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A Major Role for Memory CD4 T Cells in the Control of Lymphopenia-Induced Proliferation of Naive CD4 T Cells

Christine Bourgeois, George Kassiotis, and Brigitta Stockinger

In a state of lymphopenia, naive and memory CD4 T cells compete with each other for expansion at the expense of naive T cells. This competition prevents the proliferation as well as the phenotypic and functional conversion of naive T cells to "memory-like" T cells and may consequently prevent immune pathology frequently associated with lymphopenia-induced proliferation of naive cells. However, in T cell replete mice, memory T cells do not compete with naive T cells, indicating independent homeostatic control of naive and memory CD4 T cells in conditions that do not involve profound lymphopenia. Moreover, within the memory compartment, subsequent generation of new memory T cells precludes the survival of memory-like T cells. Thus, memory T cells have a major role in the control of lymphopenia-induced proliferation of naive cells because they inhibit both the generation of memory-like T cells and their persistence within the memory compartment. 

Control of T cell numbers in peripheral lymphoid organs is an important principle in the behavior of the immune system, which safeguards its functionality in combating infections. The term homeostasis is widely used to describe the "return-tendency" of a system, implying that any system under homeostatic control can restore itself to its original status. This principle holds true in conditions where T cell numbers exceed peripheral T cell capacity. For instance, more T cells are exported from the thymus than stably integrate into the peripheral naive T cell pool and mice grafted with multiple thymus show only a modest increase in the number of peripheral naive T cells (1, 2). However, in conditions of acute T cell loss (lymphopenia), restoration of the original status is not always achieved. In lymphopenia, naive (3) or memory T cells (4, 5) will divide extensively in the absence of their nominal Ag (6), a process which was initially considered capable of replenishing the periphery and therefore was termed homeostatic proliferation (7). However, it became clear that this proliferation does not ensure replenishment of the naive T cell population in the periphery (8), because adoptively transferred naive T cells that proliferate under lymphopenic conditions convert to a memory-like phenotype and lose their naive status (8–12). In contrast, naive T cells exported from the thymus following a lymphopenic incident retain their naive phenotype (13).

Naive cells differentiating during lymphopenia-induced proliferation exhibit a phenotype resembling that of memory cells, and although this has not been studied in every instance, may acquire functional properties matching those of memory cells (8–11, 14). Thus, the physiological role of "memory-like" T cells, generated in lymphopenic hosts, when compared with "true" memory T cells arising as a consequence of immunization is currently uncertain.

Another important principle of homeostasis, established for CD8 T cells, is the independent regulation of naive and memory T cell pools, so that generation of new memory cells results in competition for survival with existing memory cells, but not with naive T cells (15). This would ensure that generation of memory responses does not compromise the diversity of the naive T cell repertoire. However, recent observations challenged this assumption because it appeared that the survival and expansion of naive T cells is altered in the presence of memory cells (16–18), which is at odds with the notion of independent homeostatic control for the two populations. Given that most experimental protocols involve a degree of lymphopenia, which has severe destabilizing effects on peripheral T cell pools, we investigated the consequences of lymphopenia-induced proliferation on naive and memory T cells, focusing on CD4 T cells. Furthermore, we studied survival characteristics of memory-like T cells, which differentiated from naive T cells by lymphopenia-induced proliferation to gauge their potential physiological role in the immune system.

We show here that under adoptive transfer conditions into lymphopenic hosts, memory T cells prevent the expansion and conversion of naive T cells to a memory-like phenotype. In contrast, naive T cells generated by thymic export establish a normal naive T cell pool in adoptive hosts, do not influence the number of resident memory cells, and are themselves not affected by the presence of a pre-existing memory pool. Memory-like CD4 T cells that arose from lymphopenia-induced expansion and conversion show a similar phenotype and gross functional activity to bona fide memory T cells but their ability to compete for space with endogenous genuine memory T cells is severely compromised, which casts doubts on the ability of this differentiation pathway to participate in the establishment of a functional, long-lived memory pool.

