Are Major Histocompatibility Complex Molecules Involved in the Survival of Naive CD4$^+$ T Cells?

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Abstract

The exact role of major histocompatibility complex (MHC) molecules in the peripheral survival of naive T cells is controversial, as some studies have suggested that they are critically required whereas others have suggested that they are not. Here we controlled for some of the features that differed among the earlier studies, and analyzed both the survival and expansion of naive CD4$^+$ T cells transferred into MHC syngeneic, allogeneic, or MHC negative environments. We found that naive T cells transferred into MHC negative or allogeneic environments often fail to survive because of rejection and/or competition by natural killer (NK) cells, rather than failure to recognize a particular MHC allele. In the absence of NK cells, naive CD4$^+$ T cells survived equally well regardless of the MHC type of the host. There was, however, an MHC requirement for extensive space-induced "homeostatic" expansion. Although the first few divisions occurred in the absence of MHC molecules, the cells did not continue to divide or transit to a CD44^{hi} phenotype. Surprisingly, this MHC requirement could be satisfied by alleles other than the restricting haplotype. Therefore, space-induced expansion and survival are two different phenomena displaying different MHC requirements. Memory CD4$^+$ T cells, whose survival and expansion showed no requirements for MHC molecules at all, dampened the space-induced expansion of naive cells, showing that the two populations are not independent in their requirements for peripheral niches.

Key words: T cell • naive • MHC • homeostasis

Introduction

Because resting T cells promptly die when cultured in vitro, a number of studies have searched for the signals and/or trophic factors that maintain them in vivo. In some studies, MHC molecules appeared to be critical. For example, newly developing CD4$^+$ T cells failed to efficiently populate peripheral tissues in mice that expressed MHC class II molecules only in the thymus (1–5), naive TCR transgenic (Tg) CD8$^+$ T cells disappeared within days after transfer into hosts lacking MHC class I molecules (6), and both naive CD4$^+$ and CD8$^+$ T cells waned if their antigen–specific

receptors were turned off by genetic means (7, 8). In other studies, however, MHC molecules were seemingly not essential for some or all aspects of survival. Two studies showed that naive CD4$^+$ T cells survived quite well when transferred into nonirradiated hosts that did not express MHC class II molecules (9, 10), and two others showed that "naive" CD4$^+$ T cells could also initiate space-induced "homeostatic" expansion when transferred into lymphopenic MHC class II$^-$ hosts (11, 12).

Three features may have contributed to this lack of consensus. First, because resting T cells proliferate in "empty" recipients that lack T cell populations (9, 11, 13–27; for review see reference 28), the differences in cell recoveries

Abbreviations used in this paper: β2m, β2-microglobulin; Tg, transgenic.

between MHC$^+$ versus MHC$^-$ hosts could be due to selective expansion rather than selective survival. Second, because transferred T cells can be rejected by host CTL or NK cells, their disappearance in allogeneic or MHC$^-$ hosts could be due to rejection rather than to lack of survival signals. Third, the cells used to date have either been mixed populations of normal T cells (9–12, 15, 18, 23–25, 27), or TCR Tgs cloned from MHC homozygous donors (6, 14, 15, 17–21, 25, 27, 29). Therefore, they might be alloreactive to one or more foreign major and/or minor histocompatibility antigens displayed by the hosts, and could either be expanded or signaled to die by recognition of these alleles. Finally, although some authors (9, 11, 17, 18, 20–22, 25), but not all (10–12, 15, 23, 24, 27), attempted to deplete memory T cells from their transferred populations, using known markers for memory cells, such depletion protocols may nevertheless miss a small number of memory T cells whose behavior can thus obscure the behavior of truly naive T cells.

Therefore, we designed a system in which we could control for all of these features. To account for selective expansion, we labeled the transferred T cells with a dye that allows for the visualization of cell division and transferred them into both lymphopenic ("empty") and "full" hosts. To control for alloreactivity, we generated a Tg mouse (Marilyn) using a TCR isolated from an (H-2^b×H-2^k)F$_1$ CD4$^+$ clone specific for the male H-Y antigen (30). These T cells are restricted by the MHC class II molecule, A^b, and do not react to the H-2^k haplotype, or to any potential hybrid MHC class II A/E molecules. To obtain a monoclonal population of naive CD4$^+$ T cells, we crossed the Marilyn mice to RAG-2$^{-/-}$ mice to prevent the expression of other TCRs (31, 32). All of the T cells in these mice express the Marilyn TCR and, because there do not seem to be any environmental antigens that cross-react with H-Y/A^b (33, 34), they are naive until deliberate immunization. To prevent rejection by CTL or NK cells, we transferred the Tg T cells into hosts deficient for both RAG-2 (to prevent lymphocyte development) and the cytokine receptor common γ chain, γc (to prevent development of NK cells; 35), and in some experiments, into recipients that additionally lacked β2-microglobulin (β2m; to further hamper development of NK cells).

