

# Lineage relationships, homeostasis, and recall capacities of central- and effector-memory CD8 T cells in vivo

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The lineage relationships of central-memory T cells ( $T_{CM}$ ) cells and effector-memory T cells ( $T_{EM}$ ), as well as their homeostasis and recall capacities, are still controversial. We investigated these issues in a murine model using two complementary approaches: T cell receptor repertoire analysis and adoptive transfer experiments of purified H-Y-specific  $T_{CM}$  and  $T_{EM}$  populations. Repertoire studies showed that approximately two thirds of  $T_{CM}$  and  $T_{EM}$  clones derived from a common naive precursor, whereas the other third was distinct. Both approaches highlighted that  $T_{CM}$  and  $T_{EM}$  had drastically distinct behaviors in vivo, both in the absence of antigen or upon restimulation.  $T_{CM}$  clones were stable in the absence of restimulation and mounted a potent and sustained recall response upon secondary challenge, giving rise to both  $T_{CM}$  and  $T_{EM}$ , although only a fraction of  $T_{CM}$  generated  $T_{EM}$ . In contrast,  $T_{EM}$  persisted for only a short time in the absence of antigen and, although a fraction of them were able to express CD62L, they were unable to mount a proliferative response upon secondary challenge in this model.

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Abbreviations used:  $T_{CM}$ ,  
central-memory T cells;  $T_{EM}$ ,  
effector-memory T cells.

Due to the persistence of memory T and B lymphocytes and long-lived plasma cells, the immune system is able to keep track of previously encountered pathogens and to give rise to a rapid and efficient response in case of re-encounter with the same pathogen (1, 2). Memory T cells persist with an elevated frequency and display enhanced functional capacities (3). Unfortunately, our knowledge of the memory differentiation pathway is still limited, which might explain why we fail to generate efficient immune memory against some pathogens or other antigens such as tumor cells.

This difficulty may stem in part from the heterogeneity of the memory T cell pool in terms of phenotype, migration, and functional capacities (3, 4). One recently recognized heterogeneity is the existence of memory cells residing within peripheral tissues beside those recirculating between secondary lymphoid organs (5). In humans, two subsets can be defined according to their expression levels of CCR7 and CD62L (6), both markers being necessary for entry in peripheral lymph nodes through HEV (7). The  $CCR7^+CD62L^+CD45RO^+$  central-

memory T cells ( $T_{CM}$ ) recirculate through lymphoid organs and do not display immediate effector functions, whereas the  $CCR7^-CD62L^-$  cells are effector-memory T cells ( $T_{EM}$ ) that reside within, or recirculate through, peripheral tissues and have immediate effector functions. All subsets can be found in blood and in the spleen (6, 8, 9).  $T_{CM}$  and  $T_{EM}$  subsets can also be distinguished in mice depending on CD62L or CCR7 expression levels (9, 10).

The distinction between the central- and peripheral memory subsets raises two fundamental issues: (a) what is the relationship between these subsets? and (b) are both subsets equally potent to generate a recall response? Three models have recently been proposed regarding their relationship. The first one is the "progressive differentiation model" (11) characterized by three hallmarks. Depending on the strength and duration of the stimulation received by a naive T cell in the course of the immune response, this cell will either follow the differentiation pathway to acquire effector functions to its end, or stop in an intermediate differentiation state. Next, at the end of the immune response, intermediates of differentiation give rise to  $T_{CM}$ , whereas  $T_{EM}$  stem from

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fully differentiated cells. Finally, in the absence of restimulation, a proportion of  $T_{CM}$  differentiates into  $T_{EM}$  to replenish the effector–memory pool. This model is supported by several studies, both in humans and mice; only nonpolarized in vitro–generated effectors recirculate in lymph nodes (8, 12), and  $T_{EM}$  have a lower proliferative potential (9, 13) and enhanced effector functions (14–16), suggesting that  $T_{EM}$  are more differentiated. Moreover, in the presence of IL-7 and IL-15, both CD4 and CD8  $T_{CM}$  were shown to generate  $T_{EM}$  in vitro (17, 18).

In the second model, the two subsets also belong to one lineage continuum, but it is the  $T_{CM}$  that derive from the  $T_{EM}$ , the latter being viewed as a transitory state (9, 19). The rate at which these cells convert to  $T_{CM}$  depends on the magnitude of the stimulation. Consistent with this model is the observed patterns of gene expression during memory T cell differentiation (20).

No mandatory lineage relationship between the two subsets is assumed in the third model. In a previous study, we analyzed the TCR repertoires of sorted  $T_{CM}$  and  $T_{EM}$  PB-MCs in healthy donors and found that they were mainly distinct at a given time point. No conversion from one subset to the other was observed over a 9-mo period (21). Therefore, we suggested that both subsets could arise at least partly independently, appearing in distinct secondary lymphoid organs and/or under distinct primary stimulation conditions. Alternatively, if most effector cells were to give rise to both  $T_{CM}$  and  $T_{EM}$ , at least one of these memory cell population had to be transient or could not recirculate in the blood.

As a result, the differentiation pathway of  $T_{CM}$  and  $T_{EM}$  subsets remains controversial, and additional complexity arises concerning the self-renewal capacities of both subsets or the conversion of cells from one subset to the other in long-term steady-state conditions or upon restimulation (5).

The other key issue deals with the capacity of either subset to mount a recall response. Several studies showed that  $T_{CM}$  and  $T_{EM}$  may have the same effector capacities, in both human or murine CD4 and CD8 T cells (22, 23), and that  $T_{CM}$  may include fully differentiated effectors (24, 25). However, others have suggested that  $T_{CM}$  and  $T_{EM}$  might have differential protective capacities upon reencounter with their cognate antigen. Indeed, although several papers suggested that the presence of effector cells may be necessary for protection against a peripheral challenge (26, 27), other studies highlighted the crucial role of  $T_{CM}$  in host defense (9, 14, 28). The comprehension of the respective roles of  $T_{CM}$  and  $T_{EM}$  subsets in host protection in case of secondary challenge is thus still limited and needs further investigation.

The study of lineage relationships requires the in vivo production of measurable numbers of memory cells, which is best achieved by the adoptive transfer of antigen-specific naive T cells (29). Past studies have used TCR $\alpha\beta$  transgenic T cells as a homogeneous source of naive T cells. However, with this experimental design, it is impossible to determine whether two memory cells would derive from a single or two distinct naive precursors. To analyze the lineage rela-

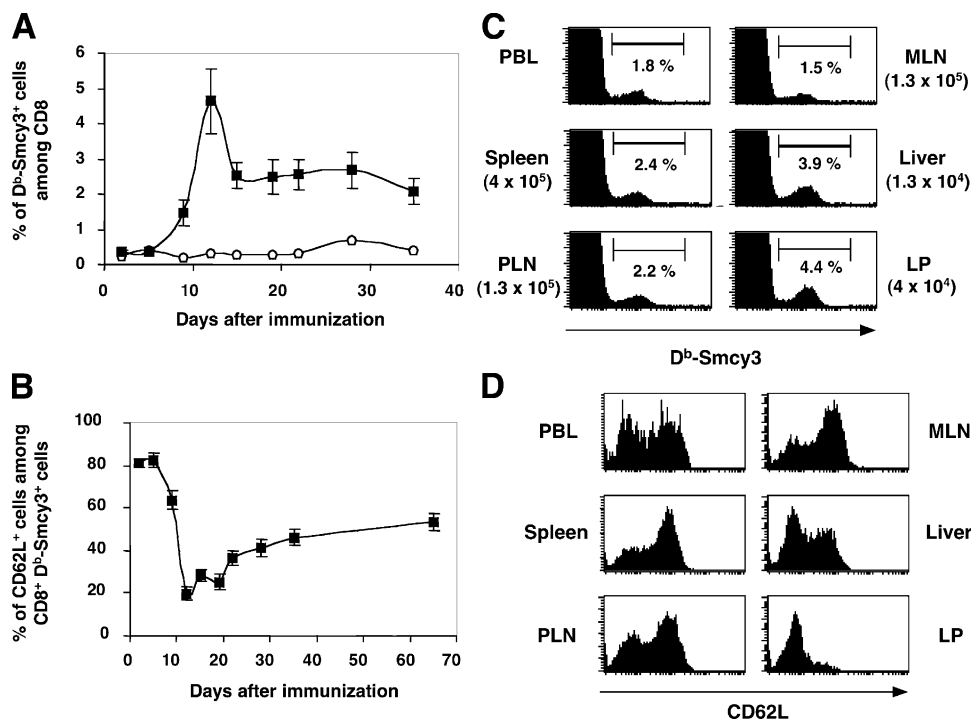
tionships between  $T_{CM}$  and  $T_{EM}$  and their respective ability to mount a recall response, we used a murine model enabling us to generate CD8  $T_{CM}$  and  $T_{EM}$  subsets specific for the H-Y male antigen with heterogeneous TCR. Mice transgenic for the  $\beta$  chain of a  $D^b$ -restricted Smcy3 peptide-specific TCR were used as a source of CD8<sup>+</sup> H-Y-specific naive T cell precursors. These cells were transferred into C57BL/6 naive female mice that were subsequently immunized intravenously with male syngeneic bone marrow cells. We have chosen this model for two reasons. First, the H-Y antigen has been described as a nonpersistent and noncross-reactive antigen (30). And second, fixing only the TCR $\beta$  chain combines the advantages of making the TCR $\alpha$  chain a signature of each T cell clone and of restricting the repertoire of  $D^b$ -Smcy3-specific CD8 T cells (31), which permits a global study of the memory TCR $\alpha$  repertoire.