Materials and Methods

Mice

A1 TCR transgenic Rag1−/− (H-2k) female mice (harboring T cells specific for the male H-Y Ag) (19) and AND TCR transgenic Rag1−/− (H-2b) with T cells specific for moth cytochrome c were used between 8 and 12 wk of age. The A1 strain was also bred onto a Thy1.1 background (A1 Rag1−/−, Thy1.1) and onto a transgenic strain expressing GFP under control of the CD2 promoter so that all T cells are GFP-positive (20) (GPFD A1 Rag1). Syngeneic recipients used for adoptive transfers were either Rag1−/− or Il2rg−/− Rag2−/− female mice. CBA, C57BL/10, and GFP- C57BL/10 female mice were used as sources for polyclonal T cells. All mice were bred...
A1 T were transferred on their own or cotransferred after transfer. Mesenteric lymph nodes were determined 7 and 21 days and T cell recovery from pooled spleen and mesenteric lymph nodes were determined 7 and 21 days after transfer. This experiment is representative of three experiments, using two to three mice per group and per time point. c. CFSE-labeled naive AND T cells (1 × 10⁶/mouse) and memory A1 T cells (0.5 × 10⁶/mouse) were transferred on their own or cotransferred into syngeneic Rag⁻/⁻ hosts. AND T cells were distinguished from A1 T cells by staining for V[G1.5]; allophycocyanin and PE-CD4 (GK1.5); allotype and PE Mel-14 and CD69; allophycocyanin, PE, and FITC-CD8 (53.6.7); biotinylated and PE-CD44; biotinylated and PE-CD4 (GK1.5); allophycocyanin and FITC-TCR (H57-597); PE-CD44; biotinylated and PE. Streptavidin-PerCP was used to develop biotinylated Abs. All Abs were purchased from BD Pharmingen.

Results

Both naive and memory CD4 T cells can undergo lymphopenia-induced proliferation, but memory T cells prevent proliferation of naive T cells

Independent homeostatic control of naive and memory T cells would suggest that cotransfer of both populations into lymphopenic hosts should result in colonization of a naive vs a memory niche. To test this, CFSE-labeled monoclonal H-Y-specific naive A1 CD4 T cells (expressing the Thy1.1 allotype) were transferred either on their own or in the presence of CFSE-labeled A1 memory T cells (expressing the Thy1.2 allotype) into lymphopenic hosts. Memory A1 T cells were previously established by transfer of naive A1 T cells together with Ag-pulsed DCs into syngeneic Rag-deficient hosts.

Abbreviation used in this paper: DC, dendritic cell.
While naive Thy1.1 A1 T cells divided and survived when transferred on their own (Fig. 1, a (top) and b (left)), the presence of memory A1 T cells completely blocked their proliferation (Fig. 1, a (middle) and b (right)). Memory T cells exhibit a higher proliferation capacity in response to lymphopenia compared with naive cells and expanded to a similar degree whether they were transferred alone (Fig. 1, a (bottom) and b (left)) or with naive T cells (Fig. 1, a (middle) and b (right)). To assess whether competition between naive and memory T cells was restricted to clonal competition, we determined the behavior of CFSE-labeled naive AND T cells transferred either on their own or in the presence of A1 petition, we determined the behavior of CFSE-labeled naive AND cells and expanded to a similar degree whether they were trans-

**Independent homeostatic control of naive and memory CD4 T cells in a T cell replete immune system**

Independent regulation of the naive and memory pool has been demonstrated within the CD8 compartment (15). We re-examined this issue for the CD4 compartment to evaluate whether the observed competition between naive and memory CD4 T cells, which contradicts independent control of the two pools, was indeed a consequence of lymphopenia or whether it indicated different principles of homeostasis within the CD4 T cell compartment. To test how naive and memory T cells are maintained under more physiological conditions, not involving cotransfer into lymphopenic hosts, we injected Rag<sup>−/−</sup> hosts with Thy1.2 A1 T cells together with Ag-pulsed DCs to generate a memory population. Four weeks later they received bone marrow from A1 mice expressing the Thy1.1 allotypic marker to reconstitute the naive T cell compartment, thereby avoiding the lymphopenia-induced proliferation following adoptive transfer of mature naive T cells. The first thymic emigrants were visible ~3 wk after transfer (Fig. 4a). They preserved their naive CD4<sup>44<sub>low</sub></sup> phenotype compared with the universal CD4<sup>44<sub>high</sub></sup> phenotype of the Thy1.2 memory A1 population (Fig. 4b). Over the course of 70 days the naive T cell pool gradually filled with thymus-derived cells, but these did not influence the number of memory cells, nor did memory cells prevent the increase in naive T cells. To verify that this phenomenon did not only apply to a transgenic system, a similar approach was used to assess competition between naive and memory CD4 T cells in a polyclonal repertoire. In this case we used bone marrow from C57BL/10 mice carrying a GFP transgene under control of the CD2 promoter, so that all naive T cells emerging from the thymus were GFP<sup>+</sup> (20). CD4<sup>44<sub>high</sub></sup>CD4 T memory T cells from normal C57BL/10 donors were transferred into syngeneic Rag<sup>−/−</sup> hosts to establish a memory pool and 4 wk later the mice received bone marrow from GFP<sup>+</sup> C57BL/10 mice to reconstitute the naive compartment. Again, under these conditions it was obvious that memory T cells do not prevent the gradual accumulation of naive T cells and that naive T cell export does not modify the number of memory T cells (Fig. 4c). Thus, it seems that under steady state conditions, when naive T cells are generated from thymic output, memory and naive CD4 T cells are indeed maintained under independent homeostatic control as previously shown for CD8 T cells. This principle is further illustrated when analyzing the immune system of aged mice in which absolute numbers of peripheral T cells decrease with age (Fig. 5, left panel). This is due to loss of naive CD4 (middle) and CD8 (right) T cells as a consequence of thymic atrophy. Although loss of naive T cells results in a proportional overrepresentation of memory T cells, the absolute numbers of memory T cells do not change at all for CD8 memory T cells.
cells and appear only slightly increased for CD4 memory T cells. On the one hand, these data confirm the by now well-established fact that maintenance of the naive T cell pool depends on thymic output (8, 24, 25). On the other hand, they provide evidence for independent homeostatic control of memory T cells, which are maintained in constant numbers rather than expanding by taking advantage of the void left by disappearance of naive T cells.

