipients remained $CD62L^{lo}$, although we did not have the means to track LCMV-specific $CD4^+$ T cells in this case (data not shown). We also noted a lack of conversion from T_{EM} to T_{CM} when we sorted endogenous OVA-specific memory $CD8^+$ T cells from VSV-OVA-infected mice into $CD62L^{hi}$ and $CD62L^{lo}$ subsets and adoptively transferred them into naive recipients. At 34 d after transfer of $CD62L^{lo}$ memory cells from spleen or liver, very few $CD62L^{hi}$ OVA-specific memory cells could be detected in either the spleen or liver of recipients (**Fig. 2b**). In addition, $CD62L^{lo}$ secondary memory cells generated by challenge of VSV-OVA-infected mice with *Listeria monocytogenes* producing OVA (LM-OVA) also did not re-express CD62L after transfer (**Fig. 2c**). These results suggested that unlike $CD62L^{lo}$ $CD8^+$ T cells generated in the TCR adoptive transfer model (**Fig. 1**), T_{EM} cells derived from endogenous $CD8^+$ T cells represented a phenotypically stable population.

To allow long-term tracking of transferred T_{EM} cells to further address whether interconversion occurs, we transferred purified T_{EM} cells and then 'recalled' these cells with infection. At 107 d after infection, $CD62L^+$ cells were still not evident (**Supplementary Fig. 1** online). Although conversion could ensue at later times and/or secondary infection could further delay conversion, given the lifespan of the mouse, it becomes difficult to see the utility of such a system. Instead, we believe that separate T_{CM} and T_{EM} lineages are generated in the primary response and that T_{CM} cells are generally sequestered in lymph nodes and recirculate preferentially through lymph nodes, where they participate in secondary responses.

Precursor frequency governs memory lineage commitment

One possible explanation for the discrepancies we noted between TCR-transgenic and endogenous T cells was the fact that the naive precursor frequency was much greater in the former than in the latter. To test whether precursor frequency affected lineage commitment, we adoptively transferred varying numbers of OT-I T cells into B6 mice, which we then infected with VSV-OVA. At 42 d after infection, we analyzed CD62L expression in transferred transgenic cells from various tissues. Although the total number of OVA-specific memory T cells in the spleen was relatively constant (including both transgenic and endogenous cells; data not shown), the absolute numbers of T_{EM} cells and T_{CM} cells differed depending on the number of OT-I T cells transferred (**Fig. 3a**). Thus, at 42 d after infection of recipients of 1×10^7, 1×10^4 or 5×10^2 OT-I T cells, the percentage of splenic T_{CM} ($CD62L^{hi}$) cells was 71%, 27% or 5%, respectively. We noted the same trend in the lung and liver (**Fig. 3a**) and obtained similar results for CCR7 expression (data not shown). At the lowest dose of 5×10^2 cells, only the memory cell population in the lymph nodes contained an appreciable population of $CD62L^{hi}$ cells. This phenotypic distribution among memory cells more closely resembled that derived from an endogenous response (**Fig. 3b**), which is likely to be initiated from a precursor pool of a few hundred cells[11].

To test whether altering the ratio of antigen-presenting cells to $CD8^+$ T cells during the primary response affected memory lineage

