# Involvement of Avidity for Major Histocompatibility Complex in Homeostasis of Naive and Memory T Cells

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## Abstract

The requirements for survival and self-renewal of peripheral T cells and the nature of mechanisms controlling the size of the naive and memory pool are not completely understood. Here, we examine the involvement of the major histocompatibility complex (MHC) in survival and homeostatic expansion of naive and memory T cells. We show that the homeostatic behavior of naive T cell receptor (TCR)-transgenic T cells can be deduced by the expression levels of TCR and CD5, a negative regulator of TCR signaling. Both these factors determine the strength of TCR stimulation by MHC-derived signals. We further show that, similarly to naive T cells, MHC-derived signals influence the homeostatic expansion capacity of memory T cells under lymphopenic conditions. In contrast to naive T cells, however, memory T cells can reach a homeostatic equilibrium, in which survival/self-renewal of each clone is dissociated from their avidity for MHC-derived signals.

Key words: major histocompatibility complex • CD4-positive T lymphocytes • immunological memory • T cell receptor

## Introduction

In an intact immune system, homeostatic mechanisms keep peripheral T cell numbers relatively constant despite incidental T cell loss and continuous T cell production in the thymus and expansion during the immune response (1). The immune system, however, has the additional task of preserving both the diversity of naive T cells, able to respond to unpredictable antigenic challenges, and immunological memory to previously encountered pathogens. Homeostatic mechanisms are thus expected to be more complex than random T cell substitution and it is thought that naive and memory T cells are regulated independently (1).

Individual cells in multicellular organisms need signals from other cells to survive and proliferate and such survival/proliferative signals may determine cell numbers, especially if cells compete with one another for limiting amounts of such signals (2). A substantial body of evidence (3–11) indicates that long-term survival and homeostatic expansion of naive T cells depend on recognition of self-peptide:MHC (sp:MHC)* complexes. In contrast, memory T cells have generally been found less reliant on MHC-derived signals for their survival and homeostatic expansion

(4, 12–14). In addition to MHC-derived signals, cytokines also have an important contribution. IL-7 is indispensable for both naive CD4 and CD8 T cell survival and homeostatic expansion (15–17), whereas IL-15 supports maintenance of memory CD8 T cells (for a review, see reference 18). However, none of the common γ chain (γ$_c$)-using cytokines seem to play a role in promoting survival of memory CD4 T cells (19, 20) and no other cytokine with such function has been identified yet.

The capacity for survival and homeostatic expansion of individual T cell clones from polyclonal mice is unknown, but the use of TCR-transgenic mice in the study of homeostasis has made apparent that there is substantial variability in the behavior of peripheral naive T cells. For instance, the total number of peripheral T cells in various TCR-transgenic strains ranges over two orders of magnitude (21, 22). Furthermore, TCR-transgenic strains differ in the extent of cell density-dependent homeostatic expansion. For example, after transfer into lymphopenic hosts, some TCR-transgenic T cells divide extensively, while others divide only marginally (22–26). Although the factors responsible for such variability remain elusive, in recent studies, the extent of naive T cell survival and homeostatic expansion has been correlated with avidity for sp:MHC complexes (27–29).

In this study we show that the survival and homeostatic expansion capacity of naive TCR-transgenic CD4 T cells

can be deduced by the level of TCR transgene expression, together with that of CD5, a negative regulator of TCR signaling. These two parameters are responsible for the heterogeneity in avidity for sp:MHC that is displayed by naive TCR-transgenic CD4 T cells. We further show that, similarly to naive T cells, the homeostatic expansion of memory T cells observed under lymphopenic conditions is strongly influenced by the availability of MHC-derived signals. In contrast to naive T cells however, the survival/self-renewal capacity of memory T cells in T cell–replete hosts is dissociated from avidity for sp:MHC, favoring long-term stability of immunological memory.

## Materials and Methods

*Mice.* C5-specific A18 (30), HY-specific A1 (31), and pigeon cytochrome C (PCC)-specific AND (32) TCR-transgenic mice were kept on an $H2^k$ $Rag1^{-/-}$ ($C5^{-/-}$) genetic background. Wild-type A mice ($H2^a$) were used as donors of control polyclonal T cells. CD4 overexpression in A18 TCR-transgenic mice (4A18 mice) was achieved by crossing the A18 line to a newly generated line, expressing mouse CD4 under the human CD2 promoter, and the resulting mice were also kept on the $H2^k$ $Rag1^{-/-}$ ($C5^{-/-}$) background. Lymphopenic recipients of naive T cells were syngeneic $H2^k$ $Rag1^{-/-}$ ($C5^{-/-}$) mice. In some experiments syngeneic $H2^a$ $Rag2^{-/-}$ $Il2rg^{-/-}$ ($C5^{-/-}$) mice were used in addition to compare the effect of the IL-2 receptor γ chain (common γ chain) deficiency. Lymphopenic recipients of memory T cells were either allogeneic $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ (referred to as $H2^b$) or syngeneic $H2^a$ $Rag2^{-/-}$ $Il2rg^{-/-}$ ($C5^{-/-}$) mice (referred to as $H2^a$). All animal experiments were performed according to institutional guidelines and Home Office regulations.

*Generation of Memory T Cells and Cell Transfer.* Lymph node cells from A18, A1, or 4A18 TCR-transgenic mice were transferred together with syngeneic bone marrow–derived dendritic cells (DCs; 1:2 ratio) into either allogeneic $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ or syngeneic $H2^a$ $Rag2^{-/-}$ $Il2rg^{-/-}$ ($C5^{-/-}$) adoptive hosts. In allogeneic hosts, activation of A18 or 4A18 T cells occurred by presentation of host-derived C5 by syngeneic DCs, while in syngeneic hosts the cotransferred DCs were prepulsed with the C5 peptide epitope (C5p, VVSKHFSKSKKIPIT). In one experiment A18 and 4A18 T cells were activated in syngeneic hosts by an intravenous injection of 10 nmol C5p and 50 μg LPS, to compare different immunization protocols. For activation of A1 T cells in both types of hosts, the cotransferred DCs were prepulsed in vitro with an HY peptide (HYp, REEALHQFRSGRKPI). Memory AND T cells were generated by activation of naive AND T cells in vitro with 10 μM cytochrome C peptide epitope (CCp, ANERADLIAYLKQATK – sequence corresponds to moth cytochrome C) for 3 d, followed by transfer of effector cells in antigen-free allogeneic $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ adoptive hosts. All memory T cells were isolated and used for secondary transfer >5 wk after primary transfer and immunization. Thymic reconstitution by splenic stem cells was avoided in all experiments involving transfer of naive T cells into syngeneic lymphopenic hosts, by using only lymph node cells as a source.