Characteristics of memory and memory-like CD4 T cells generated by adoptive transfer

Although lymphopenia-induced proliferation does not contribute to homeostasis of the naive T cell pool, it nevertheless may serve as a differentiation process generating additional memory T cells, because naive CD4 T cells transferred into lymphopenic mice expand and gradually convert to a memory-like phenotype. To compare homeostatic expansion and Ag-driven expansion leading to memory-like and genuine memory T cells, respectively, we transferred CFSE-labeled naive CD4 T cells into syngeneic female Rag-/- hosts either without a source of Ag or in the presence of H-Y peptide-pulsed DCs. Fig. 6a (left panels) shows the CFSE profiles 7 and 21 days after transfer. Although naive A1 T cells transferred without a source of Ag show some division within 7 days after transfer, their proliferation is much reduced compared with A1 T cells that received antigenic stimulation and already underwent more than eight divisions (the limit of detection with CFSE) within the first 7 days, as previously described for CD8 T cells (10, 26). Even 21 days after transfer, A1 T cells without antigenic stimulation still have not lost their entire CFSE label. When A1 T cells were transferred into syngeneic polyclonal hosts, no division was visible after transfer without Ag; but antigenic activation led to only slightly less CFSE dilution than seen in Rag-/- hosts and the recovery of A1 T cells was similar on day 7 (0.47 ± 0.02 in empty hosts compared with 0.53 ± 0.07 in polyclonal hosts). Comparing absolute T cell numbers recovered after transfer into lymphopenic hosts (Fig. 6b), it is obvious that purely homeostatic proliferation results in much reduced expansion compared with Ag-driven proliferation. While Ag activated CD4 T cells undergo a slow contraction phase following activation, homeostatically activated CD4 T cells continue to slowly expand until eventually they reach a similar plateau ~100 days later. Memory-like A1 T cells compared with Ag-experienced A1 memory cells exhibited the same CD44 profile, and a similar proportion of IFN-γ producers 6 wk after transfer (Fig. 6, c and d) in agreement with the observation of similar gene expression profiles in the two types of memory cells (27).

Memory-like A1 T cells are inferior to genuine memory A1 T cells and do not persist in competition with a polyclonal memory repertoire

Memory T cells are not only defined by their enhanced functional capacity in terms of proliferation and cytokine production, but also by their enhanced survival capacity. Although CD4 T cells that acquire a memory-like status through homeostatic proliferation appear phenotypically and functionally similar to bona fide memory cells, it remained to be tested how they survive under steady-state conditions. To test their survival ability upon naive T cell export, we transferred naive Thy1.2 A1 T cells into syngeneic Rag-/- hosts without Ag to generate a memory-like compartment. Four weeks later, these mice receive bone marrow from Thy1.1 A1 mice to reconstitute the naive T cell pool. In this setting establishment of the naive T cell pool from thymic export barely affected the resident pool of memory-like A1 T cells that formed in the course of homeostatic proliferation (Fig. 7a), demonstrating independent maintenance as seen with genuine memory A1 T cells (Fig. 4a).