We report here on the analysis of the TCR repertoires of both subsets in lymphoid organs as well as peripheral tissues, their evolution in steady-state conditions or after a secondary challenge, and the fate of the transferred purified  $T_{CM}$  or  $T_{EM}$  populations after this challenge.

## RESULTS

### In vivo generation of T CD8 memory subsets after intravenous immunization

First, we verified that we could generate measurable numbers of memory T cells of both subsets in our model. Naive C57BL/6 female mice received  $10^5$  CD8 naive T cells from a female mouse transgenic for the  $\beta$  chain of TCR H-Y, corresponding to  $\sim 3 \times 10^4$  CD8 T cells specific for the H2- $D^b$ -restricted Smcy3<sub>738-746</sub> peptide (H-Y antigen; reference 31). The transferred mice were immunized intravenously with  $5 \times 10^6$  male syngeneic bone marrow cells (32), and we used H2- $D^b$ -Smcy3 MHC tetramers to measure the immune response in vivo. In peripheral blood, we observed a classical T CD8 response beginning with an expansion of specific CD8 cells, followed by a contraction phase prolonged by a plateau that was maintained throughout the memory phase (Fig. 1 A). Using CD45.1<sup>+</sup> C57BL/6 mice as hosts, we showed that the vast majority of  $D^b$ -Smcy3-specific cells generated derived from the transferred  $\beta$ -tg CD45.2<sup>+</sup> CD8 cells (see Fig. 4 C). CD62L expression by  $D^b$ -Smcy3-specific PBLs cells was first down-regulated during the expansion phase, almost all specific CD8 effector cells being CD62L<sup>-</sup> at the peak of the response. Next, the proportion of CD62L<sup>+</sup> cells steadily increased to reach approximately half of the CD8  $D^b$ -Smcy3-specific population after 6 wk and slowly continued to increase later on (Fig. 1 B). At 6 wk, we analyzed the presence of  $D^b$ -Smcy3-specific cells in lymphoid (blood, spleen, PLN, MLN) and peripheral (liver, gut lamina propria) organs and their expression of the CD62L molecule.  $D^b$ -Smcy3-specific cells were present in all organs analyzed (Fig. 1 C), and their percentage was higher in peripheral organs such as liver or lamina propria. We observed two subsets defined by their expression level of CD62L (low/high) in all organs except lamina propria in



**Figure 1.** In vivo generation of T CD8 memory subsets after intravenous immunization with male bone marrow cells of a C57BL/6 female host transferred with naive  $\beta$ -tg CD8 cells. (A) Analysis of the percentage of D<sup>b</sup>-Smcy3<sup>+</sup> cells among CD8 PBLs for a naive C57BL/6 mouse (open circles) or immunized hosts (closed squares). Data from 12 mice were pooled. (B) Evolution of the percentage of CD62L<sup>+</sup> cells among CD8<sup>+</sup> D<sup>b</sup>-Smcy3<sup>+</sup> cells

which virtually all cells displayed a CD62L<sup>-</sup> phenotype. Thus, our murine model was able to generate two subsets of CD44<sup>hi</sup> D<sup>b</sup>-Smcy3-specific CD8 memory cells defined by their expression level of the CD62L molecule corresponding to T<sub>CM</sub> (CD62L<sup>+</sup>) and T<sub>EM</sub> (CD62L<sup>-</sup>), allowing us to study their TCR repertoires and their recall response capacities.

### Repertoires of CD8 T<sub>CM</sub> and T<sub>EM</sub> in lymphoid and nonlymphoid organs are partly distinct

We sorted the D<sup>b</sup>-Smcy3-specific T<sub>CM</sub> and T<sub>EM</sub> subsets from lymphoid (spleen, PLN, MLN) and peripheral (LP) organs from C57BL/6 mice transferred with naive  $\beta$ -tg CD8 cells and immunized at least 6 wk before, and analyzed their TCR $\alpha$  repertoires. Having previously shown that most D<sup>b</sup>-Smcy3-specific cells express a TCR $\alpha$  chain using the TCRAV9 segment (31), we focused our analysis on TCRAV9-TCRAC rearrangements, which were sequenced extensively for splenic T<sub>CM</sub> and T<sub>EM</sub> subsets sorted from several individual mice. Both D<sup>b</sup>-Smcy3-specific subsets displayed a restricted repertoire comprised of  $\leq 200$  different clones. Moreover, the different clones had a restricted usage of TCRAJ segments (Fig. S1 B, available at <http://www.jem.org/cgi/content/full/jem.20040876/DC1>) and displayed heterogeneous sizes, clone frequencies ranging from  $10^{-3}$  to  $10^{-1}$  (Fig. S1 C).

Although it was difficult to identify every clone represented within a particular sample, we could estimate the rep-

ertoire diversity and overlap of both subsets using statistical tools (see Materials and methods). Altogether, the TCR $\alpha$  repertoires of T<sub>CM</sub> subsets were more diverse than those of T<sub>EM</sub>, but only by a 1.5 factor (115 and 175 clones on average, respectively; Fig. S1 D). The low diversity observed implied at least two consequences. First, because the number of specific sorted cells analyzed by sequencing ranged between 6,000 and 27,000 cells, this sampling was large enough to study a  $>30$ -fold lower clonal diversity. Second, because we transferred  $\sim 10^5$  transgenic cells per mouse,  $\sim 30\%$  of which were specific for the Smcy3 peptide, the presence of CD8<sup>+</sup> memory T cells with the same TCR in both subsets might occur even if these cells were not derived from the same stimulated naive T cell.

To measure this probability of artifactually shared clones between the two subsets, we compared the repertoire overlap of T<sub>CM</sub> and T<sub>EM</sub> subsets from the same mouse (intra-individual overlap) or from two distinct mice (interindividual overlap), using the observed percentages of shared clones and the statistical overlap index (Table I and Fig. S1 E). The interindividual percentages of shared clones were important for both subsets ( $34.0 \pm 7.9\%$  for T<sub>EM</sub> and  $24.5 \pm 3.6\%$  for T<sub>CM</sub>), as well as the overlap index ( $0.38 \pm 0.03$ ), showing that a substantial sharing of clones between T<sub>CM</sub> and T<sub>EM</sub> subsets could be attributed to the restricted diversity of the D<sup>b</sup>-Smcy3-specific memory repertoire. However, intraindi-

**Table I.**  $T_{CM}$  and  $T_{EM}$  clone sharing

	Interindividual mean	Intraindividual mean
Overlap index	$0.38 \pm 0.03$	$0.56 \pm 0.03$
	<i>% shared clones</i>	<i>% shared clones</i>
CD62L <sup>+</sup>	$24.5 \pm 3.6$	$38.6 \pm 4.7$
CD62L <sup>-</sup>	$34.0 \pm 7.9$	$55.4 \pm 7.4$
Total	$16.5 \pm 3.1$	$29.3 \pm 3.9$

Mean interindividual and intraindividual overlap indexes and percentages of shared clones for  $T_{CM}$ ,  $T_{EM}$ , and total clones are displayed.

