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Stability of Naive and Memory Phenotypes on Resting CD4 T Cells In Vivo

Tamar E. Boursalian and Kim Bottomly

The reliable identification of naive and memory CD4 T cells is critical to understanding the cellular basis of immunological memory. In addition to exhibiting a response of more rapid onset and greater magnitude than naive cells, memory cells are thought to possess a long life span, forming the basis by which vaccines impart long term immunity. Whether a prolonged life span is due to continual stimulation or is an intrinsic property of differentiated memory cells is still in question (reviewed in Ref. 1).

Memory CD4 T cells differ from naive CD4 T cells in their expression of several cell surface molecules. Naive CD4 T cells that express high levels of L-selectin and CD45RB and low levels of CD44 (L-selectinhigh, CD45RBhigh, CD44low) (2–6; reviewed in Refs. 1 and 7) modulate their surface phenotype upon activation, leading to the reciprocal pattern of expression (L-selectinlow, CD45RBlow, CD44high) as well as an up-regulation of LFA-1, ICAM-1, CD43, and the αβ and αβ integrins (8, 9; reviewed in Refs. 1 and 7). To gain insight into whether the differential expression of these molecules contributes to the differences between primary and memory responses, it is important to know whether these phenotypic changes represent stages of differentiation of CD4 T cells, or if the changes reflect a transient state of activation resulting from recent contact with Ag. The L-selectinlow, CD45RBlow, CD44high phenotype on resting memory cells has been demonstrated in vivo and in vitro and is supported by the observation that the memory phenotype is not seen in early life, but increases with age (10–12). However, while some studies suggest that the phenotype of resting memory cells is stable, other studies indicate that a reversion from memory to naive phenotype can occur in CD4 T cells. Studies in irradiated humans suggest that there is a progressive reaccumulation of CD45RA+ (naive phenotype) CD4 T cells with time (13), as have studies in rats demonstrating reversion of memory phenotype, CD45RC+, to a naive phenotype, CD45RC−, following adoptive transfer in the absence of Ag (14). Further studies suggest that the reversion can be prevented by the presence of Ag (15). Similarly, it has been shown in mice that a significant proportion of CD4 T cells, dividing in response to Mls+ Ags, carries the naive phenotype (16). Thus, the stability of naive and memory phenotype and the role of Ag in resting CD4 T cells remain contentious issues.

While the above studies indicate that a reversion from memory to naive phenotype can occur, there is evidence that effector CD4 T cells can express phenotypic markers that are expressed on naive CD4 T cells (17) and, further, that there are no known phenotypic differences between primary effector and secondary effector CD4 T cells (7). This suggests the possibility that memory phenotype CD4 T cells that appear to reacquire the naive phenotype may, in fact, be recently activated effector cells. In addition, in the murine Mls system, it is unclear whether cells responding to Mls+ Ags acquired the naive phenotype or did not change phenotype following contact with Mls+ Ag.

In this study the long term phenotypes of resting naive and memory CD4 T cells in mice are analyzed over time in an adoptive transfer model, using both TCR transgenic (Tg) and nontransgenic donors, to determine under what circumstances the phenotype will change and how this reflects the functional capabilities of the transferred CD4 T cells. Adoptive transfer of CD4 T cells from TCR Tg mice allows the monitoring of naive and memory phenotypes in CD4 T cells expressing the transgenic TCR-αβ pair, which have little potential to respond to environmental Ag (18), and CD4 T cells bearing polyclonal TCRs, which do have the potential to respond to an environmental Ag. Our findings indicate that as a population, memory phenotype CD4 T cells, whether a
polyclonal population from normal mice or an Ag-specific population from TCR Tg mice, can retain the memory phenotype over time with no evidence of permanent reversion. Transferred populations of naive CD4 T also retain the naive phenotype over time, and a memory phenotype is only observed in CD4 T cells that have the potential for antigenic exposure. Furthermore, the phenotypically converted cells function as memory CD4 T cells by producing the mature memory cytokines IL-4 and IFN-γ. In addition, we find that the presence of Ag is not necessary for maintaining a memory phenotype.

