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Anatomical Heterogeneity of Memory CD4$^+$ T Cells Due to Reversible Adaptation to the Microenvironment[1]

George Kassiotis and Brigitta Stockinger[2]

The memory T cell pool is characterized by a substantial degree of heterogeneity in phenotype and function as well as anatomical distribution, but the underlying mechanisms remain unclear. In this study we confirm that the memory CD4$^+$ T cell pool in wild-type and TCR-transgenic mice consists of heterogeneous subsets, as defined by surface marker expression or cytokine production. Extralymphoid sites contain significant numbers of memory CD4$^+$ T cells, which are phenotypically and functionally distinct from their lymphoid counterparts. However, we show in this study that the phenotype of lymphoid and extralymphoid memory T cells is not stable. Instead, the unique properties of extralymphoid memory T cells are acquired upon migration into extralymphoid sites and are lost when memory T cells migrate back into lymphoid organs. Thus, at least some of the extralymphoid properties may represent a transient activation state that can be adopted by T cells belonging to a single memory T cell pool. Furthermore, such intermittent activation during or after migration into extralymphoid sites could provide an important signal, promoting the survival and functional competence of memory T cells in the absence of Ag. *The Journal of Immunology*, 2004, 173: 7292–7298.

D uring the immune response to infection, Ag-activated T cells acquire a battery of effector molecules, such as cytokines and chemokines, involved in orchestration of the immune response, and cytotoxic molecules with a direct effect on infected cells (1, 2). Moreover, Ag-activated T cells down-regulate the expression of cell surface molecules involved in homing into secondary lymphoid organs and up-regulate the expression of a variety of integrins, adhesion molecules, and chemokine receptors that allow migration into extralymphoid peripheral tissues (3, 4). Pathogen clearance is followed by a contraction phase, during which the majority of responding T cells disappear, and a small proportion survives long term in the absence of Ag to form the memory T cell population (5). During this transition, some of the Ag-induced changes are lost, and the Ag-experienced T cells revert back to their resting state, whereas others are permanent, representing heritable epigenetic modifications (cellular memory). However, the distinction between transient and permanent Ag-induced changes has in many cases been difficult to make. Antigenic stimulation of memory T cells by persisting or cross-reactive Ags will lead to re-expression of transient effector functions and the memory T cell pool will appear heterogeneous in phenotype and function due to the presence of recently activated, effector T cells. Thus, the mechanisms by which heterogeneity of the memory T cell pool is generated and maintained are still a matter of debate.

The exact relationship among memory T cells with different functions and among the subsets defined by location is also unclear. It has been proposed that the human memory T cell pool can be subdivided into central memory (T$_{CM}$)[3] and effector memory (T$_{EM}$) (6). According to this model, T$_{CM}$ cells, which can be identified by expression of CCR7, preferentially migrate into secondary lymphoid organs and lack immediate effector functions, such as IFN-γ production and cytolytic activity. In contrast, T$_{EM}$ cells lack expression of CCR7 and are equipped with rapid effector functions and the potential to migrate into extralymphoid sites (6). However, subsequent studies in humans failed to establish a correlation between immediate effector functions and an extralymphoid migration pattern (partly inferred by the lack of CCR7 expression) and, in contrast, suggested that the majority of cytokine-producing memory T cells are expressing CCR7 (7–9). Studies in mice have confirmed that a substantial proportion of memory CD4$^+$ and CD8$^+$ T cells reside in extralymphoid sites (10, 11). Furthermore, a functional distinction between memory T cells residing in lymphoid or extralymphoid sites (i.e., defined by location and referred to hereafter as lymphoid and extralymphoid memory T cells, respectively) was also observed (10, 12, 13), albeit not always (14). Extralymphoid memory CD4$^+$ T cells were more likely to produce IFN-γ than their lymphoid counterparts, whereas the opposite was true for IL-2 (10). Similarly, the immediate cytolytic activity of extralymphoid memory CD8$^+$ T cells was higher that that of their lymphoid counterparts (11). However, as in humans, immediate effectors functions did not correlate with loss of CCR7 or CD62L expression, because T$_{CM}$ and T$_{EM}$ cells, defined by CCR7 or CD62L expression, displayed similar effector functions (14, 15). Moreover, CD8$^+$ T$_{EM}$ cells (defined as lacking CD62L expression) gradually converted to T$_{CM}$ (CD62L$^+$), suggesting that, at least for memory CD8$^+$ T cells, the loss of CD62L or CCR7 does not define a true T$_{EM}$ population, but, rather, a population of recently activated effector CD8$^+$ T cells (14).

We studied the heterogeneity of a monoclonal memory CD4$^+$ T cell population, resting in the absence of antigenic stimulation, with emphasis to a link between phenotype and spatial distribution. In this report we confirm that memory CD4$^+$ T cells reside in both

[3] Abbreviations used in this paper: T$_{CM}$, central memory T cell; DC, dendritic cell; T$_{EM}$, effector memory T cell; WT, wild type.

lymphoid and extralymphoid sites and are characterized by substantial heterogeneity in phenotype and function. However, we also show that lymphoid and extralymphoid memory T cells do not represent distinct, stable subsets, which differ in effector function and migration pattern. Instead, the unique properties of extralymphoid memory T cells are acquired during or after memory T cell migration into extralymphoid sites and are lost when memory T cells migrate back into lymphoid organs. Thus, our results suggest that part of the characteristic properties of extralymphoid memory T cells represent a transient activation state that can be adopted by T cells belonging to a single memory T cell pool.