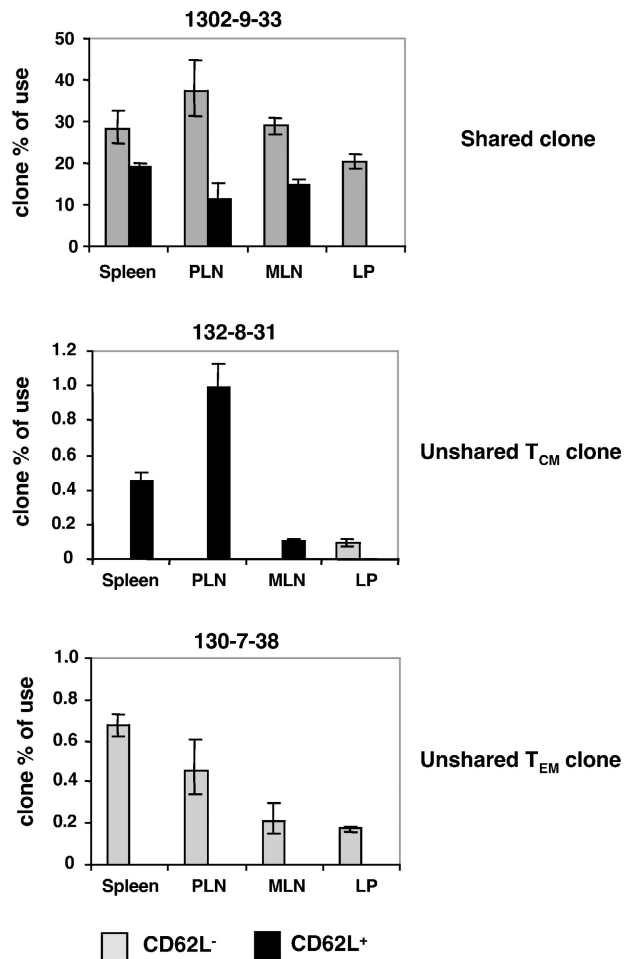
vidual percentages of shared clones were significantly higher ( $55.4 \pm 7.4\%$  for  $T_{EM}$  and  $38.6 \pm 4.7\%$  for  $T_{CM}$ ), as was the mean intraindividual overlap index ( $0.56 \pm 0.03$ ). This indicates that a significant proportion of the shared clones derived from common naive precursor T cells. Taking into account the baseline percentage of sharing due to the restricted diversity of the memory repertoire and the maximal percentage of sharing observable with our technology, we estimated that two out of three clones were shared between the  $T_{CM}$  and  $T_{EM}$  subsets (Fig. S1 E). Interestingly, most of the well-represented clones were shared between the  $T_{CM}$  and  $T_{EM}$  subsets (Fig. S1 C).

To confirm that the presence of unshared clones in the subset repertoire was not due to an insufficient accumulation of sequences, nor to sequestering of some clones within particular lymphoid or peripheral organs, and to have a broader idea of the repartition of clones in the body, we measured by quantitative clonotypic PCR the frequency of individual clones in  $T_{CM}$  and  $T_{EM}$  subsets sorted from the spleen, PLN, MLN, and LP from two individual mice transferred and immunized as described before (Fig. 2 and Fig. S2 B, available at <http://www.jem.org/cgi/content/full/jem.20040876/DC1>). Out of eight clones detected by sequencing, only in the  $T_{CM}$  or  $T_{EM}$  subset, we confirmed that six of them were readily detected in one subset only. In addition, this observation could be extended to other organs; when a clone was present in one subset of the spleen only, it was either undetected in other lymphoid organs, or remained within the same subset in these organs.

Altogether, 6 wk after immunization, approximately two thirds of total memory T cell clones were shared between both subsets, so that  $T_{CM}$  and  $T_{EM}$  repertoires could be considered as only partly distinct. Clonotypic analyses confirmed the presence of unshared clones and showed no sequestration of clones in particular organs, suggesting that the situation observed in the spleen was representative of the whole body.

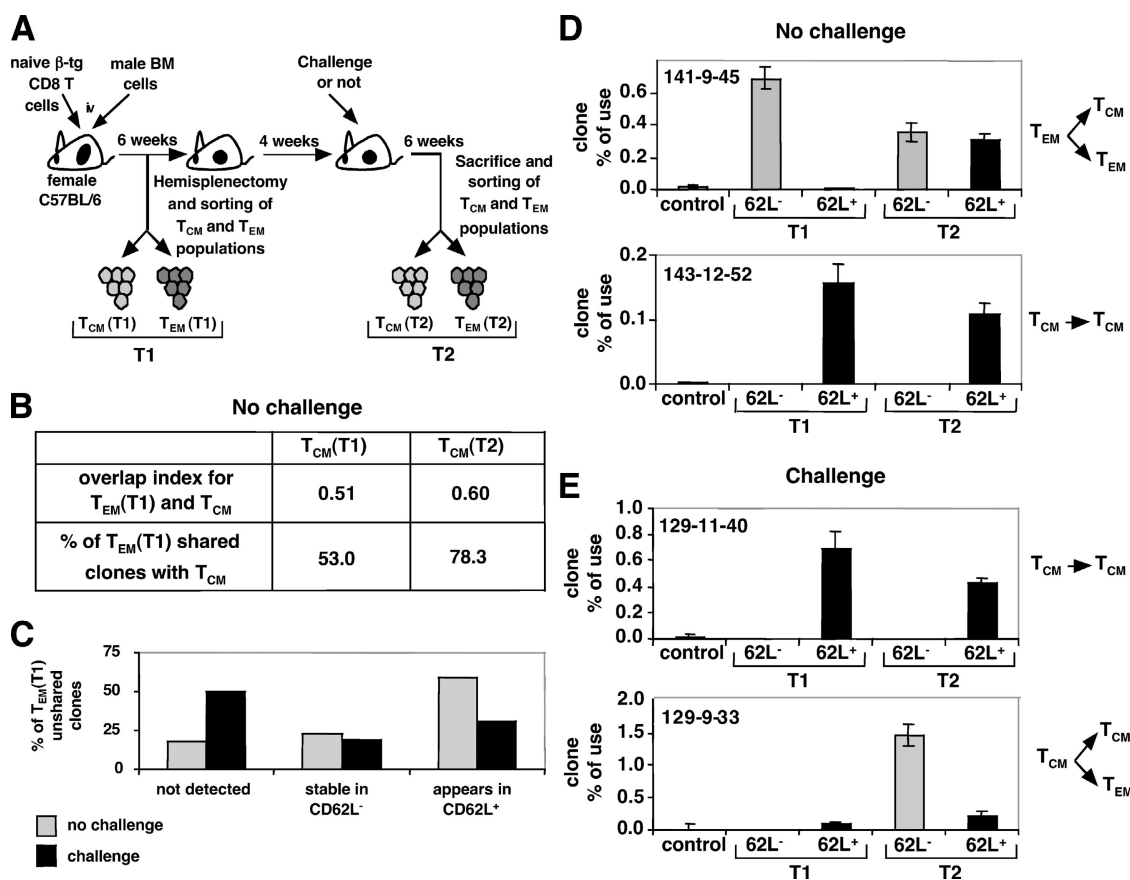
#### Evolution of $T_{CM}$ and $T_{EM}$ TCR $\alpha$ repertoires in physiological conditions or after restimulation

This repertoire analysis was performed at a given time point. It has been proposed that  $T_{EM}$  can generate  $T_{CM}$  (9) and vice versa (17, 18). Therefore, we analyzed the stability of both populations in the same mouse, with or without challenge. For this purpose, we immunized mice after transfer of transgenic naive T cells as described before, and performed



**Figure 2.** Frequency of individual clones in lymphoid and peripheral organs of an immune mouse. The frequency of each clone among TCRAV9<sup>+</sup> cells was measured by quantitative clonotypic PCR within each subset. An example of each type of clone (shared, unshared  $T_{CM}$ , unshared  $T_{EM}$ ) is displayed. The frequency observed in the LP CD62L<sup>-</sup> subset for clone 132-8-31 corresponds to several cells <10 and, therefore, is considered as nonspecific amplification.

hemisplenectomy 6 wk after immunization (T1), the other spleen half being recovered 10 wk later (T2), with or without a secondary intravenous immunization 4 wk after hemisplenectomy (Fig. 3 A). Thus, we could perform repertoire analyses of both subsets on sorted specific splenocytes at two time points distant of 10 wk. We extensively sequenced TCRAV9-TCRAC rearrangements and analyzed the percentages of shared clones and the overlap indexes between each mouse (four samples) with the aim of assessing possible passages of clones between T1 and T2 from one subset to the other. The only significant modification was observed in the nonimmunized mouse (Fig. 3 B). In that case, the percentage of shared clones between the  $T_{EM}$  (T1) and  $T_{CM}$  (T2) subsets was significantly higher than that between  $T_{EM}$  (T1) and  $T_{CM}$  (T1) subsets (78.3 vs. 53.0%). Accordingly, the overlap index also increased (from 0.51 to 0.60). This evolution strongly suggested that a sizable fraction of  $T_{EM}$  (T1) clones had reexpressed the CD62L molecule between



**Figure 3. TCR $\alpha$  repertoire analysis of splenic D<sup>b</sup>-Smcy3-specific T<sub>CM</sub> and T<sub>EM</sub> subsets at two time points (T1 and T2), with or without a challenge in between.** TCRAV9-TCRAC rearrangements were sequenced and analyzed. (A) Illustration of the experimental protocol. (B) In the non-challenged mouse, evolution of the overlap index and of the percentage of

T1 and T2, also consistent with the observed increase of the estimated diversity in the T<sub>CM</sub> subset from T1 to T2 (from 150 to 201 estimated clones), and with an increase of the percentage of CD62L<sup>+</sup> cells among D<sup>b</sup>-Smcy3-specific sorted cells (37% at T1 vs. 78% at T2). Interestingly, not all cells of these T<sub>EM</sub> clones had apparently converted to a CD62L<sup>+</sup> phenotype because the estimated diversity of the T<sub>EM</sub> (T1) and T<sub>EM</sub> (T2) repertoires remained close (111 and 121, respectively).