**Materials and Methods**

**Mice**

The AND TCR-αβ transgenic mice (TCR specific for pigeon cytochrome c) were derived from a heterozygous mouse obtained from J. Kaye (Scripps Institute, La Jolla, CA) (19). Subsequent generations of TCR transgenic mice were maintained as heterozygotes on a (B10.A(5R) x C3H)F1 background. B6.PL-Thyl1a/CyLy5.1 (CD45RB high, L-selectin high) were isolated from cytochrome c and a memory phenotype is only observed in CD4 T cells that have undergone considerable expansion when transferred in relatively small numbers (22–26), with memory T cells showing a greater potential for expansion than naive T cells (26–28). As it has been shown that the final peripheral pool size will reconstitute to the same degree regardless of the starting cell number (23, 25), we transferred a relatively large number of CD4 T cells (1.5 × 10^7) to our T-deficient hosts to establish stable populations with minimal expansion.

**Adoptive transfers**

Recipient mice used in adoptive transfer experiments underwent a surgical thymectomy at 5–6 wk of age and were allowed to recover for at least 2 wk before being used in experiments. On the day of transfer, recipient mice were lethally irradiated (900 rad) and reconstituted with 8 × 10^3 syngeneic T-depleted bone marrow cells and 1.5 × 10^7 naive or 1–1.5 × 10^8 memory CD4 T cells. Quantitation of cell recovery was estimated at various time points for individual animals as follows.

Single cell suspensions of spleen and a pool of mesenteric, inguinal, axillary, and brachial lymph nodes were prepared. Spleen and lymph node suspensions were counted to determine the total number of cells. The total number of donor CD4 T cells was calculated from the frequency of donor CD4 T cells determined by flow cytometric analysis and the total number of cells from each organ. Cell numbers in the PBLs of recipient mice were estimated as follows. Samples of blood from individual animals were counted using Turk’s solution to exclude RBCs to determine total cell numbers per microliter of blood. The number of donor CD4 T cells per microliter of blood and subsets thereof were calculated from the frequency of the cell subsets determined by flow cytometric analysis and the total number of cells per microliter of blood.

**Flow cytometry**

mAbs used to stain cell surface molecules were: FITC-labeled anti-Vα11 TCR (clone RR8-1; PharMingen), FITC-labeled anti-CD45.1 (clone A20; PharMingen), FITC-labeled anti-Thy-1.1 (clone OX-7; PharMingen), biotin-labeled anti-CD45RB (clone 16A; PharMingen), PE-labeled Vβ3 TCR (clone KJ25; PharMingen), PE-labeled anti-CD4 (clone IM7; PharMingen), and Quantum Red-labeled anti-CD4 (clone H129.19; Sigma, St. Louis, MO). Bound biotin-labeled anti-CD45RB was detected with Texas Red-avidin (Vector Laboratories, Burlingame, CA). RBCs were eliminated from PBLs before staining by osmotic lysis. Cells were fixed in 1% paraformaldehyde before analysis. Four-color analysis and FACS sorting were performed on a FACStar system (Becton Dickinson, Mountain, View, CA). FACS data were analyzed using LYSIS software and CellQuest software (Becton Dickinson).

**Cell cultures for cytokine assays**

Fifteen weeks posttransfer, CD4 T cells were purified as described above from pooled spleens of mice that received naive CD4 T cells from TCR transgenic mice. The CD4 T cells from these animals were FACS sorted into two populations: naive phenotype donor cells (Ly5.1+1, CD45RBlow, CD44+low) and memory phenotype donor cells (Ly5.1+, CD45RBhigh, CD44+high). The sorted cells were 97% and 94% pure, respectively. APCs were syngeneic T-depleted spleen cells prepared as described above. The purified naive and memory donor cells were each cultured with APCs at a 1:2 ratio of T:APC in the presence of pMCC (5 μg/ml) or Con A (1 μg/ml; Pharmacia Biotech, Piscataway, NJ) in EHAA medium supplemented with 5% FBS. To measure proliferation, cultures were pulsed after 24 h with 1 μCi/well of [3H]Tdr (ICN, Irvine, CA) in flat-bottom 96-well plates (Costar, Cambridge, MA) and harvested at 48 h onto glass-fiber filters. The incorporated radioactive thymidine was measured by liquid scintillation counting. From parallel cultures, supernatants were collected after 72 h and assayed for IL-4 and IFN-γ using ELISA kits (Endogen, Woburn, MA).