However, a different picture emerged when bone marrow from polyclonal hosts was transferred into syngeneic Rag-/- hosts containing memory-like or genuine memory cells from A1 mice.
already evident at day 50 after transfer (Fig. 7, b). This resulted in rapid erosion of the memory-like A1 T cell pool (Thy1.2). Resident Thy1.2 memory A1 T cells and bone marrow-derived naive CD44 expression related to Thy1.2 expression on cells gated for CD4 and represented by the open and filled fractions, respectively. Four weeks later they received bone marrow from Thy1.1 A1 mice to generate a naive A1 T cell compartment. The bars show absolute numbers recovered from pooled spleen and mesenteric lymph nodes at different time points after reconstitution with bone marrow. Using their differential Thy 1 allotypic marker, naive and memory A1 T cell respective numbers are represented by the open and filled fractions, respectively. b. Dot plot of CD44 expression related to Thy1.2 expression on cells gated for CD4 and TCR. Resident Thy1.2 memory A1 T cells and bone marrow-derived naive Thy1.1 A1 T cells illustrates high CD44 expression on memory cells and CD44low expression on naive T cells. c. Sorted CD44high T cells from C57BL/10 mice were transferred into syngeneic Rag−/− hosts to establish a polyclonal memory pool. Four weeks later, T cell-depleted bone marrow from GFP−/−C57BL/10 mice was transferred to reconstitute a naive polyclonal T cell pool. The bars show absolute numbers of T cells recovered from pooled spleen and mesenteric lymph nodes at different time points after reconstitution with bone marrow and the respective numbers of GFP+ T cells issued from BM (open) and memory GFP− T cells (filled fraction).

FIGURE 5. Independent homeostatic control of the memory pool in aged mice. Absolute numbers of total T cells from spleen, all lymph nodes and the peritoneal cavity (left panel), CD4 T cells (middle) and CD8 T cells (right) were determined for C57BL/10 female mice at the indicated ages. Total T cell numbers were determined by addition of CD4 and CD8 T cell numbers determined by gating on CD4+ TCR+ or CD8+ TCR+, respectively. In each panel, respective number of CD44+ (filled bars) and CD44− T cells (open bars) are shown representing three to five mice per group and per time point.

Discussion

The immune system has devised ways to control numbers of naive and memory T cells independently of each other, thus safeguarding both a diverse repertoire for control of newly emerging pathogens as well as a reservoir of memory cells to speedily eliminate pathogens it has encountered before. Perturbations from this norm are likely to compromise full functionality of the immune system. Incidents of lymphopenia, which can be caused by clinical interventions such as chemotherapy, radiation, or treatment with immunosuppressive drugs (28), as well as many viral or bacterial infections (29–33), have a profound effect on the immune system. Lymphopenia results in an oligoclonal repertoire of a few clones that may become irreversibly dominant and cause pathology (34, 35).
and transient lymphopenia arising as a consequence of viral infection causes attrition of pre-existing CD8 memory clones (36).

Although the independent regulation of naive and memory T cells was firmly established for CD8 T cells (15), recent observations cast doubt on whether such a separation exists for CD4 memory T cells. For instance, the report of IL-7 as a survival factor for memory CD4 T cells (37, 38) seems at odds with its important survival role for naive T cells and does not fit with the notion of independent homeostatic control. However, it is conceivable that IL-7 is a pool-independent, general survival factor for T cells, rather than a specific homeostatic factor for either naive or memory T cells. Our data indicate that independent homeostatic control of CD4 naive and memory T cells is guaranteed only in conditions that do not involve acute lymphopenia. It appears therefore that homeostatic control mechanisms serve primarily to avoid excessive expansion of the peripheral T cell pool in both naive and memory compartments. In conditions following extreme lymphocyte depletion without continuous thymic output, homeostatic mechanisms are unable to restore the naive T cell compartment to its original status (8, 24, 25, 39) and it is unclear whether generating memory-like cells from the naive pool is beneficial for the immune system. Lymphopenia-induced proliferation reduces T cell diversity within the naive pool, but, in contrast, one could argue that transition to a memory status increases the frequency and functional activity of any given specificity. The fact that memory T cells will outcompete naive T cells for expansion under lymphopenic conditions and thus inhibit their activation and phenotypic conversion to memory-like T cells may be a means to prevent the immune pathology that is observed following adoptive transfer of naive T cells into lymphopenic mice lacking not only naive, but also memory T cells.