*Carboxyfluorescein Diacetate Succinimidyl Ester Labeling and Flow Cytometry.* For carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling cells were incubated with 2.5 μM CFSE for 15 min at 37°C in PBS and washed twice with fetal calf serum-containing medium before transfer into adoptive hosts. Recipients of naive T cells were syngeneic $Rag1^{-/-}$ hosts. Recipients of mem-

ory T cells were syngeneic $H2^a$ $Rag2^{-/-}$ $Il2rg^{-/-}$ ($H2^a$), allogeneic $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ ($H2^b$) or $H2^q$ $Rag2^{-/-}$ $Il2rg^{-/-}$ ($H2^q$) and MHC-deficient $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ $H2Ab^{-/-}$ $b2m^{-/-}$ ($H2^-$) hosts. The mean number of CFSE-visualized divisions was calculated as the sum of the percentage of cells at each division multiplied by the division number and divided by 100. For flow cytometry cells were stained with fluorescent or biotin-labeled monoclonal antibodies (all from BD Biosciences unless otherwise stated) for 15 min on ice. IL-7Rα expression was assessed with biotin-labeled A7R34 monoclonal antibody. Four-color cytometry was performed on a FACSCalibur™ flow cytometer (Becton Dickinson).

*In Vitro Stimulation and IL-2 Production Assay.* Spleen and lymph node cells from naive TCR-transgenic mice were stimulated in 96-well plates (5–10 × $10^3$ naive T cells per well in the presence of $10^3$ bone marrow–derived syngeneic DCs per well) with the indicated amount of C5p or plate bound anti-CD3 antibody (145–2C11). IL-2 production was assessed on day 2 in culture supernatants, by an alamar blue-based CTLL-2 assay.

## Results

*TCR Density and Survival/Homeostatic Expansion Capacity of Naive T Cells.* The homeostatic behavior of different TCR-transgenic T cells varies considerably. We focused on three different TCR-transgenic strains selecting CD4 T cells with distinct antigenic specificity, namely the C5-specific A18 strain, the HY-specific A1 strain and the PCC-specific AND strain, all of which carry H2-E$^k$-restricted TCR-transgenes. The absolute number of peripheral T cells (splenic plus inguinal, axillary, and mesenteric lymph node T cells) in these strains ranges from just $6.0 \times 10^5$ ($\pm 1.5 \times 10^5$, $n = 12$) in A18 mice to $3.7 \times 10^7$ ($\pm 0.5 \times 10^7$, $n = 5$) in AND mice, while A1 mice are intermediate with $1.0 \times 10^7$ ($\pm 0.2 \times 10^7$, $n = 9$) T cells. Peripheral T cell numbers can be taken as measure of naive T cell survival, despite the fact that the various TCR-transgenic strains may also differ in their relative thymic output. This is because thymic production usually exceeds the requirements for naive T cell replenishment and many thymic emigrants are not incorporated into the peripheral T cell pool. In fact, thymic production even in the A18 strain is at near normal levels (21), which substantially exceeds the rate of thymocyte generation that has been calculated as the minimal requirement for populating the peripheral naive T cell pool (33). Thus, self-renewal of peripheral naive T cells is not required and this is supported by the lack of division of naive T cells in T cell–replete hosts (1). T cells from these TCR-transgenic strains also differ in the extent of homeostatic divisions they undergo upon transfer into lymphopenic hosts, which correlates with their survival in the original donors. A18 T cells rarely divide and AND T cells divide extensively, while A1 T cells divide more than A18 T cells (Fig. 1 A). Thus, the three TCR-transgenic T cell clones form a gradient in their capacity to survive and homeostatically expand.

Evaluation of TCR levels in naive T cells from the three TCR-transgenic strains revealed that each strain expresses a characteristic level of surface TCR (Fig. 1 A), with A18
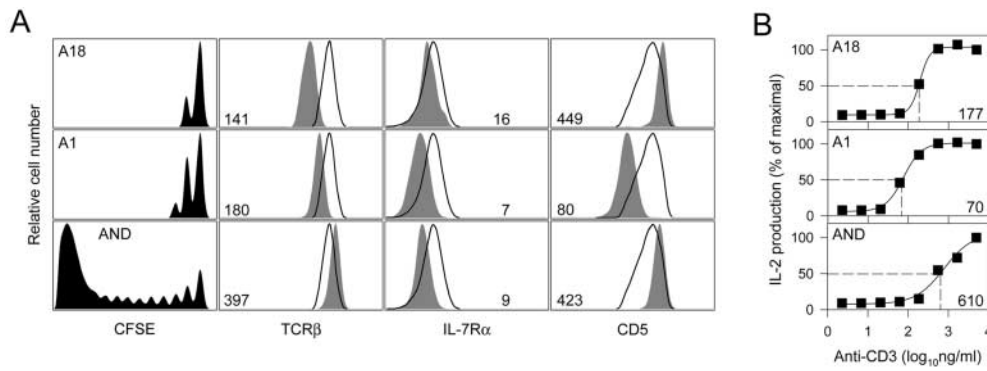
**Figure 1.** Homeostatic expansion capacity of naive TCR-transgenic T cells and TCR and CD5 density. (A) Comparison of lymphopenia-induced cell division, and expression levels of TCRβ, IL-7Rα, and CD5 in naive A18 (top row), A1 (middle), and AND T cells (bottom row). A18 and A1 T cells were analyzed for their CFSE profile at day 14 after transfer into syngeneic $Rag1^{-/-}$ hosts, while for AND T cells day 7 is shown. Levels of TCRβ, IL-7Rα, and CD5 in TCR-transgenic T cells (filled histograms) are compared with those in polyclonal T cells from A mice (open histograms). Numbers within the histogram plots represent the mean fluorescence intensity (MFI) of 4–5 mice of each strain. (B) IL-2 production (as % of maximal) in culture supernatants by TCR-transgenic T cells in response to the indicated amount of plate-bound anti-CD3. Numbers within the plots represent the mean concentration of anti-CD3 (ng/ml) that led to half the maximal IL-2 production in 3–4 mice of each strain.

and AND T cells expressing the lowest and highest levels, respectively, compared with polyclonal T cells. This correlation between TCR density and the absolute number of peripheral T cells (survival) or the number of homeostatic divisions in the three TCR-transgenic strains, strongly suggests that survival and homeostatic expansion capacity of naive TCR–transgenic CD4 T cells is influenced by TCR density. In contrast, the level of IL-7 receptor α chain (IL-7Rα) on TCR-transgenic T cells, which could be indicative of responsiveness to IL-7, was generally lower than that on polyclonal T cells and did not show any correlation with survival/homeostatic expansion capacity (Fig. 1 A).