To further analyze the possibility of T<sub>EM</sub> converting to a CD62L<sup>+</sup> phenotype, we studied the fate of the clones found at T1 in the T<sub>EM</sub> subset only (Fig. 3 C). In the absence of a second challenge, most T<sub>EM</sub> (T1) unshared clones (82%) were readily detected at T2, and 59% of these T<sub>EM</sub> (T1) unshared clones appeared in the T<sub>CM</sub> subset, confirming that cells from a substantial fraction of T<sub>EM</sub> (T1) clones had converted to a CD62L<sup>+</sup> phenotype in the 10-wk interval, although most of them were still present in the T<sub>EM</sub> (T2) subset. These observations were further confirmed by quantitative PCR clonotypic analyses. We measured the frequency of five clones present in the T<sub>EM</sub> subset at T1 (Fig. S2 C, top). Two of them were only detected in the T<sub>EM</sub> subset at T1 and both

shared clones between T<sub>EM</sub> (T1) and T<sub>CM</sub> at T1 and T2. (C) Evolution of T<sub>EM</sub> (T1) unshared clones at T2 in both conditions. (D and E) Evolution of the frequency of T1 unshared clones (D) in the absence of restimulation or (E) in the presence of an intravenous challenge. Frequencies were determined as in Fig. 2.

converted, at least partly, to a CD62L<sup>+</sup> phenotype in the absence of restimulation (Fig. 3 D, T<sub>EM</sub>→T<sub>CM</sub> and T<sub>EM</sub>). The other three clones were already shared by both subsets at T1, and two of them displayed a decrease of their frequency in the T<sub>EM</sub> subset at T2, though without a corresponding increase of their T<sub>CM</sub> frequency.

We also analyzed the fate of T<sub>EM</sub> (T1) unshared clones in the reimmunized mouse. In contrast with what was observed in the absence of restimulation, 50% of the unshared T<sub>EM</sub> (T1) clones were no longer detected at T2, neither in the T<sub>EM</sub> nor in the T<sub>CM</sub> subset, suggesting that a sizable fraction of T<sub>EM</sub> (T1) clones had either migrated into peripheral tissues or died.

Likewise, we investigated the possibility that some T<sub>CM</sub> clones could acquire a T<sub>EM</sub> phenotype over time. We analyzed the variation of the percentage of shared clones between T<sub>CM</sub> (T1) and T<sub>EM</sub> clones. No significant difference could be observed in both conditions (unpublished data), which could receive two explanations: either T<sub>CM</sub> clones were very stable and did not convert to T<sub>EM</sub>, or the T<sub>CM</sub> clones that converted to T<sub>EM</sub> were already shared at T1. To further analyze this point, we measured in both conditions

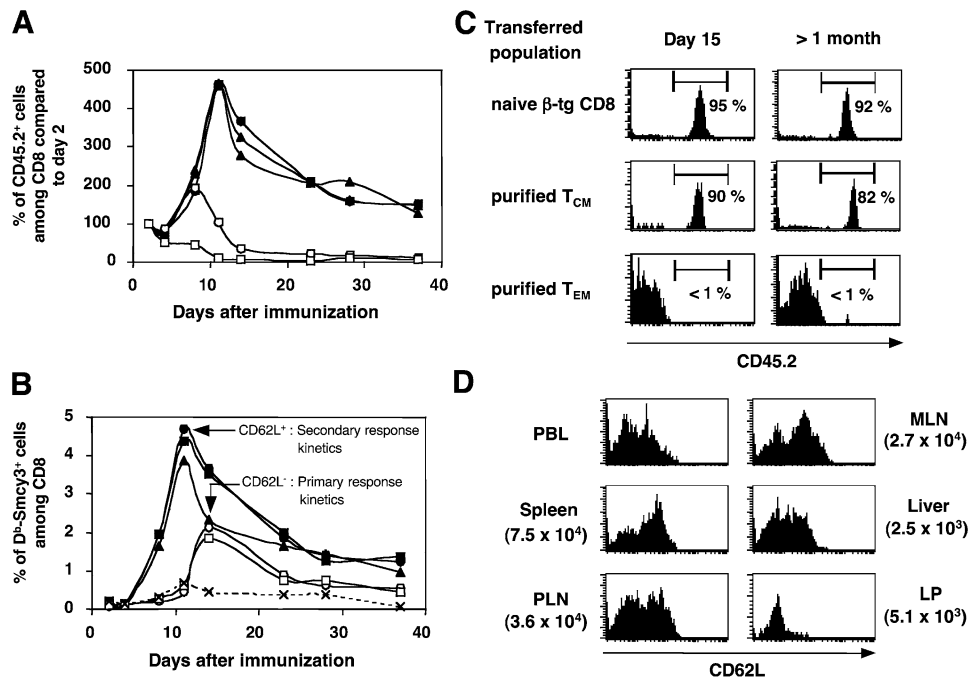
the frequency of several unshared  $T_{CM}$  (T1) clones in both subsets at T1 and T2 (Fig. S2 C). In the absence of restimulation, out of six analyzed clones, five were stable and only found in the  $T_{CM}$  subset at T2 (the last one was undetectable at T2 in both  $T_{CM}$  and  $T_{EM}$  subsets), which shows that  $T_{CM}$  clones were quite stable in the absence of restimulation (Fig. 3 D,  $T_{CM} \rightarrow T_{CM}$ ). In the reimmunized mouse, out of five  $T_{CM}$  (T1) unshared clones, four were stable in the  $T_{CM}$  subset at T2 (Fig. 3 E,  $T_{CM} \rightarrow T_{CM}$ ), whereas the last one gave rise to  $T_{EM}$  at T2 (Fig. 3 E,  $T_{CM} \rightarrow T_{CM}$  and  $T_{EM}$ ), indicating that the antigenic stimulation mainly drove already shared  $T_{CM}$  clones to differentiate into  $T_{EM}$  clones, and that few new clones appeared in the  $T_{EM}$  subset upon restimulation.

This protocol using hemisplenectomies to analyze antigen-specific  $T_{CM}$  and  $T_{EM}$  repertoires at two time points in the same mouse in the presence or absence of a secondary challenge thus enabled us to point out several features of the fate of  $T_{CM}$  and  $T_{EM}$  in vivo. First, in the absence of antigen, whereas  $T_{CM}$  clones were quite stable, cells of  $\sim 25\%$  of the observed  $T_{EM}$  clones converted to a  $CD62L^+$  phenotype. Second, in the case of restimulation,  $T_{CM}$  and  $T_{EM}$  subsets had a drastically different behavior; whereas  $T_{CM}$  clones either remained stable or gave rise to  $T_{EM}$ , a majority of  $T_{EM}$  unshared clones at T1 disappeared.

**$T_{CM}$ , but not  $T_{EM}$ , can mount a recall immune response after an i.v. challenge**

This last observation raised the question of the memory capacity of  $T_{EM}$ . To refine our analysis of the properties of  $T_{CM}$  and  $T_{EM}$  subsets, we examined their behavior after an adoptive transfer in a new host, immediately followed by a secondary intravenous immunization. At least 6 wk after the primary immunization, we purified  $CD62L^+$  and  $CD62L^-$  cells from the spleen and PLN of C57BL/6 hosts (Fig. S1 A). Each of these populations containing  $3 \times 10^4$   $D^b$ -Smcy3-specific  $T_{CM}$  or  $T_{EM}$  was transferred into a new female C57BL/6 host expressing the CD45.1 allele. These hosts were immunized with  $5 \times 10^6$  bone marrow cells from male C57BL/6 CD45.1 mice and the  $D^b$ -Smcy3-specific response was followed by flow cytometry in blood.

