Naive T Cells Transiently Acquire a Memory-like Phenotype during Homeostasis-driven Proliferation

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Abstract

In a depleted lymphoid compartment, naive T cells begin a slow proliferation that is independent of cognate antigen yet requires recognition of major histocompatibility complex-bound self-peptides. We have followed the phenotypic and functional changes that occur when naive CD8+ T cells undergo this type of expansion in a lymphopenic environment. Naive T cells undergoing homeostasis-driven proliferation convert to a phenotypic and functional state similar to that of memory T cells, yet distinct from antigen-activated effector T cells. Naive T cells dividing in a lymphopenic host upregulate CD44, CD122 (interleukin 2 receptor β) and Ly6C expression, acquire the ability to rapidly secrete interferon γ, and become cytotoxic effectors when stimulated with cognate antigen. The conversion of naive T cells to cells masquerading as memory cells in response to a homeostatic signal does not represent an irreversible differentiation. Once the cellularity of the lymphoid compartment is restored and the T cells cease their division, they regain the functional and phenotypic characteristics of naive T cells. Thus, homeostasis-driven proliferation provides a thymus-independent mechanism for restoration of the naive compartment after a loss of T cells.

Key words: lymphopenia • transgenic • Ly6C • CD44

Introduction

In the immunocompetent adult, the size and subset composition of the peripheral T cell pool is maintained despite the ongoing input of naive T cells from the thymus, the expansion and subsequent death of antigen-specific T cells during immune responses, and the graduation of T cells to the memory compartment after antigen encounter. The homeostatic mechanisms that control the size of the lymphocyte compartment remain largely a mystery (1). Evidence suggests that TCR/MHC-derived signals are essential for regulating homeostasis of the naive T cell pool at multiple stages. For example, specific interactions between the newly rearranged TCR of immature thymocytes and self-peptide/MHC molecules in the thymus are vital to the selection and export of a useful T cell repertoire. In addition, MHC-bound foreign peptides are crucial to the initiation of an adaptive immune response, resulting in the expansion of antigen-specific T cells and their differentiation to effector cells. Recently, it has become clear that naive T cells depend on self-peptide/MHC-derived signals for their peripheral homeostasis (2). Interactions between naive T cells and MHC molecules are important for the delivery of survival signals once cells enter the periphery; both newly generated CD8+ and CD4+ T cells die in the absence of peripheral expression of MHC class I and II, respectively (3–10). Furthermore, the homeostasis-driven expansion of naive T cells, which is the proliferation of cells in a lymphopenic environment without cognate antigen, requires specific TCR interactions with self-peptide/MHC (10–14).

The lymphoid system has great resiliency. After destruction of the hematopoietic system with whole body irradiation, ablative chemotherapy, or viral immunodeficiency, the peripheral T cell count can “bounce back” (15–17). In most cases, it has not been clear whether this recovery is due to new thymic output or to the expansion of the few surviving peripheral T cells. In human and animal studies, the recovery of CD4+ and CD8+ T cell numbers occurs faster in younger compared with older individuals (18, 19). This may indicate a more efficient thymic contribution in young animals, or may reflect the peripheral environment. One common finding in studies of the regenerating T cell compartment is that most of the peripheral T cells have an activated or memory phenotype, as defined by surface markers such as CD44 and CD45 (15, 16). This has led to the notion that only memory T cells have the ability to re-
plenish the peripheral T cell pool, whereas recovery of the naive compartment must depend on thymic output. This is at odds with recent studies using labeled naive T cells adoptively transferred into lymphopenic hosts, which clearly illustrate the ability of naive T cells to expand under these conditions (10–14, 20).

Some recent reports have noted a change in the surface phenotype of naive T cells undergoing homeostasis-driven proliferation (11–13, 21). In our previous study of CD8⁺ T cells, for example, we reported early upregulation of CD44 expression, but no change in other surface markers such as CD62L, CD25, or CD49d (11). These data implied that naive T cells undergoing homeostasis-driven proliferation may convert to a memory or activated state. To address the issue of whether homeostasis-driven proliferation may be important in the regeneration of the naive T cell compartment, we followed the phenotypic and functional characteristics of naive CD8⁺ T cells over a time course. This included an assessment of actively dividing cells early after their introduction into a depleted lymphoid compartment, as well as later, when the compartment was restored to normal numbers and the transferred cells had ceased dividing.