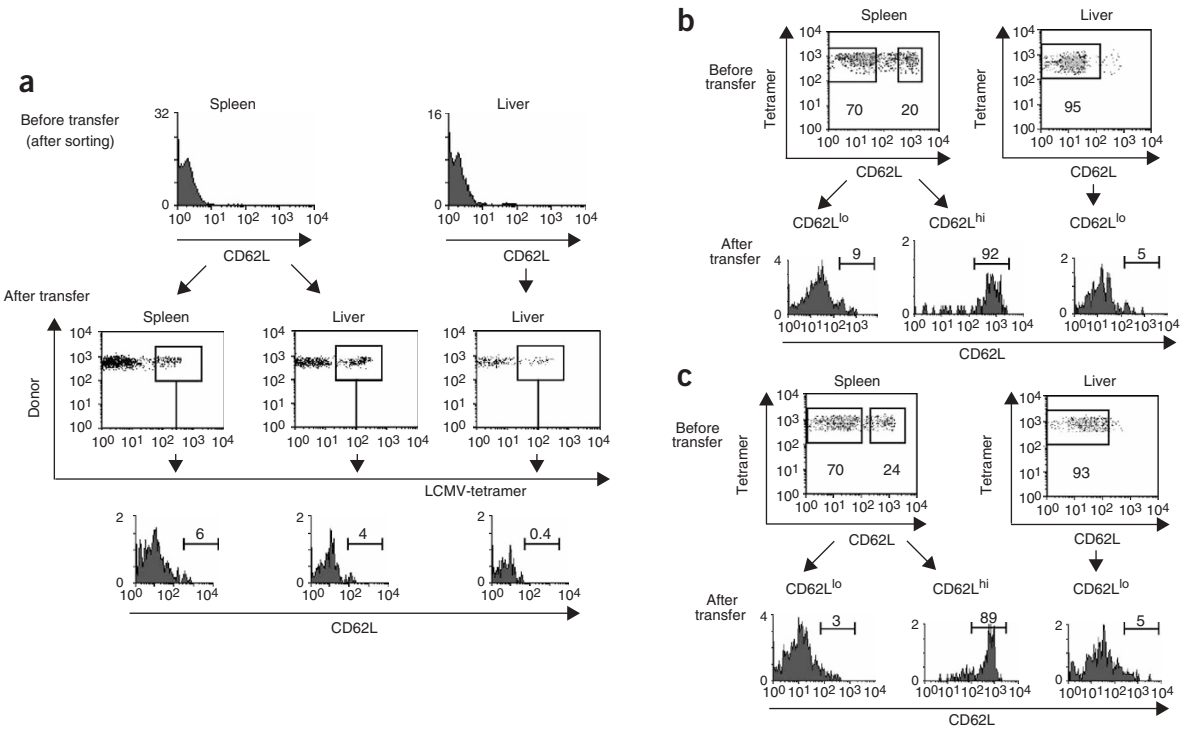

Figure 2 Endogenous CD8$^+$ T cells generate distinct T_{CM} and T_{EM} lineages in response to infection. (**a**) CD45.2 B6 mice were infected intraperitoneally with LCMV Armstrong (2×10^5 PFU) and, 111 d after infection, CD62L^{lo} CD8$^+$ memory T cells from the spleen or liver were purified by flow cytometry and were transferred into naive CD45.1 B6 mice. At 34 d after transfer, CD62L expression was assessed in tetramer-positive memory CD8$^+$ T cells (boxes indicate the population analyzed). (**b**) CD45.2 B6 mice were infected intravenously with 1×10^6 PFU of VSV-OVA and, 90 d later, purified CD62L^{lo} and CD62L^{hi} OVA-specific memory CD8$^+$ T cells were transferred into CD45.1 B6 mice. Then, 34 d later, CD62L expression was assessed in the OVA-specific memory CD8 T cells. (**c**) CD45.2 B6 mice originally infected intravenously with 1×10^3 CFU of LM-OVA were allowed to 'rest' for 132 d and were reinfected intravenously with 1×10^6 PFU of VSV-OVA. At 75 d after reinfection, CD62L^{lo} and CD62L^{hi} memory T cells from the spleen or liver were purified by flow cytometry and were transferred into CD45.1 B6 recipients; 34 d later,CD62L expression was assessed in the transferred tetramer-positive population. Data represent three to four mice per time point from three to four individual experiments. Vertical axes, relative cell number; numbers above bracketed lines indicate the percentage of cells expressing CD62L in that area. Boxes (**b**,**c**) indicate the gates used for cell sorting; numbers beneath boxes indicate the percentages of the initial populations within the sort gates.

'choice', we artificially increased dendritic cell numbers by treatment with the ligand for the cytokine receptor Flt3. Administration of Flt3 ligand resulted in an increase of about five- to sevenfold in dendritic cells in the spleen and lymph nodes (data not shown). We transferred 1×10^5 OT-I cells into mice treated with Flt3 ligand that we then infected with VSV-OVA. Then, 67 d later, we assessed the expression of CD62L by OT-I cells. In control mice, 60% of the memory cells expressed CD62L, compared with only 7% of memory cells in mice treated with Flt3 ligand (**Supplementary Fig. 2** online). Thus, increasing dendritic cell numbers enhanced T_{EM} cell generation. Although the precise mechanism that resulted in enhancement of T_{EM} cell generation has yet to be defined, an increase in dendritic cells should result in a situation in which T cell competition for antigen is decreased, thereby allowing more complete T cell activation. These data further support our contention that T cell precursor frequency and competition for resources are important physiological regulators of memory CD8$^+$ T cell lineage commitment.