As naive T cells depend on MHC-derived signals for their survival and homeostatic expansion, it could be argued that the heterogeneity in their capacity to survive and homeostatically expand reflects differential avidity for sp:MHC. Analysis of TCR density (Fig. 1 A) would thus suggest that the three TCR-transgenic strains differ in their avidity for sp:MHC as a result of differential TCR density. It has been hypothesized that T cell avidity for sp:MHC is reflected in the expression of negative regulators of TCR signaling, such as CD5, with the high avidity clones expressing high CD5 levels. Furthermore, CD5 expression could potentially influence the homeostatic behavior of T cells, by interfering with TCR stimulation by sp:MHC. We therefore analyzed CD5 expression in the three TCR-transgenic strains. CD5 levels in naive A18, A1, and AND T cells varied considerably, with A1 and AND T cells representing the lower and higher range of CD5 expression in polyclonal T cells (Fig. 1 A), as would be expected if their avidity were determined by TCR density. A18 T cells, however, expressed exceptionally high CD5 levels (Fig. 1 A), despite low TCR expression. The potential effect of CD5 expression on TCR signaling was assessed in vitro by calculating the amount of anti–CD3 that is required for half the maximal activation ($ED_{50}$) of each TCR-transgenic clone (Fig. 1 B). A1 T cells showed a low $ED_{50}$, while AND T cells were much less sensitive with almost ninefold higher $ED_{50}$ (Fig. 1 B). A18 T cells also showed an intermediate $ED_{50}$ (Fig. 1 B), indicating that the unexpectedly

high CD5 levels do not closely correlate with impaired responsiveness. However, responsiveness to anti-CD3 stimulation can also be influenced by TCR density and given the substantial differences in TCR levels between the TCR-transgenic T cells compared in this analysis, responsiveness to anti-CD3 stimulation cannot be taken as readout for CD5 function alone. Nevertheless, it is clear that the homeostatic behavior of TCR-transgenic T cells cannot be predicted by their sensitivity to in vitro antigenic stimulation or by their CD5 levels. Thus, although CD5 levels in A1 and AND T cells would indicate low and high avidity for sp:MHC, respectively, CD5 levels in A18 T cells seem more indicative of high avidity interaction, and therefore are difficult to reconcile with their low TCR expression and severely compromised survival and homeostatic expansion capacity. Two mutually exclusive hypotheses could explain this apparent inconsistency. (a) High CD5 levels are indicative of high avidity for sp:MHC in the periphery but the differential survival/homeostatic expansion capacity of naive T cells is not the result of differential avidity for sp:MHC. (b) The differential survival/homeostatic expansion capacity of naive T cells does result from differential avidity for sp:MHC but CD5 levels are not reflective of such avidity in the periphery.

*Survival/Homeostatic Expansion Capacity as a Function of CD5 Density.* To resolve whether the unexpectedly high levels CD5 in A18 T cells are indeed the result of high avidity for sp:MHC in the periphery, or alternatively, the result of fixing of CD5 levels during thymic development, we attempted to artificially enhance their avidity for sp:MHC. A18 mice were crossed with mice expressing a mouse CD4 transgene under the control of the human CD2 promoter. In the resulting cross, thymic selection gave rise to both $CD8^-$ and $CD8^+$ peripheral T cells (the latter being also $CD4^+$ due to the CD4 transgene), referred to as CD4 and CD8 4A18 T cells, respectively (Fig. 2 A). TCR density was slightly reduced in both CD4 and CD8 4A18 T cells, compared with the original A18 T cells, while CD4 levels were slightly increased (Fig. 2 A). Surprisingly, 4A18 T cells expressed considerably lower CD5
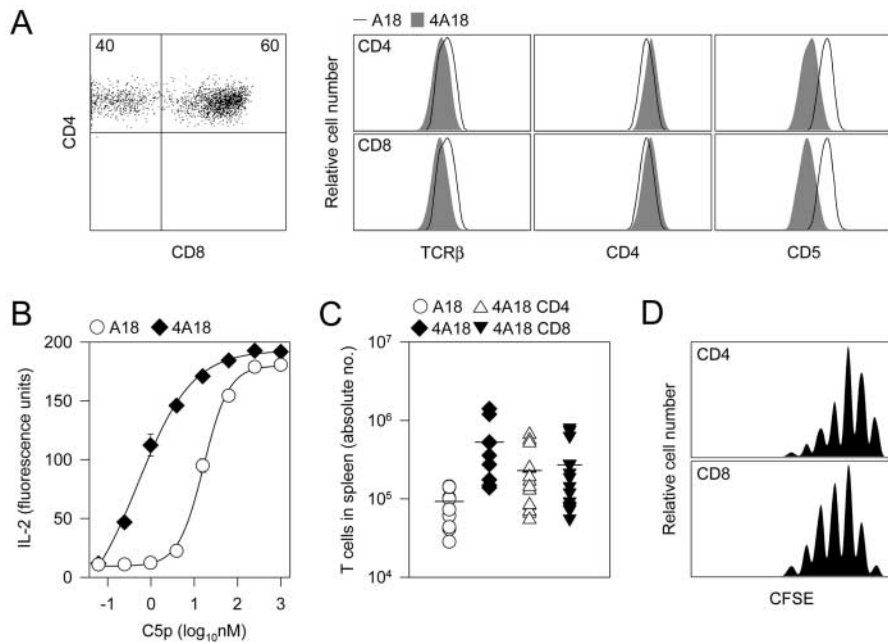
**Figure 2.** Effect of CD4 overexpression on homeostatic behavior of naive A18 T cells. (A) Gated TCR$^+$ 4A18 T cells can be further subdivided into CD8$^-$ and CD8$^+$, referred to as CD4 and CD8 4A18 T cells, respectively. Numbers within the quadrant represent the mean percentage of CD4 and CD8 4A18 T cells in splenic T cells. Histogram plots compare TCRβ, CD4, and CD5 levels between CD4 (top row) or CD8 (bottom row) 4A18 T cells (filled histograms) and the original A18 T cells (open histograms). (B) IL-2 production (fluorescence units) in culture supernatants by naive A18 (○) and 4A18 T cells (◆) in response to the indicated amount of C5p. (C) Absolute number of splenic T cells in the original A18 mice (○) and 4A18 T cells, either total (◆) or separated as CD4 (△) and CD8 (▼). Each point represents an individual mouse (P < 0.03 between A18 and either CD4 or CD8 4A18). (D) CFSE profiles of CD4 (top row) and CD8 4A18 T cells (bottom row) at day 7 after transfer into syngeneic $Rag1^{-/-}$ hosts.