**Results**

Changes in phenotype following adoptive transfer of a population of naive CD4 T cells

To determine whether cell surface phenotypes of naive CD4 T cells are stable over time, purified populations of naive CD4 T cells were adoptively transferred into adult thymectomized, lethally irradiated, bone marrow-reconstituted (ATxBM) Ly5.1+ recipients. It is known that adoptively transferred T cells can undergo considerable expansion when transferred in relatively small numbers (22–26), with memory T cells showing a greater potential for expansion than naive T cells (26–28). As it has been shown that the final peripheral pool size will reconstitute to the same degree regardless of the starting cell number (23, 25), we transferred a relatively large number of CD4 T cells (1.5 × 10^7) to our T-deficient hosts to establish stable populations with minimal expansion.

Following adoptive transfer, the surface phenotype of PBLs was monitored over time by FACS analysis, as determined by expression of CD45RB and L-selectin. Naive Ly5.2+ CD4 donor cells (CD45RBhigh, L-selectinhigh) were isolated from cytochrome c-specific TCR Tg mice. While the vast majority (88–94%) in all experiments) of donor CD4 T cells expressed the Vα11/Vβ3 transgenic TCR whose Ag is not present in the environment, these mice were not crossed onto a recombination-activating gene-1 background; therefore, the starting population of donor cells contains a very small proportion of CD4 T cells that does not express the transgene, allowing us to examine the influence of environmental Ag on these donor naive CD4 T cells. At the time of transfer,
CD4+ lymphocytes. Naive cells were defined as CD45RB^{high}, L-selectin^{low} (upper right quadrant), and memory cells were defined as CD45RB^{low}, L-selectin^{low} (lower left quadrant). The percentage of donor CD4 T cells falling into each category is shown in each quadrant. The quadrants were determined using unmanipulated control animals stained on the same day. The starting population of transferred cells (left) in a naive CD4 T cell transfer from TCR transgenic donors is compared with PBL staining of a representative recipient animal 7 wk posttransfer (right). B, The percentage of donor (Ly5.1^+) CD4 T cells (x-axis) that display the naive phenotype, CD45RB^{high}, L-selectin^{low} (left panel), and those that display the memory phenotype, CD45RB^{low}, L-selectin^{low} (right panel), as defined in A are plotted over time (x-axis). Results from individual animals in five naive CD4 T cell transfers (n = 2, 3, 4, or 6) are shown.

FIGURE 1. Phenotypic changes are seen in donor naive CD4 T cells. A, Naive and memory phenotypes were defined by FACS dot plots depicting expression of CD45RB vs L-selectin, gated on donor (Ly5.1^+) CD4^+ lymphocytes. Naive cells were defined as CD45RB^{high}, L-selectin^{low} (upper right quadrant), and memory cells were defined as CD45RB^{low}, L-selectin^{low} (lower left quadrant). The percentage of donor CD4 T cells falling into each category is shown in each quadrant. The quadrants were determined using unmanipulated control animals stained on the same day. The starting population of transferred cells (left) in a naive CD4 T cell transfer from TCR transgenic donors is compared with PBL staining of a representative recipient animal 7 wk posttransfer (right). B, The percentage of donor (Ly5.1^+) CD4 T cells (x-axis) that display the naive phenotype, CD45RB^{high}, L-selectin^{low} (left panel), and those that display the memory phenotype, CD45RB^{low}, L-selectin^{low} (right panel), as defined in A are plotted over time (x-axis). Results from individual animals in five naive CD4 T cell transfers (n = 2, 3, 4, or 6) are shown.

approximately 90% of the CD4 T cells expressed high levels of both CD45RB and L-selectin (Fig. 1A). Thymectomy of the recipients appeared to be complete, as no host-derived CD4 T cells could be detected by staining of PBLs with Ab against the Ly5.1 allele during the analysis period (data not shown). By 7 wk posttransfer, analysis of CD4^+ PBLs showed that half of the total donor population (CD4^+, Ly5.1^+) had lost the naive phenotype, becoming CD45RB^{low}. Of these, 26% acquired the memory phenotype, defined here as CD45RB^{low}, L-selectin^{low}. The remaining donor CD4 T cells had an intermediate phenotype, perhaps indicating transitional stages. A summary of multiple time points from individual animals in five experiments is shown in Fig. 1B. Phenotypic changes occur as early as 3 wk posttransfer, with a dramatic loss of both CD45RB and L-selectin expression by 15 wk posttransfer. It should be noted that the change appears stable, in that the naive phenotype of the donor cells is not significantly restored over time.