With these controls in place, the data account for the previous differences and indicate that the situation is more complex than originally thought. Naive CD4$^+$ T cells survive equally well and initiate "homeostatic" space-induced expansion when transferred into syngeneic or allogeneic MHC$^+$ hosts or into MHC$^-$ hosts, as long as the hosts have no CTL or NK cells that can reject or compete with them. After several rounds of cell division, some of the cells transit to a new state, marked by an increase in CD44 expression that allows for continued proliferation. Although this transition is MHC dependent, it is not allele specific, suggesting that the signaling events may bypass the antigen/MHC-specific portions of the TCR, or may be triggered through non-TCR molecules, such as CD2, Thy-1, or CD4.

Materials and Methods

Mice

The Marilyn TCR Tg mice have recently been described (31). The (TCR-Vα1.1, Vβ6) T cells are specific for the male antigen H-Y (peptide NAGFNSNRANSSRSS from the Dby gene) presented by A^b (31, 36). C57BL/6 Marilyn TCR Tg founder mice were crossed to RAG-2–deficient mice (RAG-2$^-$, ninth backcross to B6, N9B6). They were also crossed to B10.BR mice and then backcrossed to H-2^b Marilyn TCR Tg-RAG-2$^-$ mice, and the resulting F$_1$ progeny were intercrossed to obtain H-2^k Marilyn TCR Tg RAG-2$^-$ mice. B10.BR mice were crossed to RAG-2$^-$ B6 mice and the resulting F$_1$ progeny were crossed to the F$_1$ progeny of crosses between mice deficient for the common interleukin receptor γ chain (γc, fourth backcross to B6) and B6.RAG-2$^-$, to obtain either H-2^b or H-2^k RAG-2$^-$/γc$^-$ mice after further intercrosses. Class II–deficient (H-2A$_\beta$$^{b-}$) mice were obtained from the CDTA Centre National de la Recherche Scientifique central animal facility on an N9B6 background and were crossed to RAG-2$^-$ B6 mice to obtain RAG-2$^{-/-}$/A$^{b-/-}$ double-deficient mice. H-2^k RAG-2$^-$ Marilyn TCR Tg mice were also crossed to H-2A^{b-} RAG-2$^-$ mice to obtain F$_1$ H-2^k × H-2A^{b-} RAG-2$^-$ mice. In parallel, the H-2A^{b-} RAG-2$^-$ mice were further crossed to the RAG-2$^-$/γc$^-$ to obtain triple-deficient mice. β2m-deficient mice (N9B6) were also obtained from CDTA and crossed to RAG-2$^-$/γc$^-$ to obtain triple-deficient mice. The two triple-deficient strains (H-2A^{b-} or β2m$^-$ on RAG-2$^-$/γc$^-$ background) were then intercrossed to get quadruple-deficient mice. All crosses were tested by PCR genotyping with the appropriate primers. All Marilyn Tg mice used in this study were RAG-2$^-$ and thereby expressed only monoclonal populations of T cells of naive CD4$^+$ T cells (31, 32). Congenic CD45.1 RAG-2$^-$ Tg Marilyn mice were also obtained.

The CD4$^+$ Rachel clone, specific for complexes of H-Y/H-2A^b, was obtained from an H-2^{bxk} F$_1$ female mouse that had been immunized with male cells (37). The TCR α and β chains (Vα 1.3-Jα35 and Vβ15-Jβ2.3) were cloned into shuttle vectors and TCR Tg mice were obtained in a B6.RAG-2$^-$ background as described for Marilyn (31).

Flow Cytometry

Four color cytometry was performed on a FACSCalibur™ flow cytometer (Becton Dickinson) using directly conjugated antibodies (Becton Dickinson) according to standard techniques, and the results were analyzed with CELLQuest™ software. The following Abs were used: anti–CD3-FITC, anti–CD2-PE, anti–CD4-PE, APC, or tricolor; anti–CD8-APC, anti–Vβ6-PE, anti–CD44-PE, anti–CD45.1-PE, or biotin; and anti–Vβ6-biotin, anti–CD45.2-biotin, anti–β TCR–CyChrome, or FITC (all from BD Biosciences). Biotinylated Abs were revealed with SA-Tricolor or SA-APC (Caltag).