levels than the original A18 T cells (Fig. 2 A). CD4-transgene expression in 4A18 T cells greatly enhanced their 'functional avidity' compared with the original A18 T cells, measured as a reduction of antigen concentration needed for half their maximal in vitro stimulation (Fig. 2 B). Notably, CD4-transgene expression restored the survival defect of A18 T cells as 4A18 mice had a ∼6-fold increase in the number of peripheral T cells, with the CD4 A18 T cells alone being responsible for a ∼2.5-fold increase (Fig. 2 C). Furthermore, the homeostatic expansion defect of A18 T cells was also restored and CFSE-labeled 4A18 T cells proliferated extensively upon transfer into syngeneic $Rag1^{-/-}$ hosts (Fig. 2 D). Together, these results indicate that the homeostatic defects of the original A18 strain can be attributed to excessively high CD5 expression. Furthermore, since their homeostatic defects could be alleviated by expression of a CD4 transgene, it seems unlikely that the high levels of CD5 in A18 T cells reflect high avidity for sp:MHC.

*Survival/Homeostatic Expansion Capacity as a Function of TCR Density.* Homeostatic behavior of the three TCR-transgenic strains is in good correlation with their respective TCR density. However, these strains also differ in CD5 expression, as well as in TCR usage, with presumably different fine-specificity for sp:MHC. To verify that survival and homeostatic expansion capacity of TCR-transgenic T cells can indeed be influenced by TCR density, we studied TCR-transgenic T cells with different TCR density but with the same TCR sequence, keeping all other parameters constant. A1 TCR-transgenic mice homozygous for the TCR transgene (A1$^{hom}$) were compared with heterozygous mice (A1$^{het}$), in which TCR density is approximately half of that in A1$^{hom}$ mice (Fig. 3, A and B). A similar reduction was also obvious with anti-CD3 staining (unpublished data). Interestingly, A1$^{het}$ T cells expressed

equivalent CD5 levels compared with A1$^{hom}$ T cells (Fig. 3 A), despite the reduction in TCR levels. A1$^{het}$ mice exhibited a reduction in peripheral T cell numbers (survival), measured either as percentage in peripheral blood or absolute number in secondary lymphoid organs, in comparison with A1$^{hom}$ mice (Fig. 3 B), indicating that homeostatic behavior of TCR-transgenic T cells is linked to their TCR expression levels.

*Predictive Value of TCR and CD5 Density in Homeostatic Behavior of Naive T Cells.* Alterations in both TCR and CD5 density can greatly influence survival and homeostatic expansion capacity of naive TCR-transgenic T cells. TCR density would be expected to have a positive influence on sp:MHC recognition. Although, it has been suggested that CD5 levels are reflective of high avidity interaction with sp:MHC and correlate directly with survival and homeostatic expansion capacity (34), the homeostatic behavior of A18 and 4A18 T cells does not support this view. In contrast, CD5 density would be expected to have a negative effect in the translation of MHC-derived signals and correlate inversely with survival and homeostatic expansion capacity. We have analyzed the relative effect of CD5 expression by each TCR-transgenic T cell clone studied here, by plotting the absolute number of splenic T cells in each strain (as a measure of survival capacity) against the respective CD5 levels (Fig. 4 A). Such analysis revealed that CD5 levels alone are not predictive of the homeostatic behavior of TCR-transgenic T cells (Fig. 4 A). In contrast, the homeostatic behavior of naive TCR-transgenic T cells can be modeled more accurately by a simple two-variable regression, in which the absolute number of splenic T cells is a function of both TCR and CD5 density. Indeed, by plotting the absolute number of splenic T cells in each strain against the respective TCR and CD5 density we obtained a linear correlation involv-
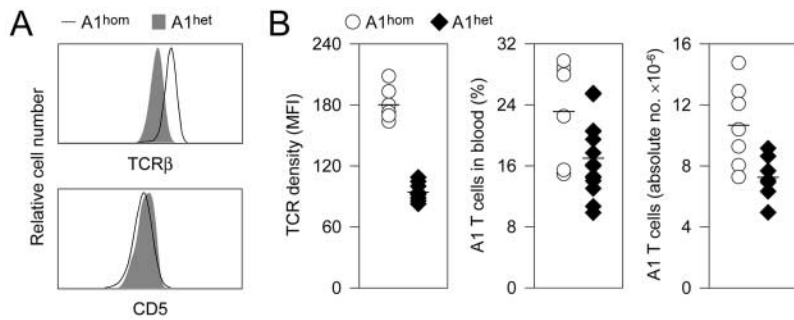
**Figure 3.** Comparison between naive A1 T cells homozygous (A1[hom] and heterozygous (A1[het]) for the TCR transgene. (A) TCRβ and CD5 levels assessed by flow cytometry on A1[hom] (open histograms) and A1[het] T cells (filled histograms). (B) Comparison of TCR density (MFI; $P < 0.0001$), % of T cells in peripheral blood ($P = 0.021$), and absolute number of peripheral splenic and lymph node T cells ($P = 0.011$) between A1[hom] (O) and A1[het] T cells (◆). Each point represents an individual mouse.

ing all TCR-transgenic strains (Fig. 4 B). It should be noted that the regression coefficient for CD5 has a negative value, which is in agreement with the negative impact of CD5 on TCR signaling. Therefore, only the combination of TCR and CD5 expression levels in naive T cells may predictably determine their homeostatic behavior.