These findings indicate that as a population, naive CD4 T cells derived from TCR Tg mice gradually lose the naive phenotype, acquire the CD45RB^{low}, L-selectin^{low} memory phenotype, and maintain this memory phenotype over time. Of particular interest was whether the cells that express the TCR transgene show an alteration in their phenotype.

Donor CD4 T cells that maintain expression of the Va11 TCR transgene maintain the naive phenotype following transfer

The starting population of naive CD4 donor T cells expresses the Va11/N/3 transgenes on 88–94% of cells and can respond well to moth or pigeon cytochrome c. However, the appropriate Ag is not present in the environment in that few, if any, memory cells expressing the Va11/N/3 transgenes develop in these mice (18). CD4 T cells with a memory phenotype can be isolated from TCR Tg mice; however, they express transgenic Vβ-chains paired with endogenous Vα-chains, creating TCRs that may be responsive to environmental Ags (18, 29). Therefore, it was of interest to determine whether the accumulation of donor CD4 T cells with a memory phenotype following transfer of naive CD4 T cells was derived from donor CD4 T cells expressing endogenous Vα-chains. To determine this, we further dissected the naive CD4 T cell donor population at each time point posttransfer to determine whether cells displaying the memory phenotype expressed the Va11 transgene. In Fig. 2A, the expression of Va11 in the starting population of naive donor CD4 T cells is compared with Va11 expression in the donor cells of an animal 12 wk posttransfer. The majority of the starting donor CD4 population has high expression of Va11, which is greatly diminished 12 wk following transfer. There is a large proportion of Va11-negative donor CD4 T cells as well as a population of donor CD4 T cells exhibiting an intermediate level of expression of Va11. These intermediate Va11-expressing cells may have also up-regulated expression of a second TCR that has been documented in these transgenic mice (18, 30) (our own unpublished observations) as well as in other transgenic systems (29, 31–33). For this reason, the Va11 intermediate cells were treated as Va11-negative CD4 T cells in examining expression of the transgene within the total donor population in all subsequent analyses. Fig. 2B (left panel) shows that the percentage of Va11^{high} donor CD4 T cells within the total donor population drops very early following adoptive transfer and continues to fall over time. This was not due to a selective and continual expansion of Va11-negative CD4 T cells within the donor population while the Va11^{+} population remained constant, as quantitation of donor cell recovery at various time points showed that the total donor population remained relatively stable and actually slightly declined at later time points (Fig. 2B, right panel). This suggests that the shift to the memory phenotype was a property of donor cells expressing endogenous Vα-chains. However, most interestingly, those donor...
cells that maintained the Vα11 transgene were primarily naive by phenotype. This is illustrated in Fig. 3, which compares the numbers of naive and memory phenotype donor cells among the Vα11+ and Vα11− donor CD4 T cells recovered from spleen and lymph nodes of recipients at a few representative time points after transfer. There is a decrease in the number of Vα11+ donor cells expressing the naive phenotype (Fig. 3A, solid bars). Most of these cells are not entering the Vα11+ memory pool in that only a few Vα11+ donor cells (<7% of the total donor population) have converted to the memory phenotype (Fig. 3A, hatched bars). Furthermore, there is an increase in the number of Vα11− cells expressing the memory phenotype (Fig. 3B, hatched bars), while a similar increase in memory phenotype is not seen in the Vα11+ donor cells (Fig. 3A, hatched bars). This suggests that in the absence of Ag, naive CD4 T cells will maintain the naive phenotype, and only those donor cells that have the capability of responding to environmental Ags will take on and maintain the memory phenotype. At this time, it is unclear whether the Vα11− cells that have the memory phenotype arose from naive donor cells that were stimulated and changed to the memory phenotype or were a small population of memory phenotype cells that accompanied the original naive cell inoculum, which subsequently expanded.