When  $T_{CM}$  were transferred, a rapid increase of the percentage of the  $CD45.2^+$  transferred cells was observed in blood (Fig. 4 A), correlated with the increase of the percentage of  $CD8^+$   $D^b$ -Smcy3-specific cells (Fig. 4 B). This  $D^b$ -Smcy3-specific response was more rapid than that observed in hosts after primary immunization of naive  $\beta$ -tg CD8 cells, and was characteristic of a recall response. On the contrary, when  $T_{EM}$  were transferred, after a possible initial increase, the percentage of  $CD45.2^+$  transferred cells among host



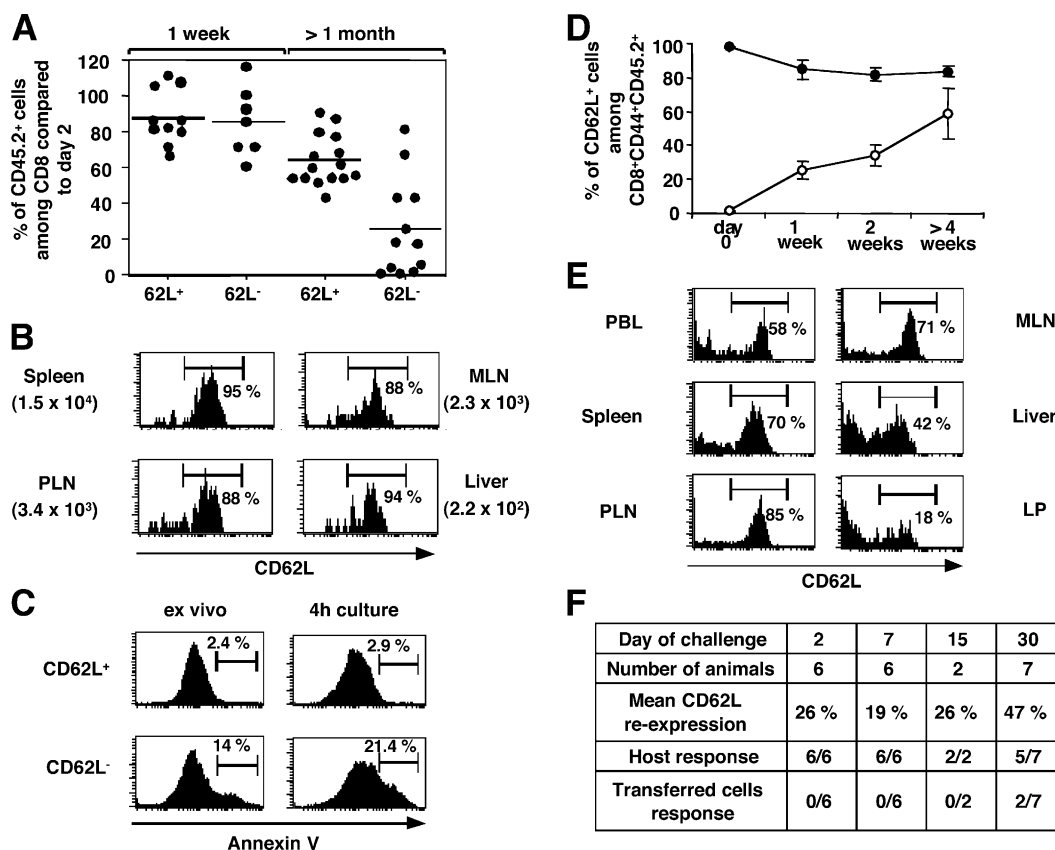
**Figure 4.  $T_{CM}$  but not  $T_{EM}$  can mount a recall response after transfer and challenge.**  $CD62L^+$  and  $CD62L^-$  cells were purified from the spleen and PLN of hosts transferred with naive  $\beta$ -tg CD8 cells and immunized at least 6 wk earlier (see Materials and methods) and equal numbers of  $D^b$ -Smcy3-specific  $T_{CM}$  (closed symbols) or  $T_{EM}$  (open symbols) were transferred into naive  $CD45.1^+$  female hosts that were immediately immunized with syngeneic  $CD45.1^+$  male bone marrow cells. (A) Evolution of the percentage of  $CD45.2^+$  transferred cells among host blood CD8 cells. The percentage observed at day 2 is normalized as 100%, and all subsequent

percentages are expressed as a percentage of this standard. (B) Analysis of the percentage of  $D^b$ -Smcy3 $^+$  cells among blood CD8 cells. (C) Expression of the CD45.2 molecule by  $CD8^+$   $D^b$ -Smcy3 $^+$  blood cells at day 15 or  $>1$  mo after challenge, after transfer of naive,  $T_{CM}$ , or  $T_{EM}$ . (D)  $CD62L$  expression profiles and absolute numbers (in parentheses) of  $CD8^+$   $D^b$ -Smcy3 $^+$  cells in organs of a  $CD45.1^+$  female host transferred with  $T_{CM}$  and challenged at least 6 wk before analysis. All results shown are representative of at least three experiments.

CD8<sup>+</sup> cells dramatically decreased until these cells became barely detectable (Fig. 4 A), in agreement with our conclusion on the spleen repertoire study. However, a D<sup>b</sup>-Smcy3 response did occur in blood (Fig. 4 B), but it was due to the CD45.1<sup>+</sup> naive cells of the host (Fig. 4 C). These results were further extended to all organs analyzed after 6 wk. In particular, the CD45.2<sup>+</sup> CD8<sup>+</sup> cells were barely detectable in all organs analyzed including liver and gut lamina propria when CD62L<sup>-</sup> cells had been transferred (unpublished data), indicating that the decrease in the percentage of CD45.2<sup>+</sup> cells observed in the blood was not due to their migration to peripheral tissues, but most likely to their death.

The sequencing and clonotypic PCR studies performed in the spleen at two time points with a secondary immunization in between indicated that only a fraction of the T<sub>CM</sub> clones could give rise to T<sub>EM</sub> clones after a secondary re-

sponse. However, this fraction was difficult to determine from the hemisplenectomy experiments. Therefore, we analyzed the CD62L phenotype of the D<sup>b</sup>-Smcy3-specific memory cells generated upon transfer with, and subsequent challenge of, T<sub>CM</sub>. As shown in Fig. 4 D, the T<sub>CM</sub> transferred cells expanded and gave rise to both T<sub>CM</sub> and T<sub>EM</sub> in the different organs analyzed. To determine the actual proportion of the transferred clones that generated the T<sub>EM</sub>, we sorted the D<sup>b</sup>-Smcy3-specific T<sub>CM</sub> and T<sub>EM</sub> splenic subsets of two individual C57BL/6 CD45.1 hosts after transfer and challenge, and analyzed their TCRV9 repertoire. Once again, the T<sub>CM</sub> subset repertoire was more diverse. Moreover, a majority of T<sub>EM</sub> clones ( $56 \pm 3\%$ ) had a counterpart in the T<sub>CM</sub> subset, suggesting that most T<sub>CM</sub> clones that gave rise to T<sub>EM</sub> also remained within the T<sub>CM</sub> subset. However, only  $47 \pm 7\%$  of the T<sub>CM</sub> clones were also detected within



**Figure 5. Adoptive transfer into naive hosts of CD62L<sup>+/-</sup> subsets purified from immune mice: overall T<sub>CM</sub> stability versus T<sub>EM</sub> death and partial CD62L reexpression.** (A, B, and D) T<sub>CM</sub> and T<sub>EM</sub> were purified from spleen and PLN of immune hosts and transferred into naive female CD45.1 hosts. (A) Analysis of the persistence of CD8<sup>+</sup>CD62L<sup>+/-</sup> transferred cells 1 wk or >1 mo after transfer. The percentage of the CD45.2<sup>+</sup> transferred cells among CD8 compared with day 2 is displayed. Each circle represents an individual transferred host. The mean value is shown for each transferred population. (B) CD62L expression and absolute numbers (in parentheses) of CD8<sup>+</sup> D<sup>b</sup>-Smcy3<sup>+</sup>CD45.2<sup>+</sup> cells 6 wk after transfer of purified CD62L<sup>+</sup> cells. (C) Analysis of the propensity of T<sub>CM</sub> and T<sub>EM</sub> to undergo apoptosis. CD62L<sup>+/-</sup> subsets were purified from the spleen and PLN cells from an old C57BL/6 mouse, and the expression of annexin V by