Materials and Methods

Mice

HY-specific A1 (16) TCR-transgenic mice were kept on an $H2^k$ $Rag1^{-/-}$ genetic background. To obtain GFP-tagged A1 T cells, A1 TCR-transgenic mice were crossed to a transgenic mouse line expressing GFP under control of the human CD2 promoter (17), also kept on an $H2^k$ $Rag1^{-/-}$ genetic background. Recipients of adoptively transferred A1 T cells were $H2^b$ $Rag2^{-/-} Il2rg^{-/-}$ mice, resulting from crossing $Rag2^{-/-}$ (18) with $Il2rg^{-/-}$ (19) mice. Wild-type C57BL/6 and CD45.1-congenic C57BL/6 mice were also bred at the National Institute for Medical Research. All experiments were performed under specific pathogen-free conditions, according to institutional guidelines and Home Office regulations.

Generation of memory T cells and cell transfers

Memory A1 T cells were generated as previously described (20). Briefly, naive A1 T cells, isolated from lymph nodes of A1 TCR-transgenic mice, were transferred i.v. ($1–2 \times 10^6$/recipient) into $H2^b$ $Rag2^{-/-} Il2rg^{-/-}$ recipient mice together with an equal number of HY peptide-pulsed syngeneic dendritic cells (DCs). DCs were prepared from day 6 bone marrow cultures from A ($H2^a$) mice, supplement with GM-CSF (21) and were pulsed with 1 μM HY peptide (REEALHQFRSGRKPI) for 2 h before adoptive transfer. Recipients of the adoptive transfer were used for isolation of memory A1 T cells 9–32 wk after transfer. For secondary transfers, memory A1 T cells isolated from the lymph node, spleen, or peritoneal cavity of primary hosts were retransferred by i.v. injection into secondary $H2^b$ $Rag2^{-/-} Il2rg^{-/-}$ recipients (0.5×10^6/recipient) and analyzed 5 wk later. Polyclonal memory T cells used for cell transfers were isolated from the lymph node or peritoneal cavity of C57BL/6 mice by magnetic depletion of CD62L$^+$ cells. Cell suspensions were incubated with anti-CD62L microbeads, and positive cells were removed with an AutoMACS (Miltenyi Biotec, Auburn, CA), according to manufacturer's instructions. Memory T cells were transferred into CD45.1-congenic C57BL/6 mice ($0.5–1 \times 10^6$/recipient) and analyzed 2 wk later.

Flow cytometry and cytokine production

For flow cytometry, cells were stained with the following fluorescent or biotin-labeled Abs (all from BD Biosciences (Franklin Lakes, NJ) unless otherwise stated): CD4 (RM4-5), TCRβ (H57-597), CD44 (IM7), CD62L (MEL-14), and CD49d (SG31). Intracellular cytokine production was assessed after a 4-h stimulation of memory A1 T cells with phorbol dibutyrate and ionomycin (both at 500 ng/ml) in the presence of brefeldin A (10 μg/ml). Cells were subsequently fixed with 4% paraformaldehyde and permeabilized with 0.1% Nonidet P-40 before staining with Abs against IL-2 (JES6-5H4) and IFN-γ (XMG1.2). Four-color cytometry and cell sorting were performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Results

To examine the heterogeneity of a CD4$^+$ memory T cell population, we used an adoptive transfer system of in vivo memory T cell generation (20, 22). Naive A1 TCR-transgenic CD4$^+$ T cells, specific for the male Ag (HY) presented by H2-E^k, were cotransferred with Ag-bearing syngeneic bone marrow-derived DCs into alymphoid, Ag-free, allogeneic hosts ($H2^b$ $Rag2^{-/-} Il2rg^{-/-}$). The cotransferred syngeneic DCs provide the only source of antigenic stimulation for the A1 T cells and become reliably undetectable by the third week after transfer. The host's haplotype ($H2^b$) is nonselecting and nonrestricting for A1 T cells, precluding their antigenic stimulation by cross-reactive Ags after disappearance of the

cotransferred DCs. Also, the host's haplotype does not support the survival and homeostatic expansion of naive A1 T cells, such that after transfer (even together with a limited number of syngeneic DCs), A1 T cells steadily decline, with a half-life of 1–2 wk, whereas the majority retain a naive phenotype for up to 7 wk after transfer (data not shown). This system thus allows study of a pure population of Ag-experienced CD4$^+$ T cells surviving in a resting state in the absence of antigenic stimulation (i.e., memory T cells). The memory A1 T cell population is skewed toward a Th1 phenotype because, on the average, 60% of them produce IFN-γ upon stimulation (20), whereas no IL-4 production is detected. In the resting phase, memory A1 T cells can be found in multiple extralymphoid sites, but their absolute numbers in most of these locations is difficult to accurately measure with conventional methods (10). However, a sizable proportion of memory A1 T cells (~5% of the splenic memory T cell pool) can be found in pleural and peritoneal cavities (20), which, in contrast to solid tissues, can be accurately sampled; thus, the peritoneal cavity was chosen as a representative extralymphoid site.