Regardless, taken together, the above results indicate that within a population of adoptively transferred naive CD4 T cells from a TCR Tg donor, a change from naive phenotype to memory phenotype occurs over time, but the resulting memory pool is expressing little to no Vα11 transgene. The vast majority of Vα11+ donor cells maintains the naive phenotype in the absence of the nominal Ag. This suggests that the phenotypic change from naive to memory is due to antigenic challenge present in the environment, and maintenance of the naive phenotype is mediated by maintenance of the transgenic TCR whose Ag is not present in the environment. Furthermore, the transferred cells that displayed the memory phenotype retained this phenotype over time without evidence of reversion.

Naive donor CD4 T cells that convert to a memory phenotype function as memory CD4 T cells, while those that retain the naive phenotype still function as naive CD4 T cells

Because a change of phenotype was seen in the total donor population of cells transferred from a TCR Tg donor, it was important to know whether this also reflected a change in the functional capabilities of these cells. This was a particularly interesting point, since there was a small proportion Vα11+ donor cells that changed to a memory phenotype in the presumed absence of Ag. To do this, CD4 T cells were recovered from the spleens of animals that received naive CD4 T cells from a TCR Tg donor at 15 wk following transfer. At this time point, it was possible to recover splenic memory cells that were comprised of a significant enough proportion of Vα11+ memory cells to assess whether they were capable of functioning as memory CD4 T cells in response to specific Ag, cytochrome c peptide (pMCC). The donor CD4 T cells were sorted into two populations: one displaying a naive phenotype and a second displaying a memory phenotype. The sorted donor cell populations were then cultured in vitro with APCs and either pMCC to stimulate Vα11+ donor cells or Con A to stimulate the total CD4 T cell population. The functional status of the donor cells was assessed by measuring the production of IL-4 and IFN-γ, cytokines characteristic of memory CD4 T cells (reviewed in Ref. 34). Both sorted donor populations proliferated in response to pMCC and Con A (Fig. 4). When stimulated with pMCC, the sorted naive CD4 T cells did not produce significant levels of the memory cytokines IL-4 and IFN-γ, although they responded to pMCC and Con A by proliferation (Fig. 4). The memory phenotype donor cells produced high levels of IL-4 and IFN-γ following stimulation by both pMCC and Con A. These data indicate that CD45RBhigh
L-selectin high CD4 T cells respond like naive CD4 T cells, producing IL-2 without producing IL-4 and IFN-γ. When a change in phenotype to CD45RB low L-selectin low takes place, the CD4 T cells produce IL-4 and IFN-γ, characteristic of becoming a memory CD4 T cell.

Memory phenotype CD4 T cells retain the memory phenotype over time regardless of whether they are polyclonal or Ag specific

In the previous experiments, populations of donor CD4 T cells with a memory phenotype gradually appeared among donor cells that did not express the Vα11/Vβ3 transgenic TCR pair and appeared to maintain the memory phenotype. However, this population may have a limited range of specificities due to pairing of the Tg Vβ3 chain with endogenous Vα-chains, making it difficult to predict whether they are capable of responding to environmental Ags. Thus we were interested in examining whether memory phenotype is maintained in polyclonal populations of memory CD4 T cells with diverse specificities. In experiments parallel to the naive TCR Tg donor cell transfers, memory CD4 T cells (CD45RBlow, L-selectinlow) were donated from nontransgenic mice (Ly5.2+) to ATxBM recipients (Ly5.1+), and maintenance of the memory phenotype in PBLs was examined over time. As seen in Fig. 5A, approximately 85% of the donor cells were of the memory phenotype (CD45RBlow, L-selectinlow). In contrast to the changes seen in the naive donor CD4 T cells, the memory donor CD4 T cells did not change their phenotypic profile (Fig. 5, A and B).