We next examined whether alterations in TCR and CD5 levels have any effect on the in vivo homeostatic behavior of TCR-transgenic T cell clones. For this purpose, we cotransferred T cells clones, which differed in either TCR or CD5 density (keeping other parameters constant) into the same host and followed their ratio. Equal numbers of A1[hom] and A1[het] T cells (distinguished by a Thy-1 allelic difference) were cotransferred into $Rag1^{-/-}$ syngeneic female hosts. Homeostatic expansion of A1 T cells does not result in overt activation and A1 T cells retain a naive phenotype during their expansion (26). The percentage of A1[hom] and A1[het] T cells in total T cells after transfer (a readout of both survival and proliferation) was followed for 10 wk after transfer. Notably, the percentage of A1[het] T cells declined steadily with time (Fig. 5 A), confirming the overall reduced survival/homeostatic expansion capacity of these cells compared with A1[hom] T cells. Finally, cotransfer of mixtures of A18 with 4A18 T cells, which differ mainly in CD5 levels (distinguished by a Thy-1 allelic difference) into $Rag1^{-/-}$ hosts, led to a rapid decline of the proportion of A18 T cells, confirming the increased survival/homeostatic expansion capacity of CD4 4A18 T cells (Fig. 5 B). Together, these results show that naive TCR-transgenic T cell clones, which differ in either TCR

or CD5 expression, exhibit differential survival/homeostatic expansion capacity.

*Memory T Cell Homeostatic Expansion and the MHC.* In contrast to naive T cells, memory T cells have generally been found less reliant on MHC-derived signals for their survival and homeostatic expansion (12–14). Nevertheless, in at least two studies memory CD8 T cells did require sp: MHC contact for their survival and their homeostatic proliferation was greater in syngeneic than in allogeneic hosts (4, 35). Furthermore, allogeneic MHC class II conferred a competitive advantage to memory A1 T cells over MHC class II deficiency (14). We hypothesized that if allogeneic, nonselecting MHC can contribute to memory A1 T cell survival and expansion (14), then the contribution of syngeneic MHC should be greater, as avidity of T cells for syngeneic, selecting MHC is higher than for nonselecting MHC. To test this hypothesis, memory A1 T cells were labeled with CFSE and retransferred into syngeneic $H2^a$ $Rag2^{-/-}$ $Il2rg^{-/-}$ (H2[a]), allogeneic $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ (H2[b]) or $H2^q$ $Rag2^{-/-}$ $Il2rg^{-/-}$ (H2[q]) and MHC-deficient $H2^b$ $Rag2^{-/-}$ $Il2rg^{-/-}$ $H2Ab^{-/-}$ $b2m^{-/-}$ (H2[−]) hosts (Fig. 6 A). Both H2[b] and H2[q] are nonselecting haplotypes for the A1 TCR (unpublished data). Compared with allogeneic or MHC-deficient hosts, homeostatic proliferation of memory A1 T cells in syngeneic hosts was substantially greater (Fig. 6 A), indicating that syngeneic MHC-derived signals positively contribute to the homeostatic expansion of memory CD4 T cells.

To further test whether avidity for sp:MHC influences the homeostatic properties of memory T cells, we com-
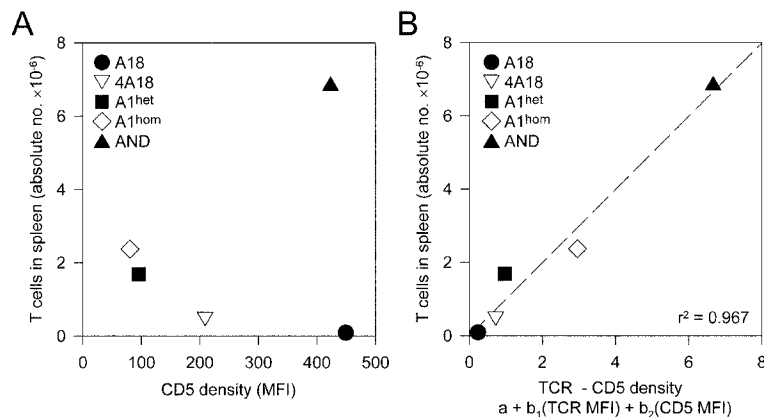


**Figure 4.** Survival/homeostatic expansion capacity of naive TCR-transgenic T cells as a function of TCR and CD5 density. Absolute number of splenic T cells in 5–12 mice from A18 (●), 4A18 (▽), A1[het] (■), A1[hom] (◇), or AND (▲) TCR-transgenic strains plotted against the CD5 density (MFI) (A), or TCR density (MFI) minus the CD5 density (MFI) of each T cell clone (B). The data in B are plotted according to a simple two-variable regression equation: $Y = a + b_1X_1 + b_2X_2$, where Y is the absolute number of splenic T cells and $X_1$ and $X_2$, the TCR and CD5 MFI, respectively. The regression coefficients were calculated as $a = -0.6299$, $b_1 = 0.0221$, and $b_2 = -0.005$, by SigmaPlot for Windows (SPSS Inc.).
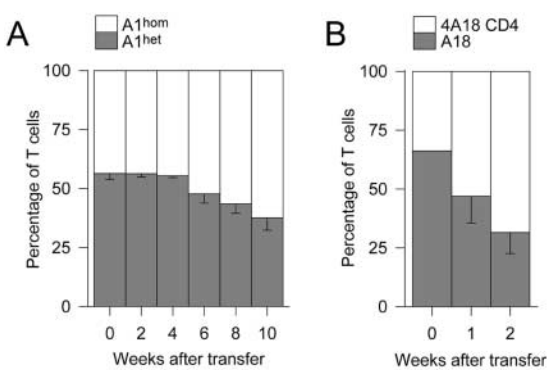
**Figure 5.** Survival/homeostatic expansion capacity of different naive TCR-transgenic T cell clones after cotransfer. (A) Percentage of A1$^{hom}$ (open bars) and A1$^{het}$ T cells (gray bars) in total T cells in peripheral blood, after cotransfer of naive A1$^{hom}$ and A1$^{het}$ T cells into syngeneic *Rag1$^{-/-}$* female hosts ($n = 5$). The 'week 0' time point represents the percentage at day 2 after transfer ($P = 0.024$, week 0 vs. week 8 and $P = 0.011$, week 0 vs. week 10). Identical results were obtained from secondary lymphoid organs at the end of the experiment. (B) Percentage of A18 (gray bars), CD4 4A18 T cells (open bars) in total peripheral blood T cells, after cotransfer of naive A18 and 4A18 T cells into syngeneic *Rag1$^{-/-}$* hosts ($n = 3$). One of two experiments with similar results is shown.