Memory lineage commitment during the primary response

The results reported above led us to investigate whether lineage commitment after transfer of low numbers of OT-I cells mimicked our observations with endogenous memory cells; that is, whether CD62L^{lo} memory cells from these mice would maintain their phenotype after adoptive transfer. We transferred 5×10^2 OT-I cells into naive mice and infected the recipients with VSV-OVA. Then, 8 d later, we isolated CD62L^{lo} effector cells and transferred them into naive mice; we used early transfer to test whether commitment to a particular lineage occurred early during the immune response. These cells remained CD62L^{lo} when examined 1 month later (**Fig. 4a**). Hence, when generated from low numbers of precursors, CD8$^+$ T_{EM} cells acquired a stable CD62L^{lo} phenotype as early as 8 d after activation. This was not dependent on transfer early after activation, however, as secondary CD62L^{lo} memory cells, derived from 5×10^2 OT-I cells after infection with VSV-OVA and rechallenge with LM-OVA, did not convert to T_{CM} cells (**Fig. 4b**). Thus, despite the fact that the precursor frequency of antigen-specific cells was substantially increased after secondary infection, the resulting T_{EM} cells did not undergo conversion to T_{CM} cells, suggesting that the lineage 'choice' was established in the primary response.

To further substantiate that commitment to the T_{CM} or T_{EM} lineages occurred during the primary response, we transferred 1×10^5, 1×10^4 or 5×10^2 OT-I (CD45.1) cells into B6 (CD45.2) recipients that we then infected with VSV-OVA. Then, 7 d later we determined the percentages of CD62L^{lo} and CD62L^{hi} OT-I T cells (**Fig. 4c**). As with the memory populations, increasing the input number of antigen-specific T cells resulted in increasingly larger

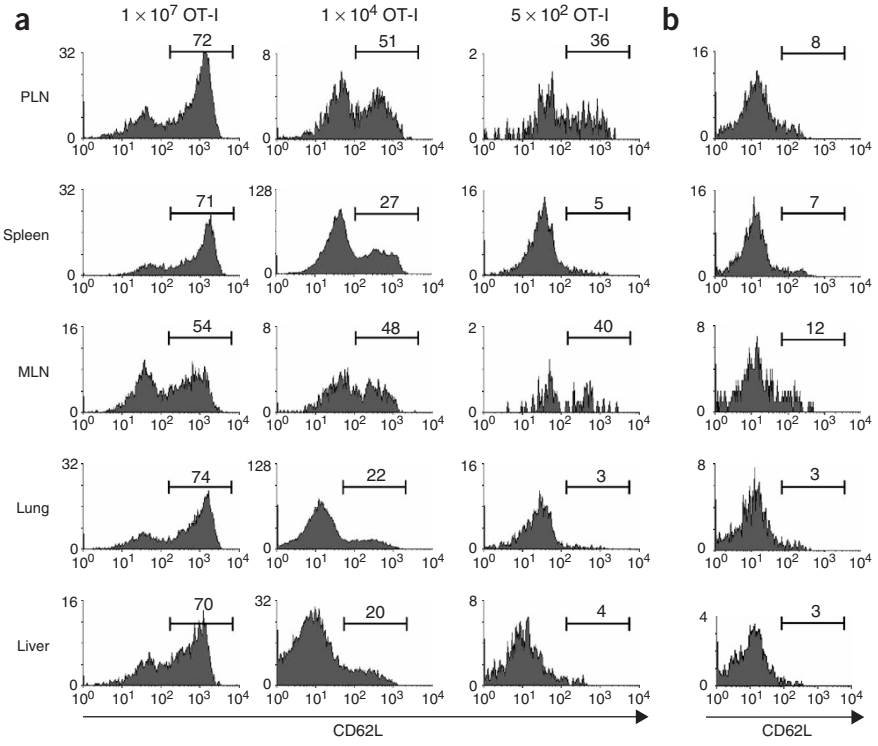

Figure 3 Initial naive CD8$^+$ T cell precursor frequency 'imprints' memory lineage commitment during the primary response. (**a**) OT-I splenocytes (1×10^7, 1×10^4 or 5×10^2) were transferred into B6 mice and 1 d later the mice were infected with 1×10^6 PFU VSV-OVA. At 42 d after infection, lymphocytes were isolated from various tissues and CD62L was measured on the OT-I T cells. (**b**) B6 mice were infected with 1×10^6 PFU VSV-OVA and 42 d after infection, CD62L was measured on the tetramer-positive memory CD8$^+$ T cells. Data represent five mice (1×10^7 and 1×10^4 OT-I) and six mice (5×10^2 OT-I) from two individual experiments. Vertical axes, relative cell number; numbers above bracketed lines indicate the percentage of OT-I cells in that area. PLN, peripheral lymph node; MLN, mesenteric lymph node.