Although the composition of the memory pool is likely to be altered due to the fact that homeostatic proliferation following a lymphopenic incident favors expansion of clones with higher avidity over those with lower avidity for self-peptide MHC complexes (5, 40), restoration of the memory pool may compromise the predominant functional role of lymphopenia-induced proliferation. The tight homeostatic control of the memory pool is demonstrated in aging mice that have very little thymic output. Although thymic output is still measurable at 6 mo of age (41) and the naive T cell compartment remains stable for the first 5–6 mo, once thymic output drops below 1% of normal values, peripheral numbers of naive T cells are severely reduced (24). The gradual shift from naïve toward CD44high memory cells during ageing is well-documented (42, 43), but conflicting data are reported concerning the maintenance of peripheral T cell numbers (44, 45). We observed that in old mice the loss of naive T cells accounts for the reduction in total T cell numbers, but irrespective of this, the number of memory phenotype T cells remains constant rather than expanding to use the “space” left by the absence of naive T cells.

It was suggested that lymphopenia-induced proliferation of naive T cells is regulated by clonal competition for self ligands, because polyclonal naive T cells proliferate when injected into TCR transgenic hosts, but not when injected into syngeneic polyclonal hosts (18, 46). Furthermore, it was shown that the diversity of the TCR repertoire of a resident memory population will determine whether injected polyclonal T cells proliferate or not, suggesting that specificity based competition underlies regulation of homeostatic division (18). However, it is worth considering that polyclonal T cells might undergo Ag-driven rather than homeostatic expansion in transgenic hosts or hosts with a restricted polyclonal repertoire, which may harbor a higher amount of environmental Ags that are not kept in control by the endogenous T cell population. In another study, monoclonal T cells from TCR transgenic mice failed to proliferate when injected into the same strain, but expanded in transgenic mice with a different specificity (47). The conclusion was that T cells ignore large numbers of competitors as long as their TCR specificity is different. Although this may well be one of the principles underlying homeostatic competition, other experiments using transfer of naive CD8 T cells into
naive or memory hosts with the same or different TCR repertoire demonstrated that lymphopenia-induced proliferation of naive cells was restricted by affinity rather than specificity, whereas naive and memory cells were competing whatever their specificity (17). Our data demonstrating competition between naive AND memory cells was restricted by affinity rather than specificity, whereas naive or memory hosts with the same or different TCR repertoire. Altogether, these data suggest that memory-like T cells generated as a consequence of lymphopenic hosts expressing allogeneic MHC molecules. Altogether, these differences between memory-like and genuine memory T cells. Differences between memory-like and genuine memory T cells were also described by Cho et al. (11) who demonstrated that genuine CD8 memory T cells exhibit higher survival capacity compared with memory-like CD8 T cells when transferred into hosts expressing allogeneic MHC molecules. Furthermore, these data suggest that memory-like T cells generated as a consequence of lymphopenia-induced proliferation will have little chance to survive long term in competition with a normal memory repertoire.

Because lymphopenia-induced proliferation of naive cells is a physiological process that regularly occurs during early postnatal life (48–50), it has been suggested that this mechanism may equip the neonate with a broad array of memory cells, and possibly make the immune response more effective during early life (49) but also in adult life (48). It is also possible that the immaturity of the immune system in neonates (51, 52) may encourage survival of memory-like cells more than the immune system found in adult lymphopenic hosts. Indeed, and in contrast with our results, Schuler et al. (48) have described the maintenance of memory-like cells until adulthood after transfer of CD8 T cells from adult mice in neonatal B6 mice. However, in this experiment, the population transferred contained both naive CD44low and memory CD44high T cells. Because CD44high T cells have a higher proliferative capacity and interfere with lymphopenia-induced proliferation of naive cells, the maintenance of transferred cells in neonates could be due to the presence of memory-like cells in the inoculum, and consequently may not reflect the persistence of memory-like cells generated from the naive pool.

Thus, lymphopenia-induced proliferation of naive T cells and their conversion into memory-like T cells may have physiological significance only in the neonatal period. During this phase, naive T cells reach a periphery totally devoid of pre-existing T cells and especially memory T cells, and their conversion to memory-like phenotype and function may therefore confer protection against early infections. In the adult, in contrast, the presence of memory T cells will not only suppress proliferation and phenotypic conversion of naive T cells, but also compete out any memory-like T cells that may have arisen in the neonatal period.

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Disclosures

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References


EFFECTS OF LYMPHOPENIA ON PERIPHERAL T CELLS

The authors have no financial conflict of interest.