Generation of Memory T Cells

Spleen and lymph node cells from CD45.2 Marilyn mice (containing 10^6 H-Y–specific naive CD4$^+$ T cells) were transferred together with 3 × 10^6 mitomycin-treated spleen cells from CD3ε^- male mice into RAG$^-$ female mice. Memory CD4$^+$ T cells were

recovered from the spleens of these mice at least 6 wk after transfer and purified as described below.

T Cell Purification and Adoptive Transfer

Thymocytes. Mature CD4^{+} thymocytes were purified by killing with a cocktail of anti-CD8 (3.168) and anti-HSA (J11.D) mAbs plus complement.

Mature T Cells. Naive (from lymph nodes of unimmunized Marilyn mice) or memory CD4^{+} T cells (from splenocytes of immunized mice) were positively purified using anti-CD2 and anti-CD4 antibodies and a FACS Vantage™ SE sorter. In two experiments, memory T cells were column purified with anti-CD4 magnetic beads (Miltenyi Biotec).

Small numbers (10^5/mouse) of CFSE-labeled CD4^{+} T cells were injected i.v. into the appropriate host mice (8–14 mice per group). Recipient spleens were harvested and studied by FACS® at different time points.

CFSE Labeling

FACS-purified CD4^{+} T cells were suspended at 5×10^6/ml in PBS-0.1% BSA and incubated with 5 μM CFSE (Molecular Probes) at 37°C for 8 min, after which the reaction was stopped by adding PBS-0.5% BSA and washing.

Prefilling Experiments

Purified memory H-2[b] CD45.2 Marilyn CD4^{+} T cells (10^5 per recipient) were transferred into the indicated CD45.2 host mice. 3–8 wk later, a second cohort of 10^5 CFSE-labeled purified naive H-2[b] CD45.1 Marilyn CD4^{+} T cells was injected into the same hosts. At the indicated time points, spleen cells were harvested and stained with either anti–CD44-PE, anti–TCRβ-CyChrome, and biotinylated anti-CD45.1 revealed with SA-APC, or anti–CD45.1-PE, anti–TCRβ-CyChrome, and biotinylated anti-CD45.2 revealed with SA-APC.

NK Depletion

Mice were injected intraperitoneally with 300 μg purified anti-NK1.1 mAb (PK136; provided by C. Carnaud, INSERM, Paris, France) at days −3 and −1 relative to the cell transfer, and then on days 3, 7, and thereafter once per week.

Online Supplemental Material

Fig. S1 shows cell division and CD44 expression of memory and naive Marilyn CD4^{+} T cells transferred into RAG-2/A [b]KO γc^{+} or γc^{−} hosts and is available at http://www.jem.org/cgi/content/full/jem.20030963/DC1.