Phenotypic heterogeneity of memory A1 T cells

To examine the extent of memory T cell heterogeneity we initially analyzed the expression of several surface markers associated with Ag-experienced T cells in memory A1 T cells recovered from the spleen, lymph node, or peritoneal cavity. Two surface molecules, namely CD62L and integrin CD49d, showed heterogeneous expression in the memory A1 T cell population. The expression of CD62L was down-regulated on the majority of (70–80%), but not all, memory A1 T cells (Fig. 1A). The proportion of CD62L$^+$ A1 T cells was similar in spleen, lymph nodes, and peritoneal cavity (Fig. 1A), arguing against a strong correlation between CD62L expression and preferential migration into lymphoid organs. The expression of CD49d was induced at low levels in a small proportion of A1 T cells in the lymph node and higher proportion in the spleen (Fig. 1A). However, a much larger proportion of A1 T cells from the peritoneal cavity expressed CD49d and at much higher levels (Fig. 1A). This pattern of CD62L and CD49d expression in memory A1 T cells was stable over time for at least 220 days after the initial adoptive transfer (data not shown).

A potential concern is the effect on the observed memory T cell heterogeneity of the adoptive transfer used to generate memory T cells and the absence of secondary lymphoid tissue (except the spleen and mesenteric lymph node) in $Il2rg^{-/-}$ hosts. To exclude these potential influences, memory A1 T cells were generated by direct immunization (using a single injection of Ag-pulsed DCs) of $Rag1^{-/-}$ A1 TCR-transgenic mice, which bear the full complement of secondary lymphoid organs, thus avoiding the adoptive transfer into lymphopenic hosts. Due to the artificially high number of Ag-specific T cells in TCR-transgenic mice, which cannot all be activated with a single immunization, and because the thymus continues to export naive T cells in the periphery after immunization, a substantial number of naive A1 T cells are still present in immunized hosts. These cells were distinguished from memory A1 T cells based on the level of CD44 expression and served as an internal control (Fig. 1B). Again, memory A1 T cells were predominantly negative for CD62L (>80%), whereas high CD49d expression was only observed in cells from the peritoneal cavity (Fig. 1B).

We next compared the phenotype of artificially generated TCR-transgenic memory T cells with that of naturally occurring Ag-experienced cells in unmanipulated, wild-type (WT) mice. Although the antigenic specificity of memory-phenotype T cells in WT mice is largely unknown, they are generally considered to be
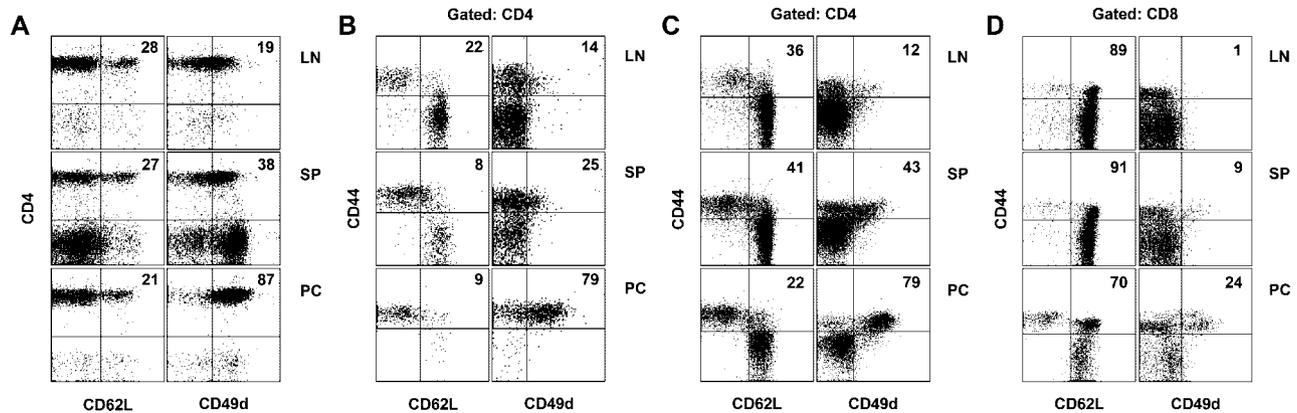
FIGURE 1. Phenotypic heterogeneity of memory T cells. *A*, CD62L and CD49d expression in memory A1 T cells, isolated from the spleen (SP), lymph node (LN), or peritoneal cavity (PC) of $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ hosts, 10 wk after transfer of naive A1 T cells together with Ag-pulsed DCs. The plots are representative of at least 15 mice analyzed between days 63 and 223 after transfer. *B*, CD62L and CD49d expression in A1 T cells isolated from A1 TCR-transgenic $Rag1^{-/-}$ mice 7 wk after immunization with Ag-pulsed DCs. The plots are gated on CD4+TCR+ A1 T cells and are representative of four mice. *C* and *D*, CD62L and CD49d expression in polyclonal CD4+ gated (*C*) and CD8+ gated (*D*) T cells from unmanipulated C57BL/6 mice. Numbers within the quadrants in all plots represent the percentage of CD4+ (*A–C*) or CD8+ (*D*) memory T cells that were positive for the expression of each surface marker.