pared the homeostatic expansion of memory AND and A1 T cells. Naive AND T cells divide more extensively upon transfer into syngeneic lymphopenic recipients than naive A1 T cells (Fig. 1 A), reflecting higher avidity for their restricting H2-E$^k$ element (H2$^a$ haplotype). We reasoned that if MHC-derived signals influenced the homeostatic expansion of memory T cells similarly to naive T cells, then the hierarchy in homeostatic expansion potential between AND and A1 T cells should be maintained also at the memory state. CFSE-labeled memory A1 and AND T cells (10$^6$ of each type, distinguished by a Thy-1 allelic difference) were cotransferred into lymphopenic recipients expressing syngeneic MHC (H2$^a$ recipients). Memory AND T cells expanded vigorously, losing CFSE labeling by day 7 after transfer (Fig. 6 B) and exceeding more than 10-fold the injected number of cells (Fig. 6 C). In contrast, the expansion of memory A1 was much less pronounced (Fig. 6, B–D), and was partially inhibited by the presence of AND T cells, at 1 wk after transfer, when compared with memory A1 T cells transferred alone (Fig. 6 C). Thus, memory T cells with higher avidity for sp:MHC show a competitive advantage over lower avidity T cells, at early time points after a lymphopenic incident. Notably however, despite the extensive initial expansion of memory AND T cells and the partial inhibition of memory A1 T cell expansion, both population reached a homeostatic equilibrium as their absolute numbers remained stable over the following 3-mo observation period (Fig. 6, C and D) and A1 T cells were not displaced by AND T cells. In fact, similar numbers of memory A1 T cells were recovered at later time points from hosts that received memory A1 T cells either alone or together with memory AND T cells, indicating that at the steady-state, both memory populations have equal chances of survival and/or self-renewal.

*Effect of Avidity for MHC on Homeostatic Equilibrium of Memory T Cells.* To further examine the contribution of MHC-derived signals on memory T cell homeostasis we adoptively cotransferred naive TCR-transgenic T cells with different survival and homeostatic expansion capacity and immunized the recipient mice at the time of transfer by coinjection of antigen-pulsed syngeneic DCs. In this model, activation of T cells depends on the presence of syngeneic DCs, which reliably disappear 3–4 wk after transfer, causing the termination of the immune response and the initiation of the memory phase (36). The relative representation of each responding clone in the early memory pool can be greatly influenced by several parameters re-
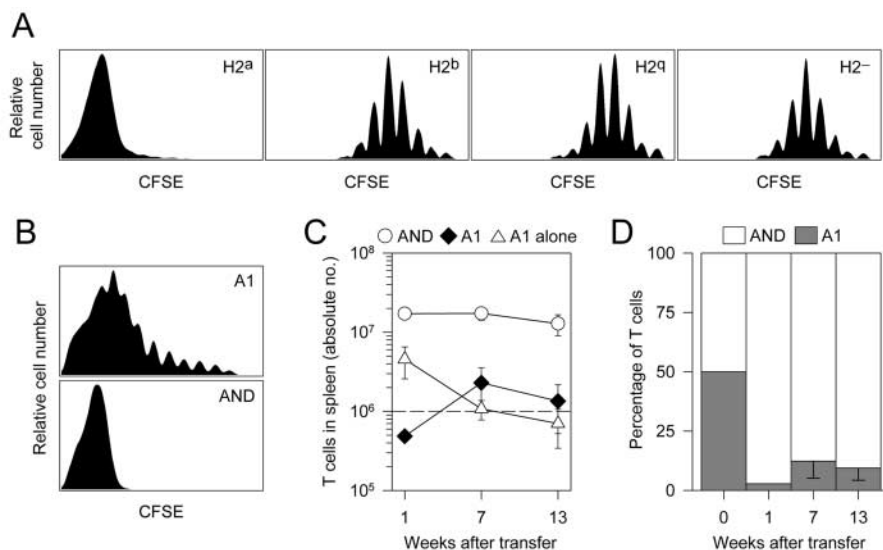


**Figure 6.** Effect of MHC-derived signals on homeostatic behavior of memory T cells. (A) CFSE profiles of memory A1 T cells 7 d after transfer (3 × 10$^6$ memory A1 T cells per recipient) into syngeneic (H2$^a$), allogeneic (H2$^b$ or H2$^q$), and MHC-deficient (H2$^-$) secondary recipients. (B) CFSE profiles of memory A1 (top) and AND T cells (bottom) 7 d after cotransfer into syngeneic H2$^a$ secondary recipients. (C) Absolute number of memory A1 (◆) and AND T cells (○) recovered from the spleen of secondary syngeneic *H2$^a$ Rag1$^{-/-}$* recipients, after cotransfer of memory A1 and AND T cells, in 1:1 ratio, or transfer of memory A1 T cells alone (△). Each time point represents the mean number of cells from the spleen of 2–3 recipient mice. The dashed line denotes the injected number of cells of each type. (D) Percentage of memory A1 (gray bars) and AND T cells (open bars) in total peripheral blood T cells of the recipients described in C. Each time point is the average of 2–5 mice. Similar results were obtained with either *H2$^a$ Rag2$^{-/-}$ Il2rg$^{-/-}$* (H2$^a$) or *H2$^a$ Rag1$^{-/-}$* recipients in additional experiments.

lating to antigen load and expansion/contraction kinetics during the effector phase. However, any changes in the composition of the long-term memory pool after the cotransferred DCs have disappeared, would reflect differential survival/self-renewal capacity of memory T cells, presumably as a result of heterogeneous avidity for sp:MHC or other survival/proliferative factors.

Naive A1$^{hom}$ and A1$^{het}$ T cells (distinguished by a Thy-1 allelic difference) were mixed in 1:1 ratio and transferred together with HYp-pulsed syngeneic DCs, into syngeneic $H2^a$ $Rag2^{-/-}$ $Il2rg^{-/-}$ (H2$^a$) lymphopenic female hosts. A1$^{hom}$ T cells expanded more than A1$^{het}$ T cells at week 1 after transfer (Fig. 7 A), indicating higher 'functional avidity'. However, from the second week after transfer, the percentage of A1$^{hom}$ and A1$^{het}$ memory T cells remained stable until the end of the observation period (Fig. 7 A). Comparable results were obtained with allogeneic (H2$^b$) or MHC-deficient lymphopenic hosts (unpublished data). In experiments similar to the one described in Fig. 7 A, naive A18 and 4A18 T cells were mixed and transferred into syngeneic $H2^a$ $Rag2^{-/-}$ $Il2rg^{-/-}$ (H2$^a$) lymphopenic hosts. To counterbalance the substantially enhanced 'functional avidity' of 4A18 T cells, a 10-fold excess of A18 T cells was injected compared with 4A18 T cells (Fig. 7 B). The recipients were immunized with a soluble C5p/LPS injection and the relative composition of the total T cell pool was monitored over time. Again, from the second week after transfer, the early memory pool remained remarkably stable until the end of the experiment (Fig. 7 B). In these experiments, similar numbers of memory T cells from each clone were recovered at the end of the experiment from hosts that received the combination of the two TCR-transgenic clones or each of the clones alone (unpublished data). Together, these results suggest that despite their differential homeostatic behavior at the naive, long-term survival/self-renewal capacity of TCR-transgenic T cells at the memory state is only minimally influenced by their avidity for sp:MHC. Thus, pairs of TCR-transgenic T cell clones with demonstrably different survival/homeostatic expansion capacity at the naive state reached homeostatic equilibrium at the memory state.

