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Anatomical Heterogeneity of Memory CD4⁺ T Cells Due to Reversible Adaptation to the Microenvironment

George Kassiotis and Brigitta Stockinger

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.
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 H2b 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 H2b Rag1−/− genetic background. Recipients of adoptively transferred A1 T cells were H2b 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 × 106/recipient) into H2b 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 (H2b) mice, supplement with GM-CSF (21) and were pulsed with 1 μM HY peptide (REALEHQFSGRKP1) 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 H2b Rag2−/−Il2rg−/− recipients (0.5–1 × 106/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 CD45− cells. Cell suspensions were incubated with anti-CD45 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 × 106/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), and CD62L (H57-597). Intracellular cytokine production was assessed after a 4-h stimulation of memory A1 T cells with phosphor 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 H2b-Ek, were cotransferred with Ag-bearing syngeneic bone marrow–derived DCs into lymphoid, Ag-free, allogeneic hosts (H2b Rag1−/−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 (H2b) 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 node, 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...
were negative for CD62L (60–80%), with only a weak correlation.

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. 1C).

In response to a 4-h stimulation with phorbol esters and ionomycin, the intensity of CD49d expression was significantly higher in cells from the lymph node or spleen (Fig. 2A). 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 recovered from secondary recipients, showed decreased TCR expression (Fig. 3A, middle) and increased CD49d expression (Fig. 3A, bottom) expression, whereas the expression of CD62L did not vary (Fig. 3B).

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 H2b Rag2–/–Il2rg–/– recipient mice (LN-origin), were retransferred into secondary H2b Rag2–/–Il2rg–/– recipient mice (PC-origin), 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. 3A). 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. 3A, top). More importantly, compared with their lymphoid counterparts, extralymphoid memory A1 T cells recovered from secondary recipients, showed decreased TCR (Fig. 3A, middle) and increased CD49d expression (Fig. 3A, bottom) expression, whereas the expression of CD62L did not vary (Fig. 3B). 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. 3C). 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 H2b Rag2–/–Il2rg–/–
recipient mice (PC-origin), generated both lymphoid and extralymphoid memory A1 T cells after being retransfered into secondary H2b 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, TCRlowCD49d− 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 TCRhighCD49d+ memory A1 T cells isolated from the peritoneal cavity after transfer of TCRlowCD49d− lymph node memory A1 T cells could originate from few TCRlowCD49d− 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 retransfered 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 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. 4A), 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 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 H2b 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
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 TEM 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 TCM cells (defined by the largely overlapping expression of CD62L and CCR7), with the TEM 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 cell pool consisting entirely of naive T cells (27, 28).

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).

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
majority of cytokine-producing memory AI 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 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.

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References