the result of an immune response to environmental Ags. The majority of memory (CD44+) CD4+ T cells from C57BL/6 mice were negative for CD62L (60–80%), with only a weak correlation between CD62L expression and anatomical distribution (Fig. 1*C*). Moreover, the expression of CD49d was almost absent in the lymph node and was very high in the peritoneal cavity, whereas spleen was intermediate (Fig. 1*C*). The remarkably similar phenotype between A1 TCR-transgenic and WT memory CD4+ T cells thus lends additional support to the validity of the model system for memory T cell generation used in this study. In contrast to CD4+ T cells, however, the majority of memory CD8+ T cells from the same WT mice were expressing CD62L (70–90%), whereas the expression of CD49d was observed at lower levels in only a small proportion of peritoneal cavity cells, indicating an important distinction between memory CD4+ and CD8+ T cells.
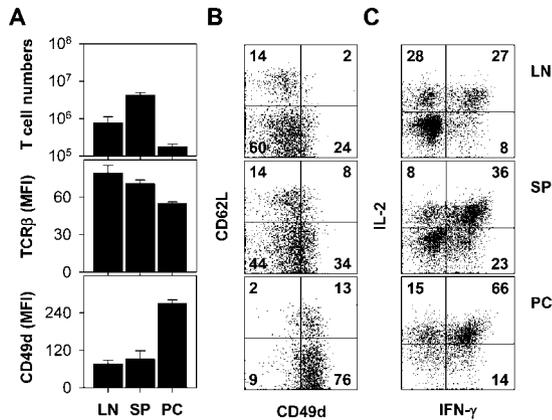
Lymphoid and extralymphoid memory A1 T cells

Although the proportion of memory CD4+ T cells expressing CD62L did not greatly vary among the various locations, the expression of CD49d appeared to be a hallmark of extralymphoid T cells, suggesting that these cells might be different from the lymphoid ones in several additional parameters. We therefore compared in more detailed the phenotypic and functional differences between lymphoid and extralymphoid memory A1 T cells (as defined by their location in spleen, lymph node, or peritoneal cavity of recipient mice). Among the three compartments, the majority (80%) of memory A1 T cells in the resting phase were found in the spleen, with small numbers in the peritoneal cavity (4%), and the remaining 16% in the single mesenteric lymph node of $Il2rg^{-/-}$ mice (Fig. 2*A*, *top*). Phenotypic analysis revealed that the level of TCR expression in memory A1 T cells from the lymph node was higher than that in the peritoneal cavity, whereas memory A1 T cells from the spleen were intermediate (Fig. 2*A*, *middle*). In contrast, the intensity of CD49d expression was significantly higher in memory A1 T cells from the peritoneal cavity compared with that in cells from the lymph node or spleen (Fig. 2*A*, *bottom*). Little or no correlation was observed between CD62L and CD49d expression (Fig. 2*B*), suggesting independent regulation of the two markers. Lymphoid and extralymphoid memory A1 T cells also differed in their immediate cytokine response. IFN-γ and IL-2 production, in response to a 4-h stimulation with phorbol esters and ionomycin,

was more pronounced in memory A1 T cells from the peritoneal cavity than in those from the lymph node, whereas the spleen was intermediate (Fig. 2*C*). Collectively, these results show that lymphoid and extralymphoid memory A1 T cells exhibit a distinct profile of TCR and CD49d expression as well as immediate IFN-γ and IL-2 production.

Stability of lymphoid and nonlymphoid phenotype

Differences in phenotype and function between lymphoid and extralymphoid memory T cells have generally been interpreted to reflect a series of distinct functional stages in a progressive differentiation program, where T cells can stay until new signals are received (differentiation compartments) (23). According to this hypothesis, the induction of full effector functions correlates with the potential for migration into extralymphoid sites, and lymphoid and extralymphoid memory T cells represent largely distinct subsets, which differ in their effector potential and migration pattern. To examine whether lymphoid and extralymphoid memory A1 T cells indeed represent distinct subsets of the memory CD4+ T cell pool, we analyzed the stability of their functional, phenotypic, and migratory properties. Memory A1 T cells, isolated from the lymph nodes of $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ recipient mice (LN-origin), were retransferred into secondary $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ recipients, and their anatomical distribution and phenotype were analyzed 5 wk later. Memory A1 T cells could be recovered from all three compartments (spleen, lymph node, and peritoneal cavity) of secondary recipients (Fig. 3*A*). Moreover, the relative distribution of memory A1 T cells among these three compartments was similar to that in the original donor mice (with 3% of the memory A1 T cells in the peritoneal cavity; Fig. 3*A*, *top*). More importantly, compared with their lymphoid counterparts, extralymphoid memory A1 T cells recovered from secondary recipients, showed decreased TCR (Fig. 3*A*, *middle*) and increased CD49d (Fig. 3*A*, *bottom*) expression, whereas the expression of CD62L did not vary (Fig. 3*B*). Furthermore, a higher proportion of extralymphoid memory A1 T cells had the potential for rapid IFN-γ and IL-2 production compared with their lymphoid counterparts (Fig. 3*C*). These results suggest that transfer of LN-origin memory A1 T cells into secondary recipients generates both lymphoid and extralymphoid memory A1 T cells. Similarly, extralymphoid memory A1 T cells, isolated from the peritoneal cavity of $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$