Materials and Methods

Mice and Cell Transfers. Transgenic mice expressing OT-I TCR have been described previously (22). The OT-I TCR utilizes a Vα2Vβ5 heterodimer to recognize a peptide derived from OVA (OVAp) presented by H2-Kb. OT-I TCR transgenic mice on the C57BL/6 (B6) background were crossed to recombinating gene (RAG)-1−/− (RAG−/−) mice (The Jackson Laboratory), also on the B6 background. Cells harvested from the spleen and lymph nodes of OT-I mice were >75% Vα2⁺Vβ5⁺CD8⁺ by flow cytometric analysis. The OT-I cells were either transferred directly or after enrichment for CD44⁺Ly6Clo cells by depleting cells coated with biotinylated anti-CD44 and anti-Ly6C antibodies with streptavidin-coated magnetic beads (Dynal). The purified cells were >90% CD8⁺Vα2⁺ and <1.5% CD8⁻CD44⁺Ly6⁺, and were injected intravenously into irradiated RAG−/− hosts or into B6 mice that had been irradiated 2 days earlier with 650 cGy.

To generate memory T cells, 3 × 10⁵ pooled spleen and lymph node cells from OT-I RAG−/− mice were transferred into B6 hosts that had been irradiated with 400 cGy 2 days earlier. Mice were injected 2 days after cell transfer with 5 × 10⁵ PFU of vaccinia virus encoding OVA. Memory cells were used >90 days later and were detected by staining with Vα2 and Vβ5 antibodies or with K⁺-OVAp tetramers (a gift of S.C. Jameson, University of Minnesota, Minneapolis, MN). To assess in vivo proliferation, mice were given drinking water with 5-bromo-2′-deoxyuridine (BrdU) at 0.8 mg/ml made fresh daily for 7 days before the indicated time point.

Phenotypic Characterization by Flow Cytometry. Pooled spleen and lymph node cells were stained for analysis by flow cytometry as follows: 10⁶ cells were first blocked with unconjugated anti-CD32/16 (2.4G2) for 5 min. Samples were then incubated with biotinylated antibodies for 20 min, washed twice and then incubated with biotinylated anti-Vα2, TR1-color-conjugated (BD Pharmingen) CD8, and biotinylated anti-CD44. Next, cells were resuspended in 0.5 ml 0.15 M NaCl, 1.2 ml of 95% ethanol was added dropwise while mixing. After a 30-min incubation, cells were washed with PBS and then incubated in 1% paraffin-aldehyde with 0.01% Tween-20 for 30 min on ice temperature, followed by 30 min on ice. Cells were washed again in PBS and then incubated for 10 min with 50 Kunitz U/ml of DNase I (Sigma-Aldrich) in 0.15 M NaCl, 4.2 mM MgCl₂, and 10 μM HCl. Cells were centrifuged, supernatants removed, and FITC-conjugated anti-Brdu (Becton Dickinson) and APC-conjugated streptavidin were added for 30 min, followed by a wash with PBS.

IFN-γ Staining. Pooled spleen and lymph node cells from the indicated hosts were incubated at 2 × 10⁶ cells per well in RPMI 10% heat-inactivated FCS alone or with 10 nM OVAp for a total of 8 h at 37°C. After the first 3 h of culture, 1 μl of Golgi Plug (BD Pharmingen) was added to each well. Cells were then harvested and stained for flow cytometric analysis. Cells were blocked with 2.4G2 for 5 min, then stained for surface molecules with PE-conjugated anti-Vα2, TR1-color-conjugated CD8, and APC-conjugated CD4. After surface staining, the cells were resuspended in 200 μl of Cytofix/Cytoperm (BD Pharmingen) and incubated for 20 min on ice. Samples were washed twice with Perm/Wash (BD Pharmingen). Samples were then blocked with 2.4G2 diluted in Perm/Wash buffer for 5 min, followed by the addition of FITC-conjugated anti-IFN-γ (BD Pharmingen), and were incubated on ice for 20 min. Samples were washed twice with 1 ml of Perm/Wash and resuspended in 200 μl of Cytofix/Cytoperm.