Since the memory donor CD4 T cells in Fig. 5 were from a nontransgenic donor and were, therefore, a polyclonal population of memory CD4 T cells capable of responding to environmental Ags, it was possible that the memory phenotype was retained by
periodic stimulation of the memory CD4 T cells. To address the question of whether changes in the phenotype of memory CD4 T cells might take place in the absence of Ag, it was necessary to obtain a population of memory CD4 T cells (CD45RB<sup>low</sup>, L-selectin<sup>low</sup>) from the cytochrome c-specific TCR Tg mice. It is known that immunization of TCR Tg mice by conventional methods such as administration of peptide with an appropriate adjuvant does not give rise to a pool of resting memory CD4 T cells (36) to generate a pool of resting memory CD4 T cells in vivo (35) (our unpublished observations). In addition, TCR Tg CD4 T cells stimulated in vitro do not appear to acquire all the characteristics of memory CD4 T cells (7) (our unpublished observations). Therefore, we adapted a previously published method to generate a pool of resting memory CD4 T cells in vivo (36) to transfer into our T-deficient hosts. Briefly, CD4 T cells from TCR Tg mice were stimulated in vitro to produce effector CD4 T cells. These cells were then parked in sublethally irradiated, euthymic, Tg mice were stimulated in vitro to produce effector CD4 T cells. Four to six weeks following the intermediate transfer, when sufficient numbers of Tg<sup>+</sup> CD4 T cells had become resting CD45RB<sup>low</sup> memory cells as judged by FACS analysis of PBLs, memory CD4 T cells were isolated from the spleens of the intermediate hosts as CD45RB<sup>low</sup> CD4 T cells. Approximately 30% of the donor memory CD4 T cells in these preparations were transgene positive, and the remaining memory cells were derived from the intermediate host. All the memory CD4 T cells in these preparations were resting cells as judged by forward and side light scatter on the FACS with a CD45RB<sup>low</sup>, L-selectin<sup>low</sup>, CD4<sup>+</sup> phenotype. These memory CD4 T cells were then transferred to their final ATxBM recipients for the phenotypic analysis over time. For these analyses, memory phenotype was assessed in terms of CD45RB phenotype alone due to the need to include markers to distinguish both donor types (Ly5.2, Thy1.2 Tg CD4 T cells and Ly5.2, Thy1.1 non-Tg CD4 T cells) from the recipient type (Ly5.1, Thy1.2). Fig. 6A shows that similar to the polyclonal TCR Tg-negative memory CD4 T cells, the TCR Tg<sup>+</sup> memory CD4 T cell population can retain the memory phenotype through the first 10 wk posttransfer. It should be noted in Fig. 6A that two mice do not fully retain the CD45RB<sup>low</sup> memory phenotype. However, this does not reflect a permanent change, as it is only seen for each animal at one time point and not at subsequent time points. This is illustrated for one such recipient in Fig. 6B, where the reappearance of the naive phenotype appears to be an outgrowth of a group of Tg<sup>+</sup> cells. These cells may have somehow become activated (perhaps through a second TCR) and may represent effector cells, which, like naive cells, can express high levels of CD45RB (7, 17) (our unpublished observations).

Unlike the studies of transferred polyclonal memory CD4 T cells, analysis of the Tg<sup>+</sup> memory cells was not conducted beyond 15 wk because the Tg<sup>+</sup> memory CD4 T cells were barely detectable beyond 7 wk (Fig. 6B). The Tg<sup>+</sup> donor CD4 T cells decrease in frequency among the total donor population with time and can barely be detected 10 wk posttransfer. This may be due to the outgrowth of the polyclonal memory cells diluting out the Tg<sup>+</sup> donor cells, thus placing them below the limits of detection in our assay. Another possibility is that the Tg<sup>+</sup> memory cells do not persist in the absence of Ag and can no longer be found in our recipients. However, without further study it is difficult to make any strong conclusions regarding this observation. The important point that can be made by these experiments is that memory CD4 T cells are capable of retaining the memory phenotype over time, independent of the presence or absence of Ag, without evidence of permanent reversions.
Discussion

We have shown that the phenotypes of naive and memory CD4 T cells are reflective of their state of priming. In the case of naive CD4 T cells (CD45RB<sub>high</sub> L-selectin<sub>high</sub>), there are two important points to be made. The first is that although changes to a memory phenotype (CD45RB<sub>low</sub> L-selectin<sub>low</sub>) are observed over time, results from individual animals in two Tg<sub>α</sub>11 memory CD4 T cell transfers (n = 3) are shown. Results from one representative experiment (n = 3) are shown.

A significant proportion of naive CD4 T cells from a TCR Tg<sub>α</sub>11/V<sub>β</sub>3 transgenic TCR. Those donor cells that express high levels of the transgene also maintained the naive phenotype. The possibility of cross-reactive Ags cannot be ruled out as a cause of the appearance of Tg<sup>+</sup> memory cells. However, we believe that this is unlikely, as this population did not increase with time and in all but one animal represented <6% of the total donor population.