FIGURE 2. Comparison of lymphoid and extralymphoid memory A1 T cells. *A*, Memory A1 T cells, recovered from the lymph node (LN), spleen (SP), or peritoneal cavity (PC) of $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ adoptive hosts after transfer of naive A1 T cells together with Ag-pulsed DCs are compared in terms of absolute numbers (*top*) and mean fluorescence intensity (MFI) of TCR (*middle*) and CD49d expression (*bottom*). *B*, Flow cytometric profiles of CD49d and CD62L expression in CD4$^+$ gated memory A1 T cells from LN, SP, and PC of the same recipients. *C*, Intracellular detection of IL-2 and IFN-γ production by CD4$^+$ gated memory A1 T cells from the same recipients after 4-h in vitro stimulation. Data are pooled from 10 (IL-2 and IFN-γ production) or 19 (absolute numbers and surface markers) mice, analyzed between days 88 and 223 after transfer.

recipient mice (PC-origin), generated both lymphoid and extralymphoid memory A1 T cells after being retransferred into secondary $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ recipients with the characteristic expression of TCR and CD49d and the potential for rapid IFN-γ and IL-2 production (Fig. 3, *D–F*). In fact, phenotypically and functionally the lymphoid and extralymphoid memory A1 T cells recovered from secondary recipients of either LN-origin or PC-origin memory A1 T cells were similar to the respective subset in the original memory A1 donor mice (compare Figs. 2 and 3). Furthermore, the absolute number and relative distribution of memory A1 T cells were indistinguishable between recipients of LN-origin and PC-origin memory A1 T cells (Fig. 3, *A* and *D*), arguing in favor of homeostatic control rather than T cell-intrinsic factors.

The ability of lymph node and peritoneal cavity memory T cells to generate both lymphoid and extralymphoid memory T cells could be due to interconversion of the two subsets. Thus, TCR^{high}CD49d$^-$ lymph node memory A1 T cells could migrate into the peritoneal cavity where they would down-regulate TCR and up-regulate CD49d expression. Alternatively, the TCR^{low}CD49d$^+$ memory A1 T cells isolated from the peritoneal cavity after transfer of TCR^{high}CD49d$^-$ lymph node memory A1 T cells could originate from few TCR^{low}CD49d$^+$ memory A1 T cells contaminating the lymph node population without the need for interconversion. To distinguish between the two possibilities, we used a GFP reporter to mark either the lymphoid (CD49d$^-$) or the extralymphoid (CD49d$^+$) memory A1 T cells. CD49d$^+$GFP$^-$ memory A1 T cells were mixed with CD49d$^-$GFP$^+$ memory A1 T cells, sorted from the spleen of donor mice, and were retransferred together into secondary recipients. If lymphoid and extralymphoid memory A1 T cells represent stable subsets, the majority of memory A1 T cells from the peritoneal cavity of secondary recipients (CD49d$^+$) should originate from the GFP$^-$ population (CD49d$^+$), whereas the reverse should be observed in the spleen. However, if lymphoid and extralymphoid memory A1 T cells interconvert, memory A1 T cells from the peritoneal cavity of secondary recipients (CD49d$^+$) should not be enriched for GFP$^-$

(originally CD49d$^+$) T cells, but, rather, should be a mixture of equal proportions of GFP$^-$ and GFP$^+$ T cells (originally CD49d$^+$ and CD49d$^-$, respectively). After transfer of CD49d$^+$GFP$^-$ and CD49d$^-$GFP$^+$ memory A1 T cells into secondary recipients, a similar ratio of GFP$^+$ and GFP$^-$ T cells was found in both spleen and peritoneal cavity (Fig. 4*A*), indicating an equal potential of CD49d$^+$ and CD49d$^-$ memory A1 T cells to generate lymphoid or extralymphoid memory T cells. The level of CD49d expression in GFP$^-$ (originally CD49d$^+$) memory A1 T cells remained slightly higher than that in GFP$^+$ (originally CD49d$^-$) memory A1 T cells recovered from the same location (Fig. 4, *A* and *B*). However, CD49d expression was significantly induced in GFP$^+$ (originally CD49d$^-$) memory A1 T cells recovered from the peritoneal cavity compared with GFP$^+$ memory A1 T cells from the spleen (Fig. 4, *A* and *B*). Similarly, CD49d expression in GFP$^-$ (originally CD49d$^+$) memory A1 T cells was significantly higher in the peritoneal cavity than in the spleen (Fig. 4, *A* and *B*). This result strongly suggest that at least some of the functional and phenotypic differences between lymphoid and extralymphoid memory T cells represent a transient effect of the migration process or the local microenvironment, rather than the result of T cell-intrinsic, distinct developmental states.