CTLA4 assay. EL4 target cells were labeled with Na²⁺ chromate in the presence or absence of OVAp at 5 μM. Labeled target cells were washed three times, and 5 × 10⁶ cells were placed in wells of a round-bottomed 96-well plate. OT-I T cells undergoing homeostatic proliferation in irradiated B6 hosts and memory OT-I cells were tested for direct ex vivo CTL killing. At the indicated time points, pooled spleen and lymph node cells were assayed; the percentage of Vα2⁺Vβ5⁺CD8⁺ cells in each sample was determined by flow cytometry and added to each well in numbers that were normalized for the number of Vα2⁺Vβ5⁺CD8⁺ cells. Primary CTL effectors were established from OT-I splenocytes by stimulation with irradiated B6 splenocytes pulsed with 1 μM OVAp, and were used on days 5–7 after stimulation.

Results

To analyze the changes that occur during the homeostasis-driven proliferation of naive CD8⁺ T cells, small numbers of peripheral cells from OT-I TCR transgenic, RAG⁻/⁻ donors (OT-I RAG⁻/⁻) were transferred into sublethally irradiated B6 hosts. During the subsequent weeks, both donor and host cells contribute to the recovery of the
T cell compartment. Both donor and surviving host T cells slowly expand, and the thymus also contributes its output of newly differentiated T cells. In this system, lymphoid homeostasis is eventually restored. In one series of experiments, we also used nonirradiated RAG-/- mice as hosts, in which case the host can make no contribution to the recovery of lymphoid cell numbers.

Homing-driven Proliferation of Naive T Cells Results in Phenotypic Conversion. Recent experiments with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled T cells have shown that all or most undergo a slow proliferation when transferred into a lymphopenic host and that this proliferation is accompanied by the upregulation of CD44 (11–13, 21). To analyze this phenotypic conversion further, naive T cells from OT-I RAG-/- mice were further depleted of CD44hiLy6C+ cells using magnetic beads, and were injected into sublethally irradiated syngeneic B6 hosts. After 17 d of expansion, the phenotype of the injected naive CD8+ T cells had changed dramatically (Fig. 1). They uniformly upregulated CD44, Ly6C, and CD122 (IL-2R β) compared with naive CD8+ T cells that express low levels of all three of these markers. This surface phenotype is similar to that of “true” memory cells activated by cognate antigen in adoptive transfer and allowed to rest for >90 d (Fig. 1). As there is no definitive marker of memory T cells, antigen-experienced cells have been identified by a combination of phenotypic and functional characteristics. A consensus has emerged in which B6 CD8+ memory T cells express high levels of CD44, Ly6C, and CD122, with high to heterogeneous expression of CD62L (24–30). Ly6C is expressed at low, heterogeneous levels on effector cells, and thus is useful to distinguish between CD8+ memory and effector T cells (Fig. 1; references 24, 30). The CD8+ T cells undergoing homeostasis-driven proliferation have a surface phenotype characteristic of memory CD8+ T cells (Fig. 1). Furthermore, unlike effectors, the cells do not significantly upregulate CD25, CD49d, or CD69, nor do they downregulate CD62L or the TCR (data not shown). Our follow-up experiments were designed to examine the kinetics and stability of the changes that occur during and after homeostasis-driven proliferation.

Figure 1. Naive T cells undergoing homeostasis-driven proliferation acquire a memory phenotype. Expression of CD44, Ly6C, and CD122 on OT-I RAG-/- T cells transferred 17 d earlier into an irradiated B6 host compared with naive, memory, and effector OT-I RAG-/- CD8+ T cells. Histogram plots show expression of the indicated marker for Vα2Vβ5+CD8+ gated cells. Before transfer, OT-I CD8+ cells were depleted of CD44hi and Ly6C+ cells. Memory cells were recovered from irradiated B6 hosts that had received 3 x 10^6 OT-I RAG-/- cells, followed by infection with vaccinia virus encoding OVA 90 d earlier. OT-I effectors were analyzed on day 5 after in vitro stimulation with OVAp-coated, irradiated splenocyte feeder cells.
RAG−/− T cells stain with Kb-OVAp tetramers, indicating that the majority of recovered Vα2+Vβ5+CD8+ cells are OT-I cells (data not shown). To determine how many of the recovered OT-I cells were dividing, mice were pulsed with BrdU for 7 d before each time point (Fig. 2 B). Between days 5 and 12 after transfer, the majority (80–88%) of the OT-I cells divided. By 37 d after transfer, only 12–15% of the OT-I cells had undergone proliferation during the 7-d BrdU pulse. The level of incorporation at day 37 and beyond is similar to the basal levels of proliferation of CD8+ T cells in unmanipulated B6 mice (23). Thus, it appears that the proliferation rate of the transferred OT-I T cells is low after their numbers stabilize and the lymphoid compartment returns to normal cellularity.