Results

Survival of Naive and Memory T Cells in the Absence of MHC Molecules and NK Cells. To set up the system, we first tested whether Marilyn T cells would behave like other published TCR Tg T cells. We transferred naive Marilyn CD4^{+} T cells into RAG-2KO (empty) recipients of either H-2[b] (the "correct" restricting haplotype), H-2[k] (allogeneic haplotype to which Marilyn does not react), or H-2A[b−] (deficient for classical MHC class II A and E mole-
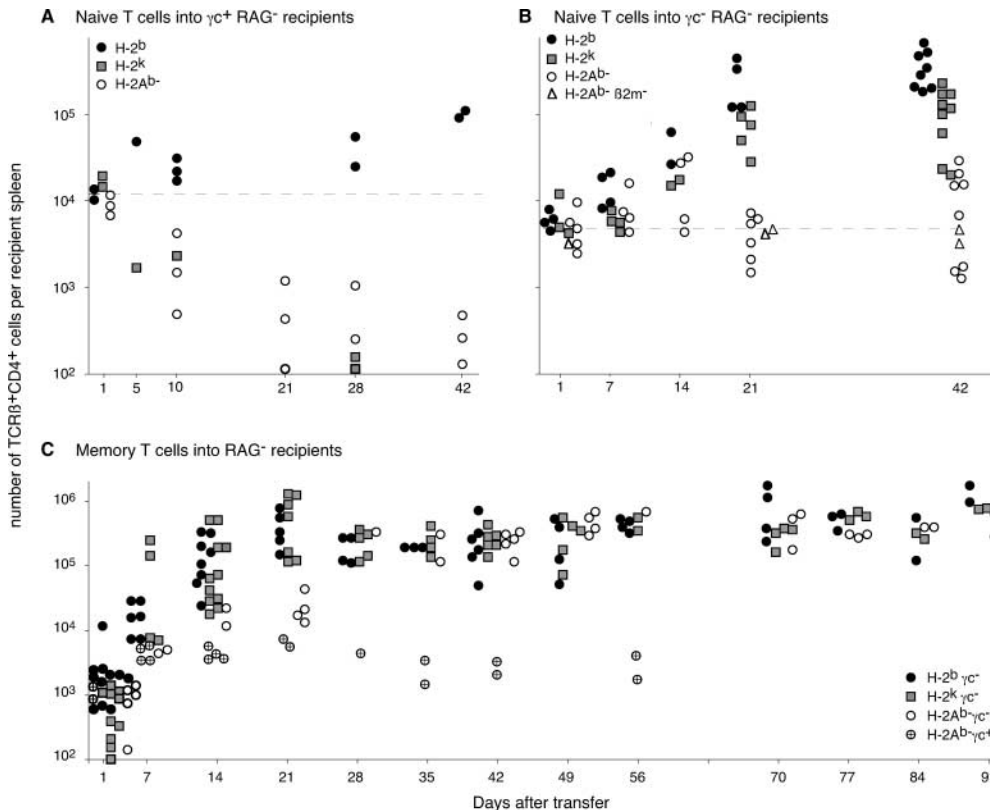

Figure 1. Recovery of naive and memory CD4^{+} T cells transferred into various hosts. (A) Absolute numbers of naive CD4^{+} Marilyn T cells recovered in NK-sufficient hosts. 10^5 CD4^{+} Marilyn T cells were labeled with CFSE and transferred i.v. into H-2[b], H-2[k], or to Aβ[b−] RAG-2^{−} hosts and their cell numbers were measured at the indicated time points. Data represent individual recipients from four independent experiments. (B) Absolute numbers of naive CD4^{+} Marilyn T cells recovered in NK-deficient hosts. 10^5 FACS®-purified T cells from lymph nodes were labeled with CFSE and transferred i.v. into the indicated RAG-2γcKO recipients. Then, 1, 7, 14, 21, and 28 d after injection, cells from the spleen of individual hosts were stained with anti–CD44-PE, anti–β TCR–CyChrome, and anti–CD4-APC. Absolute numbers of CD4^{+}/TCRβ^{+} are shown for individual recipients in six independent experiments. (C) Absolute numbers of memory CD4^{+} Marilyn T cells recovered in NK-deficient hosts. 10^5 column- or FACS®-purified memory CD4^{+} Marilyn T cells were injected into the indicated recipients. The absolute numbers of memory cells recovered in different recipients from day 1 to 13 wk after injection are shown. Data represent individual recipients from eight independent experiments.

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cules) haplotypes. As seen with other T cells (17, 18, 27, 29), we found that the naive Marilyn T cells survived only in the presence of the "correct" restricting MHC molecules (Fig. 1 A). As early as 9 d after transfer, the T cell recoveries were 10–30 times better in H-2^b than in H-2^k hosts, and by 6 wk there were nearly no recoverable T cells in the H-2^k or in the MHC class II$^-$ recipients.

Because the RAG-2KO hosts lack CTL, these differences in cell recovery could not have been due to CTL-mediated rejection. However, the RAG-2KO mice do have NK cells, which can reject donor cells differing at classical or nonclassical MHC class I loci (e.g., H-2^b Marilyn cells into H-2^k hosts; 38, 39), but do not seem to recognize MHC class II differences (e.g., Marilyn cells into H-2A^{b-} hosts).