In our adoptive transfer experiments both LN-origin and PC-origin memory A1 T cells gave rise to lymphoid and extralymphoid memory T cells, respectively. It was still theoretically possible that the apparent activation of at least LN-origin memory T cells into a extralymphoid phenotype was due to the lymphopenic state of the secondary hosts. The homeostatic expansion of memory T cells transferred into $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ hosts is drastically slower than in syngeneic hosts due to the lack of appropriate MHC class II molecules (24). Nevertheless, we repeated the adoptive transfers into T cell-replete hosts, a setting that precludes any possible homeostatic expansion and activation of the adoptively transferred T cells. Moreover, to show that interconversion between lymphoid and extralymphoid memory T cells is not restricted to TCR-transgenic T cells, memory-phenotype CD4$^+$ T cells from WT mice were used. Memory T cells from either lymph nodes or the peritoneal cavity of C57BL/6 mice were transferred i.v. into CD45.1-congenic C57BL/6 mice and their distribution and phenotype were analyzed 2 wk later. Donor-derived memory CD4$^+$ T cells were recovered from lymph nodes, spleen, and peritoneal cavity of the recipient mice. However, their frequency was too low to allow for accurate determination of absolute numbers. Nevertheless, the distribution pattern did not greatly differ between LN-origin and PC-origin T cells (data not shown). More importantly, regardless of their origin and phenotype, the transferred memory CD4$^+$ T cells adopted a phenotype similar to that of resident memory CD4$^+$ T cells in the same anatomical compartment (Fig. 5). Thus, previously CD49d$^-$ LN-origin T cells that migrated into the peritoneal cavity of recipient mice also up-regulated CD49d expression, whereas previously CD49d$^+$ PC-origin T cells lost expression of CD49d upon migration into the recipient's lymph nodes (Fig. 5).

Discussion

The mechanisms that generate and maintain heterogeneity in terms of phenotype and function as well as of anatomical distribution of the memory T cell pool are not entirely clear. Extending previous findings (25), we have shown in this study that the memory CD4$^+$ T cell pool consists of heterogeneous subsets, with respect to the expression of surface molecules. In contrast to memory CD8$^+$ T cells, which uniformly express high levels of CD62L, the majority of memory CD4$^+$ T cells have permanently lost CD62L expression. In keeping with published data (10, 11), extralymphoid sites
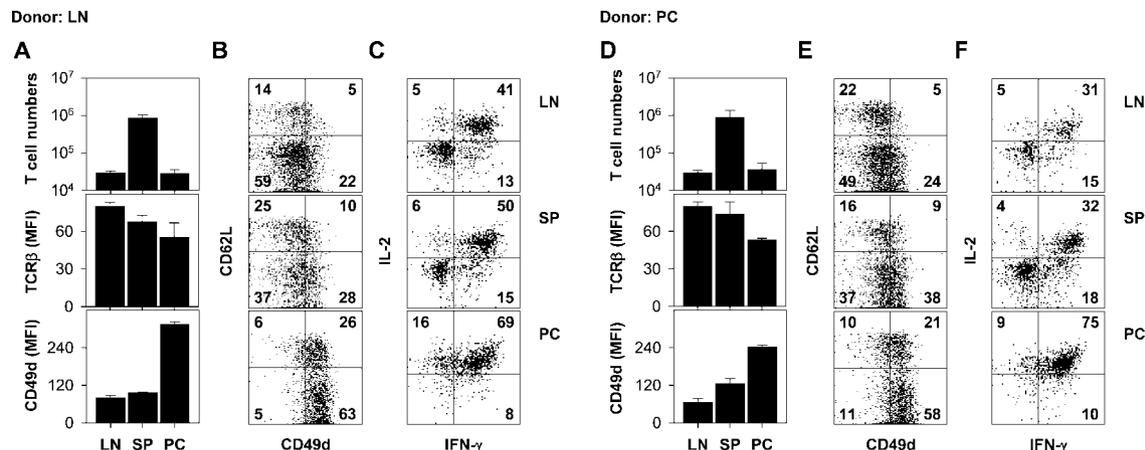
FIGURE 3. Interconversion of lymphoid and extralymphoid memory A1 T cells. Memory A1 T cells, generated by transfer of naive A1 T cells together with Ag-pulsed DCs, were isolated from the lymph node (LN-origin; *A–C*) or peritoneal cavity (PC-origin; *D–F*) of $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ hosts and re-transferred into secondary $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ hosts. Memory A1 T cells were recovered for analysis from the lymph node (LN), spleen (SP), and peritoneal cavity (PC) of secondary hosts 4–5 wk later. *A* and *D*, Absolute numbers (*top*) and mean fluorescence intensity (MFI) of TCR (*middle*) and CD49d expression (*bottom*) of LN origin or PC origin memory A1 T cells, respectively. *B* and *E*, Flow cytometric profiles of CD49d and CD62L expression in LN origin or PC origin memory A1 T cells, respectively, from LN, SP, and PC of the same secondary recipients. *C* and *F*, Intracellular detection of IL-2 and IFN-γ production by LN-origin or PC-origin memory A1 T cells, respectively, after 4-h in vitro stimulation. A total of four mice per group were analyzed in two independent experiments.