Homeostasis-driven proliferation of naive T cells is accompanied by a reversible phenotypic conversion. By 5 d after transfer, a significant percentage of OT-I cells has upregulated CD44, Ly6C, and CD122 (data not shown), and by days 12–20, the majority have homogenous expression of high levels of these markers (Fig. 3). No changes are observed in the expression of CD5, CD25, CD62L, CD69, or the TCR at any of the time points (data not shown). Interestingly, the phenotypic conversion of naive OT-I cells to a "memory-like" phenotype is not an irreversible differentiation. Between 20 and 30 d after transfer, the OT-I cells begin to de novo express all three activation/memory markers (Fig. 3). By 40–50 d after transfer, the majority of OT-I cells no longer express significantly higher levels of CD44, Ly6C, or CD122 than naive OT-I cells. Thus, the homeostasis-driven proliferation of naive T cells is accompanied by a transient conversion of the expanding cells to a "memory-like" phenotype, followed by a reversion back to a naive phenotype.

OT-I Cells Undergoing Homeostasis-driven Proliferation Transiently Acquire Ex Vivo CTL Activity and the Ability to Make IFN-γ. Naive CD8+ T cells from OT-I RAG−/− mice do not lye targets bearing their cognate antigen when tested directly ex vivo (Fig. 4 A), nor do they have the ability to produce IFN-γ during an 8-h pulse with antigen (Fig. 5 A). In contrast, effector cells that have recently been stimulated with antigen display high levels of target lysis and IFN-γ production (Figs. 4 A and 5 A). OT-I memory T cells can lyse targets ex vivo and produce IFN-γ more...
rapidly than naive T cells, though they are less efficient in lysis and IFN-γ production than effector cells (Figs. 4 A and 5 A; references 25–28, 31). When the functional activity of the OT-I cells dividing in response to a homeostatic signal is measured at day 5, no ex vivo CTL killing is observed (Fig. 4 B; references 11, 13). However, between 12 and 31 d after transfer, OT-I cells exhibit significant antigen-specific killing in a 6-h CTL assay (Fig. 4 B), and make IFN-γ during an 8-h stimulation with OVA peptide (Fig. 5 B). Thus, as observed with surface markers, OT-I cells undergoing homeostasis-driven proliferation resemble memory cells in function. As is the case for phenotypic conversion, both rapid CTL activity and IFN-γ production are transient. By day 45, the ability to perform effector functions in vitro wanes, and they become similar to naive cells (Figs. 4 B and 5 B).

OT-I T cells transferred into RAG−/− hosts divide indefinitely and Show Phenotypic and Functional Conversion, but not Reversion. When naive OT-I RAG−/− T cells were transferred into RAG−/− hosts, they proliferated and displayed phenotypic and functional changes as described for OT-I cells transferred into irradiated B6 hosts (Table I and Fig. 6). At 20 d after transfer into the RAG−/− host, the OT-I cells have upregulated CD44 and Ly6C, have ex vivo CTL activity (Table I), and produce IFN-γ after stimulation (Fig. 6 C). Therefore, OT-I cells undergoing homeostasis-driven proliferation in RAG−/− hosts acquire a "memory-like" phenotype as they do in irradiated B6 hosts.