CD8<sup>+</sup>CD44<sup>+</sup> cells was analyzed either directly ex vivo or after a 4-h stimulation of both subsets with PMA and ionomycin. (D) Kinetic analysis of the percentage of CD62L<sup>+</sup> cells among CD8<sup>+</sup>CD44<sup>+</sup>CD45.2<sup>+</sup> cells in blood after transfer of CD45.2<sup>+</sup>CD62L<sup>+</sup> (closed circles) or CD45.2<sup>+</sup>CD62L<sup>-</sup> (open circles) cells in a naive CD45.1 host. (E) CD62L expression profiles of CD8<sup>+</sup>CD45.2<sup>+</sup> remaining cells in various organs of a CD45.1 female host 6 wk after transfer of CD62L<sup>-</sup> spleen and PLN cells sorted from an immune donor. (F) Naive CD45.1 female mice were transferred with CD45.2<sup>+</sup>CD62L<sup>-</sup> cells, allowed to rest for various durations, and challenged with  $5 \times 10^6$  CD45.1<sup>+</sup> male bone marrow cells. Indicated for each duration are the number of mice analyzed, the percentage of transferred CD8 cells expressing CD62L, and the number of individual mice in which a host or a donor response was observed, respectively.

the  $T_{EM}$  subset, demonstrating that less than half of  $T_{CM}$  clones gave rise to  $T_{EM}$  clones in case of restimulation (unpublished data).

These transfer experiments of purified  $CD62L^+$  or  $CD62L^-$  memory cells showed that  $T_{CM}$  and  $T_{EM}$  had drastically different behaviors in response to a secondary intravenous immunization; whereas  $T_{CM}$  mounted a secondary response giving rise to an heterogeneous memory pool comprised of both  $T_{CM}$  and  $T_{EM}$ ,  $T_{EM}$  did not expand significantly and were even less efficient than naive  $\beta$ -tg CD8 cells to mount a proliferative  $D^b$ -Smcy3-specific response.

### In the absence of antigen, a proportion of $T_{EM}$ apparently convert to $T_{CM}$ but remain generally unable to mount a secondary response

The results of our splenocyte repertoire studies showed that, in the absence of antigenic restimulation, a fraction of  $T_{EM}$  converted to a  $T_{CM}$  phenotype. Here, we wanted to determine whether this conversion would be restricted to the mere expression of  $CD62L$ , or would permit the converted cells to regain the full capacity to respond as true  $T_{CM}$ . We used the same transfer model as for the analysis of  $T_{CM}$  and  $T_{EM}$  recall capacities and monitored the evolution of the percentage of the transferred population among  $CD8^+$  cells and its  $CD62L$  phenotype in blood in the absence of restimulation.  $CD62L^+$  cells persisted after transfer, with only a slow decrease in the percentage of  $CD45.2^+$  cells among  $CD8$  PBL cells with time (Fig. 5 A). Transferred  $CD8^+$   $CD44^+$  cells, as well as  $D^b$ -Smcy3-specific cells, remained in great majority  $CD62L^+$  in all organs analyzed (Fig. 5 B and not depicted).

When  $CD62L^-$  spleen and PLN cells were transferred, we observed a progressive decrease in the percentage of  $CD45.2^+$  transferred cells among  $CD8$  PBLs with time, which often became undetectable after 3 or 4 wk (Fig. 5 A). This result and the fact that the transferred  $T_{EM}$  were unable to mount a recall response and disappeared within a few days from reimmunized hosts suggested that they could have an increased susceptibility to apoptosis. Thus, we analyzed the propensity of purified  $CD62L^{+/-}$  cells to undergo apoptosis after in vitro stimulation. As shown in Fig. 5 C, the percentage of annexin  $V^+$  cells among  $CD8^+$   $CD44^+$  cells was already much higher in the  $CD62L^-$  than in the  $CD62L^+$  population ex vivo. Moreover, contrary to  $CD62L^+$   $CD8^+$   $CD44^+$  cells, the percentage of apoptotic annexin  $V^+$  cells in the  $CD62L^-$  population increased significantly after a 4-h stimulation, suggesting that the  $T_{EM}$  subset comprised a subpopulation of memory cells that had an increased susceptibility to apoptosis in comparison to  $T_{CM}$ .

The expression of the  $CD62L$  molecule by the transferred  $CD8$  cells was altered with time. In blood, we observed a progressive reexpression of the  $CD62L$  molecule (Fig. 5 D). In some cases, when the transferred  $CD45.2^+$  cells persisted at detectable frequencies, we could analyze the evolution of the  $CD62L$  phenotype in organs 6 wk after transfer. Due to the already low percentage of transferred

cells, we performed our analysis on the total  $CD8^+$  transferred  $T_{EM}$  population. The  $CD8^+$   $CD45.2^+$   $CD62L$  profiles obtained in various organs showed that a substantial fraction of the persisting transferred  $CD8$  cells were reexpressing  $CD62L$  (Fig. 5 E). Moreover, although the percentage of transferred  $CD8$  cells in PLN was  $1.61 \pm 0.7$  and  $2.11 \pm 0.7$  times lower than in spleen and liver, respectively, some converted  $CD62L^+$  transferred cells even gained access to these lymphoid organs. This suggested that, in the absence of antigen, although a majority of  $T_{EM}$  had died after a few weeks, a fraction of them was able to progressively convert to  $T_{CM}$ .

Next, we analyzed the capacity of the  $CD62L^+$  converted cells to mount an anamnestic response. We transferred the purified spleen and PLN  $CD45.2^+$   $CD62L^{+/-}$  populations into  $CD45.1^+$  female hosts and intravenously reimmunized these mice 2, 7, 15, or 30 d after transfer with male  $CD45.1^+$  bone marrow cells. We monitored the anti- $D^b$ -Smcy3-specific response in PBLs (Fig. 5 F). For mice transferred with  $CD62L^-$  cells 2, 7, or 15 d before immunization, although respectively  $26 \pm 7\%$ ,  $19 \pm 7\%$ , and  $26 \pm 7\%$  of the transferred cells had converted to a  $CD62L^+$  phenotype, the specific response observed after immunization was always due to host naive cells. In some cases, we did see a short and abortive proliferation of the transferred  $CD8^+$   $CD45.2^+$  cells. In five out of seven hosts transferred 30 d before immunization, the transferred cells had reached an undetectable frequency on day 30 and a host-specific response was observed after immunization. However, in the two others, the transferred cells were still fully detectable at day 30, although the percentage of  $CD62L^+$  converted cells among the  $CD8^+$   $CD45.2^+$  cells was drastically different in the two mice (35 vs. 91%). In both cases, a  $CD45.2^+$   $D^b$ -Smcy3-specific memory response was observed without a substantial host response. These two mice were from distinct experiments in which, although several other mice had been transferred with the same batch of purified  $T_{EM}$ , no other donor response was observed.

Altogether, in contrast with  $T_{CM}$  that can persist in the absence of antigenic restimulation, most of the  $T_{EM}$  were susceptible to apoptosis, and the small fraction of cells that could reexpress  $CD62L^+$  were generally unable to mount a secondary response.

## DISCUSSION

### $T_{CM}$ and $T_{EM}$ lineage relationships and homeostasis

The recent recognition that the  $CD8^+$  T lymphocyte memory pool is heterogeneous raises the fundamental issue of lineage relationship of  $T_{CM}$  and  $T_{EM}$  subsets, for which several models have been proposed. In the model of independent differentiation of  $T_{CM}$  and  $T_{EM}$  subsets, no relationship is assumed between the two subsets;  $T_{CM}$  and  $T_{EM}$  could arise independently, on a stochastic basis, based on precommitment of the individual naive T cell, or depending on its stimulation conditions. In the present work, the  $T_{CM}$  and  $T_{EM}$  repertoires analyzed 6 wk after immunization were partly distinct, some clones being found in one subset only



in all organs studied. This observation could be interpreted as the result of an independent differentiation occurring in particular locations or in response to some stimulation conditions. The TCR affinity is probably not a discriminating factor for this matter, as we observed no difference in TCR affinity between  $T_{CM}$  and  $T_{EM}$  clones based on their TCR $\alpha$  sequences (31). All stimulated cells appeared to display TCR $\alpha$  sequences considered as high affinity (unpublished data), and identical clones could be unshared  $T_{CM}$  clones in one host and unshared  $T_{EM}$  clones in another one. Among other factors, particular cytokine environments have been shown to favor the differentiation of  $T_{CM}$  or  $T_{EM}$  in vitro (8, 12) and in vivo (33), and the stimulation of naive CD8 $^{+}$  T cells in the absence of CD4 help resulted in memory cells with a  $T_{EM}$  phenotype (34), indicating that particular stimulation conditions could preferentially induce the differentiation of  $T_{CM}$  or  $T_{EM}$ .