contained significant numbers of memory CD4$^+$ T cells that were phenotypically and functionally different from their lymphoid counterparts. However, at least some of the characteristic features of lymphoid and extralymphoid memory CD4$^+$ T cells were not stable, but, rather, were interconvertible, arguing against the existence of distinct lineages or differentiation compartments of memory CD4$^+$ T cells with different migration patterns being responsible for the division of memory T cells into lymphoid and extralymphoid subsets.

Analysis of the phenotypic complexity of the memory T cell pool is confounded by the fact that resting memory T cells cannot be reliably distinguished from recently activated effector T cells by phenotype alone. In fact, changes in the expression of several surface molecules, which are currently used to identify T$_{EM}$ cells, such as loss of CCR7 or CD62L expression, for example, are also characteristic of effector T cells. Recent studies in mice have argued that the memory CD8$^+$ T cell pool consists entirely of T$_{CM}$ cells (defined by the largely overlapping expression of CD62L and CCR7), with the T$_{EM}$ subset representing a relatively transient stage in the progression of effector CD8$^+$ T cell differentiation into memory CD8$^+$ T cells (14). In this respect, loss of CCR7 or CD62L expression may only characterize an effector CD8$^+$ T cell population, with a relatively recent Ag experience, instead of a resting CD8$^+$ T$_{EM}$ cell population (14). This would be important in studies with polyclonal T cells and especially in human studies, where the absence of antigenic stimulation cannot be guaranteed, whereas it suggests that differences between subsets defined by CD62L or CCR7 expression may, in fact, relate to their recent history of Ag exposure. However, our results indicate that the majority (60–90%) of memory CD4$^+$ T cells resting in the complete absence of antigenic stimulation have permanently lost expression of CD62L regardless of their location. Thus, the expression of CD62L in memory T cells (at least of the CD4$^+$ subset) is indeed heterogeneous, although it does not necessarily correspond to functionally distinct populations.

Re-expression or permanent loss of CD62L in memory CD8$^+$ and CD4$^+$ T cells, respectively, may arise from genuine differences between the behavior of the two subsets in response to antigenic stimulation. Regulation of surface CD62L expression after
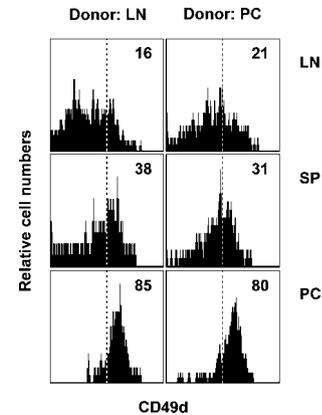
activation of both CD4$^+$ and CD8$^+$ T cells occurs at several levels, ranging from shedding of CD62L protein from the cell membrane to changes in the *Cd62l* gene transcription rate or message stability (26). Although CD62L shedding is mostly responsible for the rapid (within hours) loss of surface CD62L during activation, the failure of a proportion of memory T cells to re-express CD62L at later times is thought to result from gene silencing (26). Furthermore, the loss of CD62L expression occurs faster in CD8$^+$ than in CD4$^+$ T cells (26). This seems to be part of a more general response mode, which appears to be intrinsically different between CD4$^+$ and CD8$^+$ T cells, with CD8$^+$ T cells proliferating and differentiating into effector cells faster than CD4$^+$ T cells (27, 28). It is conceivable that the faster acquisition of effector phenotype and function by CD8$^+$ T cells does not represent stably heritable epigenetic modification at all the respective gene loci; thus, some of these Ag-induced changes that are acquired during Ag exposure are eventually lost from the memory population. In contrast, the differentiation of CD4$^+$ T cells, albeit slower and with more requirements, may be more permanent, corresponding to true cellular memory.