However, unlike the situation in B6 hosts, the OT-I cells transferred into RAG−/− hosts do not revert to a naive status by either phenotypic or functional criteria at later time points (Table I and Fig. 6). 35% of the OT-I cells transferred into RAG−/− hosts incorporated BrdU between 61 and 68 d, compared with only 7% in B6 hosts (Table I). Even at day 92, 47% of the OT-I cells divided during the previous week (Fig. 6 A). Additionally, the OT-I cells in RAG−/− hosts maintain CTL activity and the ability to make IFN-γ 68 d after transfer, unlike cells recovered from irradiated B6 hosts at the same time point (Fig. 6 C and Table I). OT-I cells recovered from RAG−/− recipients retain the "memory-like" phenotype even 92 d after transfer, displaying high levels of CD44, Ly6C, and CD122 (Fig. 6 B). This is in marked contrast to the reversion to a naive phenotype by 60–90% of the OT-I cells in B6 hosts by day 37 after transfer (Fig. 3).

Why do OT-I cells continue to divide in RAG−/− hosts? One possible explanation is that the RAG−/− lymphocyte compartment is never reconstituted, so the transferred cells continue to receive a signal for homeostatic
proliferation. In support of this idea, the number of OT-I cells recovered from the RAG^{−/−} hosts never reaches the level achieved in B6 hosts (Table I). Thus, the RAG^{−/−} hosts remain lymphopenic. It is not clear why the OT-I T cells do not accumulate in the RAG^{−/−} hosts. The absence of fully developed secondary lymphoid organs or the absence of either B cells or other T cells in RAG^{−/−} hosts may explain why OT-I numbers do not increase at the rate seen in B6 hosts.

Discussion

We have observed that during homeostasis-driven proliferation, naïve OT-I CD8^{+} T cells acquire surface markers and functional characteristics reminiscent of memory cells. As the lymphoid compartment is restored to normal cellularity because of the expansion of both donor and host cells, the signal for homeostasis-driven proliferation wanes and the T cells cease dividing. Remarkably, the cells that converted to a "memory-like" phenotype revert to a naïve phenotype as homeostasis is regained. The conversion to a "memory-like" phenotype correlates with proliferation of the OT-I cells, and their reversion back to a naïve phenotype correlates with a return to a resting state.

The dramatic changes in surface phenotype and function after transfer into an irradiated host occur in the population of OT-I T cells as a whole and are not due to the outgrowth of a small, activated population. Several observations support this conclusion. First, when purified CD4^{+}CD8^{−} OT-I cells are transferred into irradiated hosts, all of the cells upregulate CD44, Ly6C, and CD122 (Fig. 1), showing that truly naïve T cells can undergo this phenotypic conversion. Second, when CD4^{+}OT-I T cells are labeled with CFSE before transfer, division is observed in all of the transferred cells, and all of the cells divide at a similar rate without evidence of a separate, rapidly dividing subset (data not shown [11–13]). The reversion from a "memory-like" to naïve phenotype also appears to be a property of the population as a whole. It is unlikely that the reversion is due to the outgrowth of a small population that did not convert accompanied by the massive death of the "memory" phenotype OT-I cells, because the reversion occurs at a time when the total number of OT-I T cells is increasing only minimally, and very few cells incorporate BrdU (Figs. 2 and 3). For OT-I cell numbers to be maintained in the absence of significant proliferation, the majority must be reverting.

OT-I cells undergoing homeostasis-driven proliferation are similar to antigen-experienced memory cells by the phenotypic and functional assays we have used. Thus, a "memory-like" phenotype can be acquired by CD8^{+} T cells in the absence of an encounter with cognate antigen. This poses a real challenge for our ability to classify T cells, as it implies that a significant number of antigen-inexperienced T cells may be masquerading as memory cells as defined by our current phenotypic and functional definitions. This would be the case particularly for patients recovering from treatment- or disease-induced lymphopenia. When T cell populations from patients recovering from chemotherapy or HIV-induced lymphopenia are analyzed, most of the remaining T cells display an activated or memory phenotype (15, 16). The presence of these dividing memory phenotype T cells has been used to argue that naïve T cells do not contribute to the restoration of the T cell compartment during recovery from lymphopenia. In light of the data presented in this paper and recently published by others, it is reasonable to argue that some of the memory phenotype cells observed in lymphopenic patients may in fact be residual naïve T cells or recent thymic emigrants (11–13, 21). Additionally, it is possible that homeostasis-driven proliferation is ongoing at low levels in full lymphocyte compartments, resulting in a population of "fake" memory cells in normal hosts as well.