populations of CD62L^{hi} cells in the lymph node and spleen. When 5×10^2 OT-I cells were transferred, most effector cells in the spleen lacked CD62L, whereas those populations in lymph nodes contained about one third CD62L^{hi} cells. Thus, sampling only the spleen, as is often done, would provide misleading results regarding CD62L expression. Because CD62L is used by T cells to enter lymph node, enrichment of CD62L$^+$ cells in this tissue might be expected. Our results suggested that precursor frequency determined lineage commitment during the primary response.

Initial precursor frequency influences T_{EM} cell proliferation

Our results suggested that CD62L^{lo} CD8$^+$ memory cells generated from high or low numbers of naive cells were fundamentally different, with only the latter representing a phenotypically stable population. To investigate this possibility further, we assessed the kinetic activity of these cells *in vivo*. A study of mouse CD8$^+$ memory cells has suggested that T_{CM} cells have a greater capacity for proliferation than do T_{EM} cells[6]. Thus, we sought to assess whether precursor frequency during the primary response affected the proliferative capacity of the memory cells generated. We adoptively transferred 1×10^7, 1×10^4 or 5×10^2 OT-I T cells into B6 mice and infected the mice with VSV-OVA 1 d later. We infected the mice with LM-OVA 198 d later and at least 40 d after secondary infection, we provided the mice with bromodeoxyuridine (BrdU) in their drinking water for an additional 40 d. Splenic memory cell populations derived from 1×10^7 or 1×10^4 OT-I cells were about 60% CD62L^{hi}, whereas only about 25% of those derived from 5×10^2 OT-I cells expressed CD62L (**Fig. 5a**). At all input doses, more than 75% of the CD62L^{hi} memory cells had incorporated BrdU. In contrast, the BrdU incorporation of the CD62L^{lo} cells was highly dependent on the input number of T cells. Thus, 66%, 50% and 27% of CD62L^{lo} cells incorporated BrdU when 1×10^7, 1×10^4 and 5×10^2 OT-I cells were transferred, respectively. This difference in the proliferative capacity of CD62L^{lo} cells was even more substantial in the lung, with 74%, 41% and 27% BrdU$^+$ cells after transfer of 1×10^7, 1×10^4 or 5×10^2 OT-I cells, respectively (**Fig. 5b**). We also noted this difference in proliferative capacity in the CD62L^{lo} population in the peripheral lymph nodes and liver (data not shown). In addition, we also noted this difference in experiments examining proliferation of primary endogenous T_{EM} cells and T_{CM} cells and with primary memory cells generated from low numbers of transferred OT-I cells. Thus, in general it seems that T_{EM} cells have lower proliferative capacity than T_{CM} cells (data not shown). These data show that the initial precursor frequency of antigen-specific T cells 'imprinted' fundamental physiological differences on the memory cells generated and that these differences were maintained even through a secondary recall response.

Our findings suggested a model of CD8$^+$ memory T cell lineage commitment in which high initial naive T cell precursor frequency resulted in the development of T_{EM} and T_{CM} cells but also of a transitional CD62L$^-$ memory subset with the proliferative capacity of CD62L$^+$ memory cells and the ability to re-express CD62L (**Supplementary Fig. 3** online). In contrast, in physiological conditions of low initial precursor numbers, distinct T_{EM} and T_{CM} lineages developed without the ability to interconvert (**Supplementary Fig. 3** online).