## Discussion

The immune system has to accommodate naive lymphocytes with a diverse repertoire of antigenic specificity and memory lymphocytes with specificities that were proven useful to the host. As naive and memory T cells have been assigned different functions, it stands to reason that their respective pools are regulated independently (2).

While is has been hypothesized that avidity for sp:MHC influences the survival/homeostatic expansion capacity of peripheral naive T cells, the lack of a simple way of determining a T cell's avidity, makes it impossible to test this hypothesis directly. Our results argue that avidity for sp:MHC, and thus survival/homeostatic expansion capacity of naive TCR-transgenic CD4 T cells is largely determined by TCR and CD5 density. This is a surprising finding, given that, in addition to TCR interaction with sp:MHC, other factors, such as IL-7, are also indispensable for naive T cell survival and homeostatic expansion. The definition of avidity for sp:MHC however, should include parameters that influence both MHC recognition (such as T cell avidity) and translation of the MHC-derived signal (such as sensitivity of the TCR signaling machinery). T cell avidity for an antigen-presenting cell is governed mainly by two parameters: TCR avidity and ligand availability (37). TCR avidity depends on the TCR amino acid sequence (which determines TCR affinity for sp:MHC complexes) and TCR density. Ligand availability on the other hand, is determined by the abundance and the physicochemical properties of the sp:MHC complex. Given however, that the identity of self-peptide(s) recognized by any particular TCR is usually unknown, ligand availability is difficult to measure.

The sensitivity of the TCR signaling machinery is thought to be influenced by sp:MHC recognition. It has been proposed that continuous TCR contact with sp:MHC is constantly coupled to the expression of negative regulators of TCR signaling (38). For instance, high CD5 expression is believed to be reflective of high avidity interactions with sp:MHC (34, 39, 40), and predictive of the survival/homeostatic expansion capacity of naive CD4 T cells (34). However, the exceptionally high CD5 levels ex-
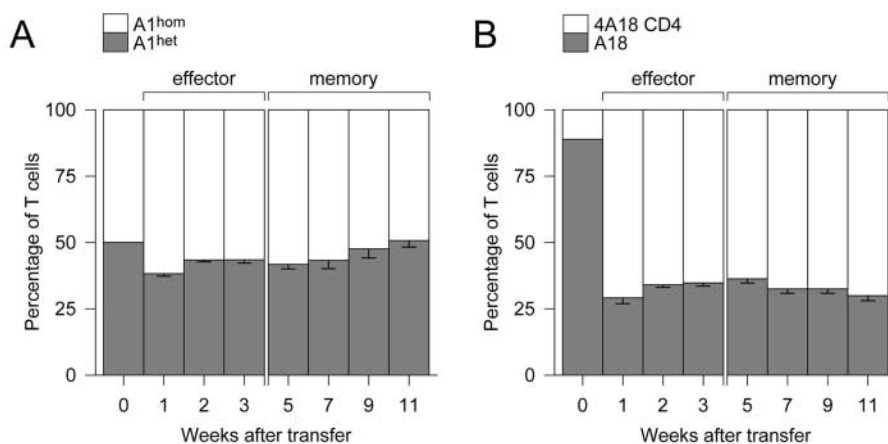
**Figure 7.** Apparent lack of competition between different memory T cell clones. (A) Naive A1$^{hom}$ and A1$^{het}$ T cells were cotransferred together with HY-pulsed syngeneic DCs into syngeneic H2$^a$ ($n = 5$) recipients and the percentage of A1$^{hom}$ (open bars) and A1$^{het}$ T cells (gray bars) in total peripheral blood T cells over time is shown. (B) Naive A18 and 4A18 T cells were cotransferred into syngeneic H2$^a$ ($n = 7$). Recipients were immunized with a C5p/LPS intravenously injection. The percentage of A18 (gray bars) and CD4 4A18 T cells (white bars) in total peripheral blood T cells over time is shown. Note that the time scale is in weeks up to week 3 and in alternate weeks for the rest of the experiment.

pressed by A18 T cells together with their severely reduced survival/homeostatic expansion capacity, argue that CD5 levels alone are not always predictive of the homeostatic behavior of naive CD4 T cells. Furthermore, rescue of the homeostatic defects of A18 T cells by a CD4 transgene makes it unlikely that high CD5 levels in A18 T cells reflect high avidity for sp:MHC in the periphery. At present, the mechanisms responsible for the differential CD5 expression by naive TCR-transgenic T cell clones are not entirely clear. CD5 levels could be continuously tuned according to the avidity of TCR interaction with sp:MHC in the periphery (34), or simply represent a consequence of thymic selection events, which is not further modified in the periphery (39). The reduction (instead of increase) of CD5 levels in naive 4A18 T cells compared with the original A18 T cells, and the essentially identical CD5 expression by naive A1$^{hom}$ with A1$^{het}$ T cells (which differ in their avidity for sp:MHC due to difference in TCR density), are more compatible with the latter possibility. It is likely that untimely onset and/or inappropriate levels of TCR transgene expression during thymic development of at least some TCR-transgenic T cells imprints on them a distinctive level of CD5 expression which is not in accordance with their avidity for sp:MHC in the periphery.