Our results clearly demonstrate that, during a systemic immunization, approximately two thirds of the stimulated naive clones can give rise to both  $T_{CM}$  and  $T_{EM}$  that are able to coexist, at least transiently, in peripheral and lymphoid organs, which argues against an exclusive model of independent differentiation pathways for  $T_{CM}$  and  $T_{EM}$ . This sharing percentage is much higher than that we had reported in a previous paper on total PBL CD8 $^{+}$   $T_{CM}$  and  $T_{EM}$  human subsets, although it was computed in a different way (21). This variation can now be explained by the fact that we had mainly analyzed whole human repertoires, irrespective of the antigenic specificity, therefore a long time after memory has probably been established for most antigens, and we know from this and another paper that many  $T_{EM}$  are able to regain the CD62L expression with time (9). Also, subsequent immunizations with other antigens could differentially alter  $T_{CM}$  and  $T_{EM}$  repertoires (35).

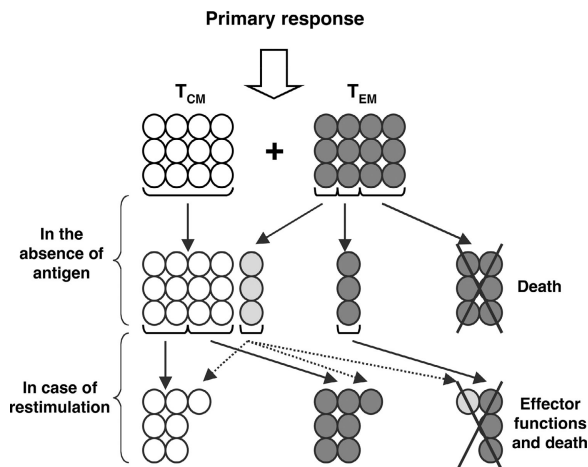
In the frequent case where a naive T cell generates both  $T_{EM}$  and  $T_{CM}$ , three models can be proposed: the two cell types could arise in parallel or sequentially,  $T_{EM}$  giving rise to  $T_{CM}$  (9) or vice versa (17, 18). We were unable to detect any significant production of  $T_{EM}$  from  $T_{CM}$  clones in the absence of antigenic challenge, neither after hemisplenectomy nor using adoptive transfer. Indeed, in both approaches, the  $T_{CM}$  subset was very stable, consistent with the known persistence of memory T cells in lymphoid organs (3). In the hemisplenectomy experiment, although we cannot rule out that the shared  $T_{CM}$  clones could produce  $T_{EM}$ , the presence of unshared  $T_{CM}$  clones indicates that at least some  $T_{CM}$  clones did not produce  $T_{EM}$ . In addition, when we transferred purified  $T_{CM}$  into naive recipients, we were unable to detect any significant number of  $T_{EM}$  in the absence of restimulation. This result challenges in vitro studies showing conversions of some  $T_{CM}$  clones to  $T_{EM}$  in the presence of homeostatic IL-15 and IL-7 cytokines (17, 18). There are two main possible explanations: first, in these studies, inflammatory DC-derived cytokines that could mimic inflammation mediated by a heterologous infection were added in most cases. Heterologous immunity has been shown to affect memory homeostasis,

with consequences such as attrition and/or expansion of cross-reactive T cells (35). Therefore, these conditions might drive the differentiation of  $T_{CM}$  clones into  $T_{EM}$ , as was the case here when  $T_{CM}$  were challenged with male cells. Similarly, we also observed some phenotype conversion of non-D $^{b}$ -Smcy3-specific CD8 cells in mice transferred with CD62L $^{+}$  cells and reimmunized with male cells, but it might also be accounted for by other H-Y epitopes' ongoing responses (32). Alternatively, this phenomenon of  $T_{CM}$  conversion to  $T_{EM}$  in the absence of antigen could be a much slower process in vivo compared with in vitro and we might have observed it at later time point.

Strikingly, in the absence of challenge, cells from  $T_{EM}$  clones were shown to reexpress CD62L over a 10-wk period, consistent with a recent paper (9). Although a high proportion of  $T_{EM}$  clones were implicated in this process, all cells of these clones did not reexpress CD62L in a synchronized manner, and most  $T_{EM}$  clones found 6 wk after immunization were still present in the  $T_{EM}$  subset 10 wk later. This is consistent with other papers showing an overall stability of the  $T_{EM}$  subset in humans (6, 13, 15, 21), although most of these studies were performed either on the total memory population, independently of antigen specificity, or in chronically infected patients. Moreover, despite the persistence of  $T_{EM}$  clonotypes over a 10-wk period in the nonreimmunized mouse, the percentage of CD62L $^{+}$  cells among total D $^{b}$ -Smcy3-specific sorted cells significantly increased from T1 to T2 in this mouse, showing that the number of  $T_{EM}$  globally decreased in the absence of restimulation. This may in part stem from the reexpression of CD62L by  $T_{EM}$  but certainly also results from the general poor survival capacity of  $T_{EM}$  that was observed after their adoptive transfer into naive recipients.

Indeed, although our findings concerning  $T_{EM}$  phenotypic conversion to  $T_{CM}$  are in line with the results obtained by Wherry et al., we found that only a fraction of the transferred  $T_{EM}$  converted to a  $T_{CM}$  CD62L $^{+}$  phenotype, whereas many others were disappearing, probably because of apoptosis. These results are in agreement with several studies showing a progressive decrease of nondividing  $T_{EM}$  in peripheral tissues (16, 26, 36) and others showing that the intrinsic ability of an effector T cell to differentiate into a memory T cell is inversely correlated with its effector functions (37).

Overall, our results show that the differentiation process of  $T_{CM}$  and  $T_{EM}$  is highly complex and heterogeneous and that several features of the different proposed models have to be taken into account. Indeed, although approximately two thirds of  $T_{CM}$  and  $T_{EM}$  clones derive from a common naive precursor, it is still possible that some  $T_{CM}$  clones arise without passing through a  $T_{EM}$  stage, as suggested by the presence of unshared  $T_{CM}$  clones and the higher repertoire diversity of the  $T_{CM}$  subset as early as 6 wk after immunization. This would be in agreement with many studies suggesting that  $T_{CM}$  contain fewer differentiated cells (6, 13, 15). Concerning the stability of both subsets in vivo in the absence of antigen, our results show that, whereas  $T_{CM}$  are



**Figure 6.** Proposed model for the differentiation pathways of T<sub>CM</sub> and T<sub>EM</sub> after a primary response, in the absence of antigen, or in the case of antigenic restimulation. T<sub>CM</sub> are depicted as white cells and T<sub>EM</sub> are depicted as dark gray cells. The three possible fates of the latter are displayed. Light gray cells represent transitory T<sub>EM</sub>→T<sub>CM</sub> reexpressing CD62L but with various recall capacities depending on their position in the T<sub>EM</sub>→T<sub>CM</sub> differentiation pathway.

quite stable, both in terms of persistence and phenotype, T<sub>EM</sub> can follow three different fates: death, persistence as T<sub>EM</sub>, or conversion to a T<sub>CM</sub> phenotype (Fig. 6). Only a fraction of these cells were prone to apoptosis, whereas the remaining cells were able to survive for several weeks, even upon transfer into a new host, therefore in the absence of antigen. This threefold fate of T<sub>EM</sub> could be stochastic, only a certain percentage of the T<sub>EM</sub> progeny of a naive precursor being able to differentiate further into T<sub>CM</sub>. This could explain why a potent recall response was observed only in 2 out of 15 recipients transferred with purified T<sub>EM</sub> and immunized at least 7 d later, whereas in none of the other mice transferred with the same batches of T<sub>EM</sub> was a donor response observed. Alternatively, some particular exogenous signals might be needed for a T<sub>EM</sub> to turn into a potent T<sub>CM</sub>. In any case, for the T<sub>EM</sub> capable of converting to T<sub>CM</sub>, the differentiation process appears to be progressive and the re-expression of CD62L is probably only a first step. Indeed, although a significant proportion of T<sub>EM</sub> transferred cells had already converted to a CD62L<sup>+</sup> phenotype 2 d only after transfer, no recall response of the transferred cells was observed in any of the 14 animals that were transferred <30 d before the challenge. This aspect is consistent with the fact that the transcriptome of activated antigen-specific cells starts stabilizing only 6 wk after immunization (20).