DISCUSSION

At present, the idea of memory T cell development is based on the finding that memory T cells can be divided into subsets distinguishable by the expression of homing molecules and chemokine receptors including CD62L and CCR7 (refs. 1–3). In addition, T_{CM} (CD62L$^+$CCR7$^+$) cells lack immediate effector function, thus functionally distinguishing them from T_{EM} (CD62L$^-$CCR7$^-$) cells[2,3]. Therefore, based on migratory abilities, T_{EM} cells 'preferentially' reside in nonlymphoid tissues, whereas T_{CM} cells can be found in the spleen and lymph node[1–3], although these phenotypes and locations are by no means absolute[12]. Nevertheless, both T_{EM} cells and T_{CM} cells are members of a blood-borne pool of memory cells able to circulate to most tissues of the body, depending on the expression of the appropriate homing molecules for entry into a given tissue[13].

The lineage relationship between T_{CM} and T_{EM} cells has been the topic of several reports. For example, based on *in vitro* studies using human T cells, it was proposed that T_{CM} cells could provide a

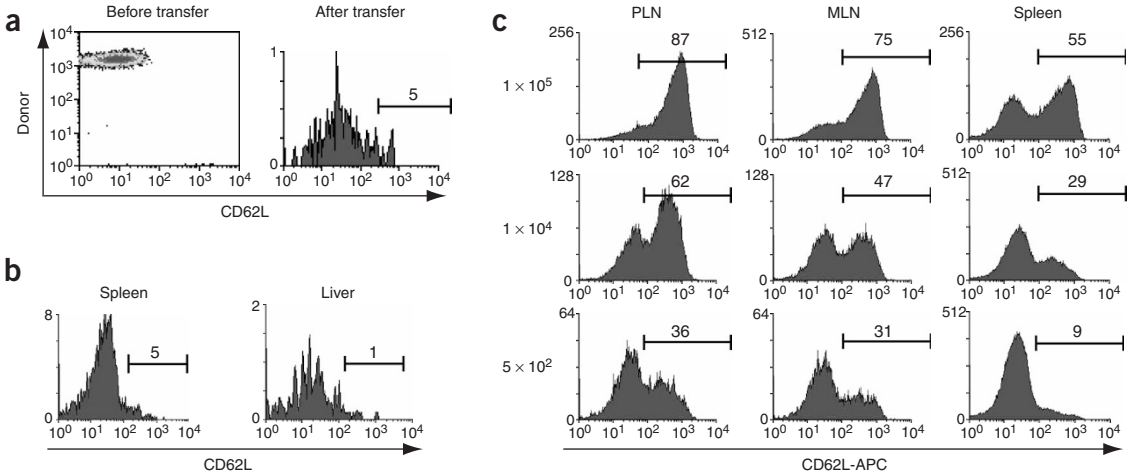

Figure 4 Commitment to the T_{CM} and T_{EM} lineages occurs during the primary response. (**a**) CD45.1 OT-I cells (5×10^2) were transferred into CD45.2 B6 mice and 1 d later the mice were infected with 1×10^6 PFU VSV-OVA. Then, 8 d later, $CD62L^{lo}$ OT-I cells were purified by flow cytometry and were adoptively transferred into naive B6 mice; 34 d later, the expression of CD62L by splenic OT-I cells was assessed. (**b**) B6 mice received an initial adoptive transfer of 5×10^2 OT-I cells and were infected with VSV-OVA, then were allowed to 'rest' for 114 d and were infected intravenously with 1×10^3 CFU of LM-OVA. Then, 48 d later, $CD62L^{lo}$ memory T cells from the spleen or liver were purified by flow cytometry and were transferred into naive recipients; 34 d after transfer, CD62L expression by OT-I cells from the spleen and liver was assessed. (**c**) OT-I splenocytes (1×10^7, 1×10^4 or 5×10^2) were transferred into B6 mice and 1 d later mice were infected with 1×10^6 PFU VSV-OVA. At 7 d after infection, lymphocytes were isolated from the peripheral lymph node, mesenteric lymph node and spleen, and CD62L was measured on the OT-I T cells. APC, allophycocyanin. Similar results were obtained in three independent experiments. Vertical axes, relative cell number; numbers above bracketed lines indicate the percentage of cells in that area.