In addition to reflecting high avidity interaction with sp:MHC (at some stage of their development), CD5 reduces the responsiveness of naive T cells to TCR stimulation. As such, CD5 could have a negative effect on TCR stimulation by both strong antigenic stimuli, such as anti-CD3, and weak ligands, such as sp:MHC, which affect the homeostatic properties of naive T cells. The finding that CD5$^{hi}$ naive polyclonal T cells exhibit a higher homeostatic expansion potential compared with their CD5$^{lo}$ counterparts, despite being less sensitive to anti-CD3 stimulation, has been taken to imply that CD5 has no effect on TCR stimulation by sp:MHC (34). However, the homeostatic behavior of the TCR-transgenic clones we have studied here can only be explained by taking into account their respective CD5 levels. The heterogeneous survival/homeostatic expansion capacity of TCR-transgenic clones can be accurately modeled by incorporating TCR and CD5 density into a simple two-variable regression. The negative value of the coefficient for CD5 implies that high CD5 levels negatively influence the translation of sp:MHC-derived homeostatic signals and that CD5$^{hi}$ clones will be inferior to CD5$^{lo}$ clones in their survival/homeostatic expansion capacity (if all other parameters are equal). Although, restoration of survival/homeostatic expansion capacity in A18 T cells by expression of a CD4 transgene (which essentially reduces the levels of CD5) fits well with this hypothesis, the higher homeostatic expansion potential of CD5$^{hi}$ naive polyclonal T cells compared with their CD5$^{lo}$ counterparts (34), is in apparent disagreement. However, small changes in TCR density will have a much greater impact on homeostatic behavior than changes in CD5 density (since the coefficient for TCR has a much greater value than the coefficient for CD5). It is therefore expected that the influence of CD5 would be maximal at low TCR density (as in the case of A18 T cells), while at normal or high TCR densities, small differences in TCR avidity would have a major impact in homeostatic behavior. In the latter case, the influence of high CD5 levels, while still negative, would be masked by the higher TCR avidity. Furthermore, this model provides an explanation for the heterogeneity in survival and homeostatic expansion capacity of TCR-transgenic clones, as their TCR density (and presumably indirectly their CD5 density) is artificially determined by factors related to the onset and level of expression of the TCR transgenes (e.g., transcriptional efficiency of transgenic promoter/enhancer, transgene copy number, timing of expression, etc.).

Currently it is uncertain whether competition between peripheral naive T cells based on their avidity for sp:MHC can cause measurable changes in the polyclonal repertoire. However, continuous thymic export of naive T cells, with the full range of avidities, would counteract such selective pressure. Although competition between naive T cells in T cell-replete hosts was not addressed in this study, such competition based on avidity for MHC has been demonstrated in other systems. Studies with fetal liver chimeras between CD4-deficient and wild-type strains have revealed a reduction in the competitive fitness of CD4-deficient T helper-lineage cells, as their proportion was further reduced from the single-positive thymocyte compartment to the periphery (28).

Study of the requirement for sp:MHC recognition for memory T cell homeostasis has yielded conflicting results. In an initial study, survival of memory TCR-transgenic CD8 T cells could be sustained by a nonspecific (allogeneic) MHC class I but not by MHC class I deficiency, while for their homeostatic expansion the correct (syngeneic) MHC allele was required (4). Subsequently however, polyclonal memory CD8 T cells were shown to persist indefinitely and divide homeostatically in MHC class I–deficient mice (12). Similarly, memory TCR-transgenic CD4 T cells were found to be long-lived in the absence of MHC class II (13). More recently, we demonstrated that, despite comparable survival (at the population level) and homeostatic expansion of memory TCR-transgenic CD4 T cells in the presence of allogeneic MHC or in the absence of MHC, allogeneic MHC conferred an advantage over MHC deficiency under competitive conditions (14). The results in this study clearly demonstrate that, similarly to naive T cells, recognition of sp:MHC is an important factor in lymphopenia-induced homeostatic expansion of memory T cell and that memory T cells can sense the quality of MHC-derived signals (exemplified by comparing syngeneic and allogeneic MHC).

Memory T cells exhibit an increased capacity for homeostatic expansion, compared with naive T cells. Furthermore, TCR–transgenic T cell clones that rarely divide homeostatically as naive cells, divide extensively as memory T cells. Such enhancement of homeostatic expansion capacity at the memory state could be due to an overall enhancement of memory T cell avidity for sp:MHC, similarly to what has been described for the 'functional avidity'

of memory T cells (41, 42). Alternatively, memory T cells might be more responsive to homeostatic cues because sp: MHC recognition is not an absolute requirement for their survival and homeostatic expansion. In contrast to their naive counterparts, memory T cells can quickly reach a homeostatic equilibrium, in which different memory T cell clones seem to have equal chances of long-term survival/self-renewal. The stability of the composition of the memory pool could be due to either lack of competition between memory clones or to the fact that all memory clones exhibit comparable competitive fitness, as similar numbers of memory T cells from different clones were recovered when memory cells were generated alone or together into the same hosts. The apparent lack of competition between memory T cell clones has important implication for the understanding of the mechanisms responsible for the stability of immunological memory, as it suggests that once they have entered the memory pool, T cells have equal chances of survival. In this study, only pairs of T cell clones with the same antigenic specificity were compared, which might be competing for the same sp:MHC ligands, especially at the naive state. This comparison emphasizes the change in homeostatic behavior with the transition from the naive to the memory state, as it reveals equalization at the memory state of the survival/self-renewal capacity of TCR-transgenic T cell clones with demonstrably different homeostatic behavior at the naive state. However, the stability of a multiclonal memory pool relies on the relative competitive fitness of various memory clones with different antigenic specificity. Therefore, it would be important to examine the ability of naive and memory T cells with different antigenic specificity to reach homeostatic equilibrium. Previous studies with polyclonal CD8 T cells responding to a virus infection (43, 44) or CD4 T cells responding to a protein antigen (45) have shown that the distribution of dominant T cell clones at the memory phase of the response was proportionally similar to that at the peak of the response.

In conclusion, our results argue that survival/homeostatic expansion capacity of naive CD4 T cells can be predicted by their TCR and CD5 expression levels. In contrast, the transition into the memory state is accompanied by a "normalization" of the homeostatic behavior of memory T cell clones, irrespective of their avidity for sp:MHC. Nevertheless, avidity for sp:MHC can significantly influence the homeostatic expansion of memory T cells during acute lymphopenia. Thus, a lymphopenic incident may considerably alter the composition of the memory pool, due to differential homeostatic expansion of different memory clones. On the other hand, dissociation of long-term survival/self-renewal from avidity for sp:MHC in memory T cells may represent an adaptation to preserve all the memory T cell clones with TCR specificity proven useful to the host and thus to maintain immunological memory to all previously encountered pathogens.

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