### Respective roles of T<sub>CM</sub> and T<sub>EM</sub>

Our results show that most T<sub>EM</sub> have a short persistence in vivo, which prompts the question of the real memory capacity of these cells in vivo. In this work, T<sub>EM</sub> were less efficient than T<sub>CM</sub> to eliminate the recall antigen, as reported recently in another model (9). In particular, they were unable to mount a sustained recall proliferative response. If they did

participate in the elimination of the male cells, their action was certainly not potent enough so as to prevent any endogenous primary host response to occur. However, despite their inability to proliferate upon restimulation, T<sub>EM</sub> probably mediated some kind of memory (Fig. 6) because the host response was somewhat attenuated in recipients transferred with T<sub>EM</sub> compared with nontransferred ones or those transferred with naive T cells (Fig. 4 and not depicted), suggesting that T<sub>EM</sub> provided a first effector wave to eliminate male cells. It should be emphasized here that we only tested splenocytes as a source of T<sub>EM</sub>, in combination with the intravenous route for injection of a nonpathogenic antigen. Therefore, we cannot exclude that the transfer of T<sub>EM</sub> from different peripheral tissues, such as LP T<sub>EM</sub> and/or the choice of a pathogenic antigen, and/or its administration locally, would lead to a different conclusion. Our model is more akin to conditions that prevail for a systemic immunological response against autologous virally infected or malignant cells.

The short-term memory role of the T<sub>EM</sub> could be a general phenomenon, as several studies suggest that peripheral T<sub>EM</sub>, and maybe recruited circulating T<sub>EM</sub>, do not divide in peripheral infected tissue, but rather provide a first line of defense against replication of the pathogen, the resolution of the recall infection being mediated by new effector cells derived from memory cells proliferating in the draining lymph node (38). Moreover, although several studies have implicated remaining effector or effector-memory cells in protection against some short-term secondary challenges, particularly for local peripheral restimulations (26, 27), most data point out the crucial role of T<sub>CM</sub> in secondary responses. Similarly, we found that transferred T<sub>CM</sub> mounted a rapid and sustained proliferative response upon secondary challenge, generating a large pool of secondary effectors that, after clearance of male cells, gave rise to a heterogeneous memory population, comprised of both T<sub>CM</sub> and T<sub>EM</sub>. However, this paper is the first to demonstrate that not all T<sub>CM</sub> clones give rise to T<sub>EM</sub> after a secondary response in vivo (Fig. 6) and that the T<sub>CM</sub> clones already shared after the primary response may preferentially differentiate into T<sub>EM</sub> upon secondary challenge.

Altogether, this paper provides new insights into the drastically different behaviors of T<sub>CM</sub> and T<sub>EM</sub> CD8 subsets in vivo and shows that vaccinologists should be careful not to generate the maximum quantity of memory T cells, but rather the right quality of memory cells, as was suggested recently by Seaman et al. (33).

### MATERIALS AND METHODS

**Mice and immunizations.** CD45.1<sup>+</sup> and CD45.2<sup>+</sup> C57BL/6 mice were purchased from CDTA and Charles River Laboratories, respectively. Female mice transgenic for the  $\beta$  chain of the H-Y TCR ( $\beta$ -tg mice) were raised in our animal facility. In these animals, ~30% of the CD8 naive cells are specific for the D<sup>b</sup>-Smcy3 complex (31). All animal experiments were performed according to institutional guidelines for animal care and use.

For the generation of T CD8 memory cells, 10<sup>5</sup> female  $\beta$ -tg CD8 naive cells containing ~3 × 10<sup>4</sup> D<sup>b</sup>-Smcy3-specific CD8 cells were adoptively transferred into C57BL/6 female hosts, which were intravenously in-

jected with  $5 \times 10^6$  bone marrow cells from male syngeneic mice. All studies were performed at least 6 wk after immunization.

**Antibodies, peptides, and apoptosis detection.** All antibodies used in this study were purchased from BD Biosciences. Smcy3<sub>738-746</sub> peptide and D<sup>b</sup>-Smcy3 tetramers were described previously (31). For apoptosis experiment, purified splenic CD62L<sup>+</sup> or CD62L<sup>-</sup> cells from C57BL/6 mice were triple stained with anti-CD8 allophycocyanin, anti-CD44 Cychrome antibodies, and annexin V solution (Boehringer) as recommended by the manufacturer, either directly ex vivo or after 4-h culture with RPMI 1640 medium supplemented with 10% FCS, 50 ng/ml PMA, and 0.5  $\mu$ g/ml ionomycin.

**Purification of T<sub>CM</sub> and T<sub>EM</sub> subsets.** Lamina propria cells were prepared as described previously (39). For repertoire analysis, splenic cells from immunized mice were enriched in CD8 cells using MACS CD8 purification kit (Miltenyi Biotec). Cells from all organs were stained with D<sup>b</sup>-Smcy3 tetramers, with anti-CD8-allophycocyanin and anti-CD44-Cy, and anti-CD62L-FITC antibodies and CD8<sup>+</sup>D<sup>b</sup>-Smcy3<sup>+</sup>CD44<sup>+</sup>CD62L<sup>+/-</sup> (T<sub>CM</sub>/T<sub>EM</sub>) cells were sorted on MoFlo sorter (DakoCytomation). For functional analyses and transfer experiments, splenic CD62L<sup>+</sup> and CD62L<sup>-</sup> subsets were purified using the automacs (Miltenyi Biotec), either by staining cells with anti-CD62L-allophycocyanin antibodies and anti-allophycocyanin microbeads, or directly with anti-CD62L microbeads. Purity data and representative CD62L expression profiles of sorted samples are provided in Fig. S1 A.

**Transfer experiments.** To analyze the behavior of T<sub>CM</sub> and T<sub>EM</sub> subsets in the absence of antigen or in case of restimulation, purified spleen and PLN CD45.2<sup>+</sup> CD62L<sup>+/-</sup> cells containing  $3 \times 10^4$  CD8<sup>+</sup> D<sup>b</sup>-Smcy3-specific memory cells were transferred into CD45.1<sup>+</sup> C57BL/6 female mice. For recall response experiments, these mice were injected intravenously with  $5 \times 10^6$  bone marrow cells from CD45.1<sup>+</sup> C57BL/6 male mice on day 0, 2, 7, 15 or 30 after transfer of the purified CD62L<sup>+/-</sup> populations.

**TCR $\alpha$  repertoire analysis.** For sequencing analysis, TCRAV9-TCRAC transcripts were amplified using TCRAV9.1-specific, 5'-GACACCGT-TGTTAAAGGCACC-3', TCRAV9.2-specific, 5'-GACGCCGTTGT-TAAAGGCAC-3', and TCRAC-specific, 5'-GGCACATTGATTTGG-GAGTCA-3' primers; cloned; and sequenced as described previously (40). For clonotypic quantitative analysis, real-time PCR was performed as described previously (40) and in Fig. S2 A using the TCRAV9-specific primers, the TCRAV9-specific nested Taqman probe TM<sub>m</sub>TCRAV9 6-FAM-ACTCGGCCTCAAAGC-MGB, and either a nested TCRAC-specific primer, 5'-CTGAGACCGAGGATCTTTAACTGG-3' or one of the clonotypic primers described in Fig. S2 (B and C).

**Statistical analyses.** To assess the repertoire diversity of a T cell subset, we computed the abundance-based coverage estimator (41) with EstimateS software (<http://purl.oclc.org/estimates>). We accumulated sequences until the estimator reached a plateau. Representative curves for T<sub>CM</sub> and T<sub>EM</sub> sorted populations are displayed in Fig. S1 D and show that, for most samples, the estimator plateaued after 300–400 sequences. To estimate the repertoire overlap between two populations, we computed the nonparametric maximum likelihood estimator (42). This index gives an estimation of the percentage of shared clones among the pooled T<sub>CM</sub> and T<sub>EM</sub> sequences, taking into account their frequencies. Two controls were performed to normalize these values (Fig. S1 E).

**Online supplemental material.** Fig. S1 illustrates the purification of CD62L<sup>+/-</sup> subsets and the analysis of TCRAV9-TCRAC rearrangements of sorted D<sup>b</sup>-Smcy3-specific T<sub>CM</sub> and T<sub>EM</sub> spleen cells. Fig. S2 illustrates the analysis of individual clonotypes by quantitative PCR (Q-PCR). Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20040876/DC1>.

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