continual source of T_{EM} cells that were considered end-stage cells[1]. In contrast, analysis of the TCR repertoires of human memory T cell subsets indicated that the lineages develop independently[5]. To directly test lineage relationships among memory T cell subsets, an experimental model using adoptive transfer of TCR-transgenic $CD8^+$ T cells and LCMV infection was used[6]. Transfer of purified $CD62L^+$ or $CD62L^-$ memory cells demonstrated that T_{EM} cells are able to convert to T_{CM} cells over several weeks. Our results presented here have corroborated those findings with transfer of LCMV- or OVA-specific TCR-transgenic T cells. However, conversion of T_{EM} cells to T_{CM} cells occurred only in a subset of the T_{EM} cells, at least in the time frame studied, suggesting that there may be lineage heterogeneity in the T_{EM} population. Indeed, our results have shown that the ability of T_{EM} cells to convert to T_{CM} cells increased with the transfer of larger numbers of naive $CD8^+$ T cells. In fact, when we transferred memory cells derived from 5×10^2 naive OT-I cells or when we used endogenous memory cells, we did not note conversion of $CD62L^-$ cells to $CD62L^+$ cells. Coupled with that result was the finding that the proliferative capacity of $CD62L^-$ cells also increased with increasing input numbers of responding $CD8^+$ T cells. Those data indicated that with nonphysiologically high numbers of responding $CD8^+$ T cells, commitment to the T_{EM} lineage seemed incomplete. This resulted in the production of

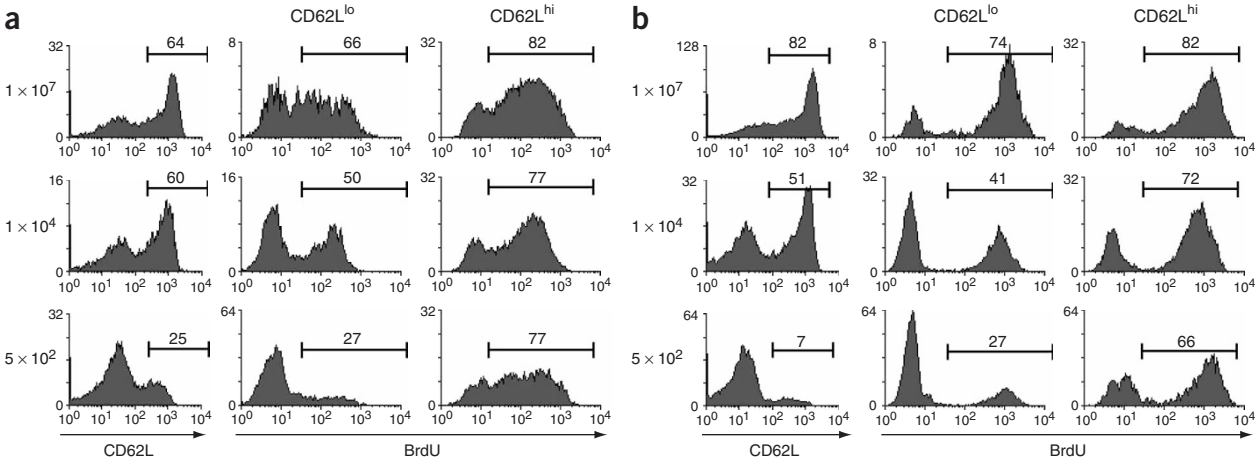

Figure 5 Proliferative capacity of T_{EM} cells is regulated by clonal competition. CD45.2 B6 mice received an initial adoptive transfer of 1×10^7, 1×10^4 or 5×10^2 CD45.1 OT-I T cells and were infected with VSV-OVA, then were allowed to 'rest' for 198 d and were infected intravenously with 1×10^3 CFU of LM-OVA. Then, 40 d later, mice were provided with BrdU in their drinking water for 40 d, at which time BrdU incorporation was analyzed in $CD62L^{lo}$ and $CD62L^{hi}$ OT-I cells from the spleen (**a**) and lung (**b**). Data represent three mice (1×10^7 and 1×10^4 OT-I cells) and five mice (5×10^2 OT-I cells) from two individual experiments. Numbers above bracketed lines indicate the percentage of cells in that area.

a 'transitional' T_{EM} population with the proliferative capacity of a T_{CM} population and the ability to phenotypically convert to T_{CM} cells. Thus, the protracted development of memory $CD8^+$ T cells proposed using gene expression analysis of responders derived from the transfer of large numbers of TCR-transgenic T cells bears reexamination[14].