The most striking of our observations is that the CD8^{+} T cells that acquire a "memory-like" phenotype during homeostatic proliferation revert to a naïve phenotype once the T cell compartment is restored to normal numbers. This implies that homeostasis-driven proliferation may provide a thymic-independent mechanism whereby naïve T cells can contribute to the reconstitution of the naïve com-

### Table I. Comparison of Homeostasis-driven Proliferation in B6 and RAG^{−/−} Hosts

|          | Day 20 |          | Day 68 |
|----------|--------|----------|--------|
|          | Percentage of BrdU * | Cell no. ‡ | Percentage of BrdU * | Cell no. ‡ |
| OT-I RAG^{−/−} → RAG^{−/−} | 54 | 3 | 30 | 35 | 9 | 66 |
| OT-I RAG^{−/−} → B6 | 36 | 46 | 43 | 7 | 83 | 7 |

*Percentage of BrdU^{+} cells out of Vα2^{+}CD8^{+} pooled spleen and lymph node cells.

†Number of recovered Vα2^{+}Vβ5^{+}CD8^{+} cells ×10^{6}.

§Specific lysis of OVAp-pulsed targets ex vivo at an E/T ratio of 100:1. The E/T ratio was normalized for the number of Vα2^{+}Vβ5^{+}CD8^{+} cells, and the lysis of targets without OVAp was <6% for all samples.
partment after lymphopenia. In support of this notion, rat T cells expressing low levels of CD45R C, typically consid-
ered to be memory cells, reexpress CD45R C after transfer
into nude hosts, thereby regaining their naive phenotype
(32). Also, in experiments in which unseparated lymph
node cells were transferred into SCID mice, both naïve and
memory T cell populations were observed 8 mo later (34).

Presumably, all of the cells initially divided and converted
to a memory phenotype in the empty lymphoid compart-
ment, implying that some of the cells reverted to generate a
pool of naïve T cells.

We note that the change in surface phenotype and func-
tion correlates with cell division: when the cells are divid-
ing, they have a "memory-like" phenotype, and when
division ceases, the cells return to a naïve phenotype.
Similarly, memory phenotype cells incorporate BrdU at
higher levels compared with naïve phenotype cells (23). In
RAG−/− hosts where the transferred cells continue to di-
vide indefinitely, the OT-I cells retain their "memory-
like" phenotype for >92 d (Fig. 6). The signal for the con-
version to a "memory-like" phenotype could be the signal
that drives the homoeostatic proliferation itself, or cell divi-
sion may actually promote the changes. The fact that naïve
T cells make IFN-γ more slowly than memory or effector
cells may be explained by data showing that naïve T cells
must undergo several rounds of division before they are
able to secrete IFN-γ (35–37). Cell cycle progression is
thought to relieve epigenetic repression of gene expres-
sion, possibly by causing changes in chromatin organiza-
tion (35, 38). In memory and effector populations, the IFN-γ locus
remains accessible because of the maintenance of a demeth-
ylated state (35, 37–39). It is provocative to consider that
the higher rate of division observed in the memory com-
partment in immunocompetent hosts could be responsible
for maintaining some of the phenotypic or functional char-
acteristics of memory cells via a cell cycle-dependent, epi-
genetic mechanism.

Why do cells undergoing homoeostasis-driven prolifera-
tion convert to a "memory-like" phenotype? As discussed
above, the changes may simply be a side effect of cell divi-
sion. On the other hand, there may be a functional ratio-

nate for this conversion. One possibility is that the rapid re-

sponse characteristics of cells in a lymphopenic situation is
of benefit to the host when few T cells are available for de-
fense against pathogens. Another possibility is that the up-

regulation of signaling or adhesion molecules may be
important for controlling homeostatic proliferation. For
example, IL-15 is thought to maintain the homoeostasis
of memory CD8+ T cells, and in its absence the memory
compartment is disrupted (40). IL-15 is known to be a
strong promoter of proliferation for memory pheno-
type CD8+ cells, and the upregulation of CD122, one of
the chains of the IL-15-receptor, could place the naïve T
cells under the influence of IL-15 (42).

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