The appearance of a large number of memory phenotype Tg<sup>+</sup> donor CD4 T cells may be due to environmental pressures causing expansion of a small contaminating population of memory phenotype Tg<sup>+</sup> CD4 T cells in the starting inoculum or may be due to exposure to Ag, driving the change in phenotype accompanied by expansion of these activated cells. Indeed, it has been suggested that homeostatic mechanisms exist to maintain a particular ratio of naive and memory CD4 T cells within an animal (14), and therefore, in situations where almost pure populations of naive CD4 T cells are transferred, there may be a need to generate a population of memory cells, whether driven by Ag or not. However, we favor the argument that the expansion of the memory phenotype cells is Ag driven due to the fact that the small population of V<sub>α</sub>11<sup>+</sup> memory phenotype CD4 T cells does not continue to grow (Fig. 3A), while the V<sub>α</sub>11<sup>+</sup> memory population continues to expand. However, regardless of the origin of these memory phenotype donor cells, the fact remains that they maintain the memory phenotype over time, without restoration of the naive phenotype.

Transferred CD4 T cells of a memory phenotype showed no significant reversion to a naive phenotype as determined in three types of experiments. First, a large proportion of the naive CD4 T cells converted to a memory phenotype without gradual reversion to the naive phenotype. Second, transfers of polyclonal memory cells led to a stable donor population in which no reversion or reaccumulation of naive phenotype was seen. This finding is in

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Ag-specific memory cells are capable of retaining the memory phenotype over time in the absence of Ag. A. The percentage of total donor CD4 T cells (open circles) that retains the memory phenotype, CD45RB<sub>low</sub>, and the percentage of Tg<sup>+</sup> donor cells (filled circles) that retains the memory phenotype are shown over time. Results from individual animals in two Tg<sup>+</sup> memory CD4 T cell transfers (n = 3) are shown.

B. The frequency of transgene-positive donor cells among the total donor cells is represented by the total bar. Each bar represents an individual animal at a given time point and is divided into those transgene-positive donor cells that display the memory phenotype, CD45RB<sub>low</sub> (bottom, solid portion of bars), and those that display the naive phenotype, CD45RB<sub>high</sub> (top, hatched portion of bars). Results from one representative experiment (n = 3) are shown.
contrasts to previous studies showing that as many as one-third of polyclonal memory phenotype CD4 T cells in rats appeared to reexpress the naive phenotype (14). One possible explanation is that CD4 T cells expressing the naive phenotype defined by CD45R expression are, in fact, only effector CD4 T cells, as effector cells can share this phenotype with naive CD4 T cells. What was not determined was whether the CD4 T cells also expressed other markers of previously activated cells. In our studies memory phenotype was assessed by more than one marker. Furthermore, the mixture used in this study were maintained in filtered cages to minimize the presence of significant numbers of activated effector cells at any one time. Third, Ag-specific memory CD4 T cells derived from ANB transgenic mice retained the CD45RBlo memory phenotype in most recipients. Two animals showed a transient re-expression of the naive CD45RBlo phenotype for one time point each. This re-expression of CD45RB may be due to an environmental Ag stimulating a second TCR on these donor cells, thus producing effector cells that express CD45RB. Still another possibility is that the change in phenotype is not Ag driven and is an effect of bystander activation, which has been reported to occur in human CD4 T cells (37) and murine CD8 T cells (38). Importantly, it should be noted that this re-expression does not reflect a permanent reversion to a naive phenotype. Taken together, these studies indicate that memory CD4 T cells retain a memory phenotype independent of Ag.

These data support the idea that naive and memory CD4 T cells can be reliably identified by their expression of a particular combination of cell surface molecules. Because expression of some molecules is shared by naive and effector or by memory and effector CD4 T cells, it is important to examine more than one of these markers to effectively characterize the stage of differentiation (naive or memory) or the state of activation (effector) of CD4 T cells. Ag appears to be required to recruit naive CD4 T cells into the memory compartment, leading to a change in cell surface phenotype, but is not necessary to maintain the memory phenotype.

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