Our findings indicated that T_{EM} and T_{CM} $CD8^+$ cells represented stable memory cell lineages when generated from physiologically low numbers of naive precursors. Based on those data, we propose a model in which naive $CD8^+$ T cells develop into effector cells and generate memory cells of two distinct lineages based on CD62L expression. Our data would support the idea that the memory lineage 'decision' is made during the primary response, as the early phenotype of responding cells was dictated by the naive precursor frequency. In fact, with low numbers of transferred cells or with assessment of the endogenous response, small numbers of $CD62L^+$ cells were present in the lymph node but not the spleen throughout the primary response. The link between precursor frequency and differentiation stage suggested that competition for limiting resources (such as access to antigen-presenting cells[15] or cytokines such as interleukin 7 or 15; refs. 16–19) may be involved. This hypothesis was further supported by the demonstration that increasing the ratio of antigen-presenting cells to $CD8^+$ T cells preferentially drove T_{EM} cell development.

The fact that greater numbers of T_{CM} cells and transitional T_{EM} cells were produced when antigen-specific T cell frequencies were high fits with the idea that relatively weak signaling favors T_{CM} cell generation, whereas production of T_{EM} cells requires strong signaling, a model that has been proposed based on *in vitro* studies[1,20]. In a published study using transfer of TCR-transgenic T cells, low-dose virus infection resulted in what was interpreted as a more rapid conversion of T_{EM} cells to T_{CM} cells, whereas high-dose infection preferentially generated $CD62L^{lo}$ T cells, and the appearance of $CD62L^{hi}$ memory cells was extended[6]. In that study, the amount of antigen was not measured and only blood was analyzed and no cell sorting or adoptive transfers were done. Thus, conversion was never directly demonstrated; the study only showed that the phenotype of the cells was altered. Reducing the viral dose would probably alter the amount of inflammation induced, which might also affect the outcome of the response. Our results would suggest that these findings may be related to the generation of different ratios of T_{CM}, T_{EM} and transitional T_{EM} cells based on antigen dose and precursor frequency. Thus, at decreasing naive T cell frequencies, which may represent relatively high antigen/T cell ratios, fewer $CD62L^+$ cells will be generated. As these cells show greater homeostatic proliferation than $CD62L^-$ memory cells[6,21,22], the $CD62L^+$ subset would gradually increase over time rather than being generated from $CD62L^-$ memory cells.

It remains possible that the proliferative ability of the memory subsets could affect the conversion of T_{EM} cells to T_{CM} cells. However, if the reduced proliferative capacity of T_{EM} cells is responsible for the reduced differentiation from T_{EM} cells to T_{CM} cells, then our example with an initial precursor frequency of 1×10^4, in which the $CD62L^{lo}$ cells also had lower proliferative capacity than the $CD62L^{hi}$ memory cells, should have resulted in less conversion of T_{EM} cells to T_{CM} cells. However, even in the face of lower proliferative capacity than $CD62L^{hi}$ cells, 50% of the cells converted to the $CD62L^{hi}$ phenotype. This scenario has also been demonstrated in another study in which 5×10^4 P14 TCR–transgenic T cells were transferred[6]. In that study, although T_{EM} cells had a reduced proliferative capacity, 50% of the T_{EM} cells nevertheless underwent reversion to T_{CM} cells. Therefore, it is unlikely that the reduced proliferative capacity of T_{EM} cells could explain the reduced differentiation from T_{EM} cells to T_{CM} cells using endogenous precursor frequencies. In neither our study here nor in published studies[6] was the actual number of 'convertible' T_{EM} cells in the overall T_{EM} population known.

Although the efficiency of conversion may also be regulated by precursor frequency, several pieces of data would suggest otherwise. In our studies, even 40 d after transfer of $CD62L^{lo}$ cells, we noted little if any conversion. In addition, when nonlymphoid tissues are monitored long term, few T_{CM} cells are present, particularly in tissues such as the intestinal mucosa. Thus, if conversion occurred in normal circumstances, it would have to occur very slowly and mainly in lymphoid tissues, whereas most T_{EM} cells are in nonlymphoid tissues. These data indicate that if conversion occurs in physiologically relevant conditions, then the process seems very inefficient. Given the lifespan of a mouse (about 2 years), this hypothetical slow rate of conversion would perhaps have a minimal effect on the overall process of memory development and reactivation.