The existence of extralymphoid memory T cells, which differ from their lymphoid counterparts in many aspects of phenotype and function, has generally been interpreted as a division of the memory T cell pool into distinct subsets, each with a characteristic effector function and homing potential (6, 29). This hypothesis is supported by the fact that subsets of memory T cells with different homing potential (usually inferred by the expression of CD62L and CCR7) differ in many ways, including global gene expression profile (30) and TCR repertoire (31). However, such a comparison might not necessarily refer to two memory T cell subsets, and in our case, the link between CD62L expression and anatomical distribution is not very strong. The comparison between lymphoid and extralymphoid memory T cells (defined by location rather than phenotype) would thus be more informative. Our results are in agreement with previous studies demonstrating that lymphoid and extralymphoid memory T cells are phenotypically and functionally different (10, 11), although in our case, IL-2 and IFN-γ production was not a characteristic of lymphoid and extralymphoid memory T cells, respectively. Instead, both cytokines were coexpressed in the
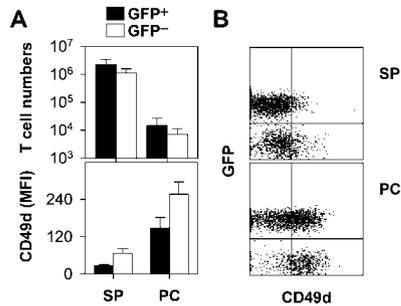
FIGURE 4. Plasticity of CD49d expression by memory A1 T cells. *A*, Absolute numbers and MFI of CD49d expression in GFP$^+$ (■) and GFP$^-$ (□) memory A1 T cells, isolated from the spleen (SP) or peritoneal cavity (PC) of $H2^b$ $Rag2^{-/-}Il2rg^{-/-}$ secondary hosts 5 wk after cotransfer of sorted CD49d$^+$GFP$^-$ and CD49d$^-$GFP$^+$ memory A1 T cells. *B*, Flow cytometric profile of GFP and CD49d expression in CD4$^+$ gated memory A1 T cells from the same recipients. A single experiment with three mice per group is shown. Similar results were obtained when the CD49d$^+$ memory A1 T cells were marked with the GFP reporter (i.e., CD49d$^+$GFP$^+$ and CD49d$^-$GFP$^-$ memory A1 T cells) in an additional experiment.

FIGURE 5. Plasticity of CD49d expression by polyclonal memory CD4$^+$ T cells. Memory-phenotype CD4$^+$ T cells were isolated from the lymph node (LN) or peritoneal cavity (PC) of C57BL/6 mice and retransferred into CD45.1-congenic C57BL/6 recipients. A flow cytometric profile of CD49d expression in CD45.2$^+$ gated memory CD4$^+$ T cells from the LN, SP, or PC of secondary recipients is shown. Donor-derived memory CD4$^+$ T cells were recovered for analysis from two or three recipient mice analyzed individually 2 wk after transfer.

majority of cytokine-producing memory A1 T cells, which were enriched in extralymphoid rather than in lymphoid sites. However, despite the observed differences, either subset in isolation could reconstitute the full memory T cell pool (consisting of both lymphoid and extralymphoid memory T cells), suggesting a substantial degree of interconversion or reversibility. Although reversibility was observed in both cytokine production and level of TCR and CD49d expression by memory T cells, these features do not exhaust the list of properties that could be different between lymphoid and extralymphoid subsets, and it is therefore possible that the two subsets differ in other characteristics. However, the observed reversibility in at least these features provides a proof of principle for the interconverting nature of lymphoid and extralymphoid memory T cell subsets. Furthermore, a strict correlation between absolute numbers of lymphoid and extralymphoid memory T cells was also observed. These results are compatible with a model, in which both lymphoid and extralymphoid subsets belong to a single pool of memory T cells. Although the expression of certain homing receptors in the memory T cell pool is heterogeneous (4), the presence of memory T cells in extralymphoid sites may simply reflect a given probability of all memory T cells migrating into these sites. A highly mobile nature of lymphoid and extralymphoid memory T cells is also supported by a recent study using adoptive transfer and parabiosis models, in which it was demonstrated that blood-borne resting memory CD8$^+$ T cells were capable of relocating into lymphoid and extralymphoid tissues (32). Such a mechanism can explain why the absolute number of extralymphoid memory T cells is always closely related to the absolute number of the lymphoid ones and suggests that the total memory T cell pool (lymphoid and extralymphoid) is under the same homeostatic control. The interconvertible nature of the phenotypic pattern that characterizes each subset indicates that this pattern is acquired during or after migration of memory T cells into the respective anatomical compartment. It is reasonable to assume that the resting, inactive phenotype of memory T cells in lymphoid organs is modified when these cells migrate into extralymphoid environments, which differ from the lymphoid ones in many respects, including the density and quality of lymphocytes and APCs, cytokines, and components of the innate immune system. Alternatively, memory T cells may adopt a more activated phenotype as a result of transendothelial migration, which is a necessary step for entry into extralymphoid sites. In fact, in vitro studies with

human memory CD4$^+$ T cells have demonstrated several phenotypic and functional consequences of noncognate interaction with endothelial cells, which included up-regulation of CD49d and hyper-responsiveness to antigenic stimulation (33). Thus, all memory CD4$^+$ T cells could continuously recirculate between lymphoid and extralymphoid compartments, being transiently activated after transendothelial migration and reverting to a resting state after entry into lymphoid organs. Furthermore, such an intermittent activation as a result of noncognate interaction with the endothelium of extralymphoid sites could provide an important signal promoting the survival and maintaining the functional competence of memory T cells in the absence of Ag.

Acknowledgments

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