Although our experiments have identified precursor frequency as one factor controlling memory lineage development, further studies are needed to elucidate the precise molecular mechanisms involved in memory lineage commitment. Nonetheless, the ability to manipulate memory T cell lineage 'choice' at will could provide new therapeutic avenues for vaccine development and immunotherapy. Thus, memory development could potentially be intentionally skewed toward T_{EM} or T_{CM} cells, depending on the infection type or perhaps location of a tumor. It might be expected that T_{CM} cells would be more suitable for protection against infectious agents that prime responses preferentially in lymph node, whereas T_{EM} cells would provide better protection against agents focused in spleen or nonlymphoid tissues. For example, although T_{EM} cells respond poorly to LCMV infection[6], T_{EM} cells generate a robust response to pulmonary virus infection[23]. In any case, ongoing delineation of the signals regulating memory development will continue to provide support for rational vaccine design.

METHODS

Mice and infections. B6 (CD45.2) mice were purchased from the Jackson Laboratory. B6 (CD45.1) mice were obtained from Charles River. B6D2 P14 mice bearing the H-$2D^b$–gp33–specific TCR were maintained in the specific pathogen–free unit of the Institute for Animal Health (Compton, UK), and OT-I recombination-activating gene 2–deficient mice bearing the H-$2K^b$–OVA–specific TCR were bred and maintained in specific pathogen–free conditions at the University of Connecticut Health Center (Farmington, Connecticut). B6 mice were infected with LCMV Armstrong (2×10^5 plaque-forming units (PFU)) by intraperitoneal injection or with VSV-OVA (1×10^6 PFU) intravenously or were challenged intravenously with 1×10^3 colony-forming units (CFU) of LM-OVA[24,25]. For recall responses, mice originally infected with VSV-OVA were 'recalled' with LM-OVA or vice versa.

Isolation of T cell subsets and adoptive transfer. Lymphocytes were isolated from lymphoid and nonlymphoid tissues as described[2]. T_{CM} and T_{EM} cells were purified by flow cytometry on a FACSVantage SE (Becton-Dickinson) or with anti-CD62L magnetic beads (Miltenyl Biotec) according to the manufacturer's instructions. The purity of samples sorted by flow cytometry was 91% for spleen T_{EM} cells, 96% for spleen T_{CM} cells, 92% for liver T_{EM} cells and 90% for liver T_{CM} cells and ranged from 89% to 99% for spleen and liver T_{CM} and T_{EM} cells purified by magnetic beads. Purified T_{EM} or T_{CM} cells were adoptively transferred intravenously.

Flow cytometry. For staining, lymphocytes were resuspended at a density of 1×10^6 to 1×10^7 cells/ml in 0.2% BSA and 0.1% NaN_3 in PBS, followed by incubation for 1 h at 25 °C with tetramer-allophycocyanin plus the appropriate dilution of peridinine chlorophyll protein–conjugated antibody to CD8 (anti-CD8; clone 53.6.7; BD PharMingen)[26–28]. Cells were washed with 0.2% BSA and 0.1% NaN_3 in PBS and were stained with fluorescein isothiocyanate–conjugated anti-CD11a and phycoerythrin-conjugated anti-CD62L (clone Mel-14; BD PharMingen) and were incubated for at least 20 min at 4 °C,

then were washed again and were fixed in 3% paraformaldehyde in PBS. For adoptive transfer experiments, donor cells were 'tracked' with anti-CD45.1–fluorescein isothiocyanate (PharMingen). For staining for CCR7, cells were incubated for 1 h at 37 °C with CCL19–immunoglobulin (1 μg/ml), followed by goat anti-human immunoglobulin–biotin and streptavidin-phycoerythrin as described[29]. For BrdU incorporation studies, mice infected with VSV-OVA were given water containing BrdU (0.8 mg/ml; Sigma) daily for 4 weeks[30]. BrdU staining was done after cell surface staining[31]. Cells were treated for 15 min at 25 °C with FACS Lysing Solution (Becton Dickinson), followed by overnight fixation at 4 °C in 1% paraformaldehyde and 0.05% Nonidet-P40 in PBS. Cellular DNA was then denatured for 30 min at 37 °C with 50 Kunitz units of DNase I (Boehringer Mannheim) before being stained for 45 min with anti-BrdU (Becton Dickinson) in 5% FBS and 0.5% Nonidet-P40 in PBS. Cells were washed twice before flow cytometry. Relative fluorescence intensities were measured with a FACSCalibur (Becton Dickinson). Data were analyzed with WinMDI software (J. Trotter, The Scripps Clinic, La Jolla, California).

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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