What is not generally known, however, is that the MHC haplotype of the H-2A^{b-} mice (made from strain 129 stem cells) is not exactly H-2^b. Strain 129 was designated H-2^{bc} (40) and differs from the standard H-2^b haplotype (found in B6 and Marilyn mice) in the nonclassical MHC region, telomeric of the D locus (41), a region known to code for NK targets (42). Therefore, we repeated the survival studies in hosts that were doubly deficient for both CTL, because of the RAG deletion, and for NK cells, because of deletion of the cytokine receptor common γ chain, γc. In these hosts (Fig. 1 B), we recovered nearly equivalent numbers of Marilyn T cells in both H-2^b (closed circles) and H-2^k environments (gray squares). In fact, the T cells survived in the complete absence of any MHC class II molecules (open circles). To test whether such survival might be due to an unusual cross-reaction with MHC class I molecules, we also used quadruply deficient hosts lacking expression of both MHC class I and II, in addition to RAG and γc. Even in this environment, without any expressed MHC molecules, the naive T cells survived without any

losses for 6 wk (triangles). Thus, when transferred T cells are not alloreactive to the host, and when the host cannot reject the transferred T cells, naive CD4$^+$ T cells seem to survive without a need to recognize MHC molecules.

Memory Marilyn cells not only survived but also expanded in the absence of MHC for at least 13 wk when transferred into RAG/γc doubly deficient hosts expressing H-2^b, H-2^k, or no class II molecules at all (Fig. 1 C), but they did far less well in A^{b-} RAGKO hosts, which contain NK cells (crossed circles). In these hosts, they hardly expanded at all (see Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20030963/DC1), and the numbers came to only ∼1% of those in the RAG/γc doubly deficient hosts. Thus, for both memory and naive T cells, the processes that promote survival do not seem to require the recognition of MHC molecules as long as the hosts cannot reject the donor cells.

Previously, two groups used antibodies to deplete NK cells and found that their removal did not significantly enhance the survival of naive T cells transferred into allogeneic hosts (6, 17). Antibody depletion, however, may not always be complete. Therefore, we compared the survival of naive cells in hosts depleted of NK cells genetically or by treatment with anti-NK antibody. Fig. 2 shows that treatment with anti-NK antibodies increased the survival of naive T cells transferred into H-2A^{b-} hosts, although it was not effective across a strong allogeneic MHC difference (H-2^k). In general, the survival of transferred naive T cells was better in recipients that were genetically deficient of NK cells than in those that were treated with anti-NK antibodies, regardless of haplotype, although the differences were most striking when an MHC difference existed, suggesting that anti-NK antibody treatment may not be sufficient to abolish all NK activity. The data suggest that the presence of NK cells may interfere with the survival of na-
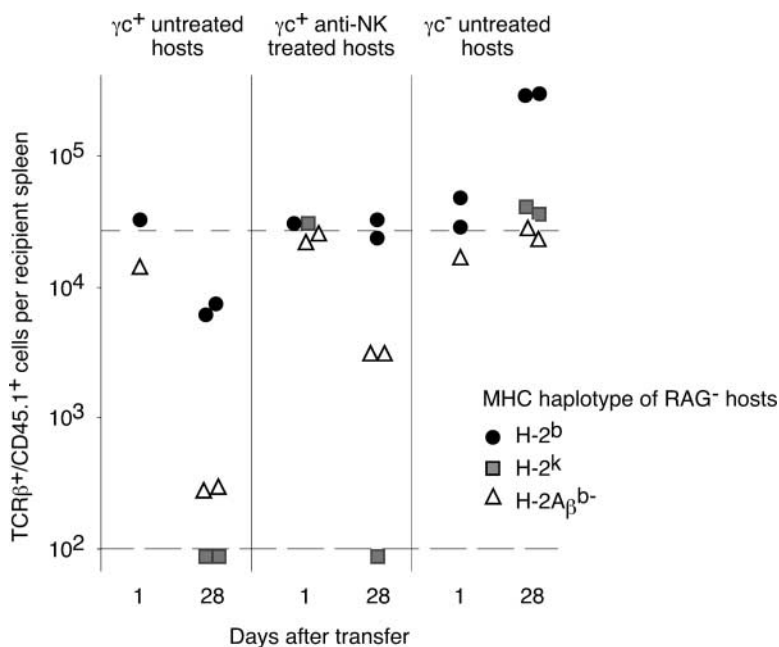

Figure 2. NK cells interfere with the survival of naive CD4$^+$ T cells in MHC class II–deficient and in allogeneic hosts. 10^5 naive Marilyn CD4$^+$ T cells were transferred, as in Fig. 1, into untreated RAG-2$^-$ or RAG-2/γcKO hosts, or into RAG-2$^-$ hosts that were treated with anti-NK antibody at days −3, −1, +3, and +7, and then once a week. CD45.1$^+$/TCRβ$^+$ spleen cell numbers were measured after 1 d and 1 mo. The dashed line at 10^2 represents the limit of detection.
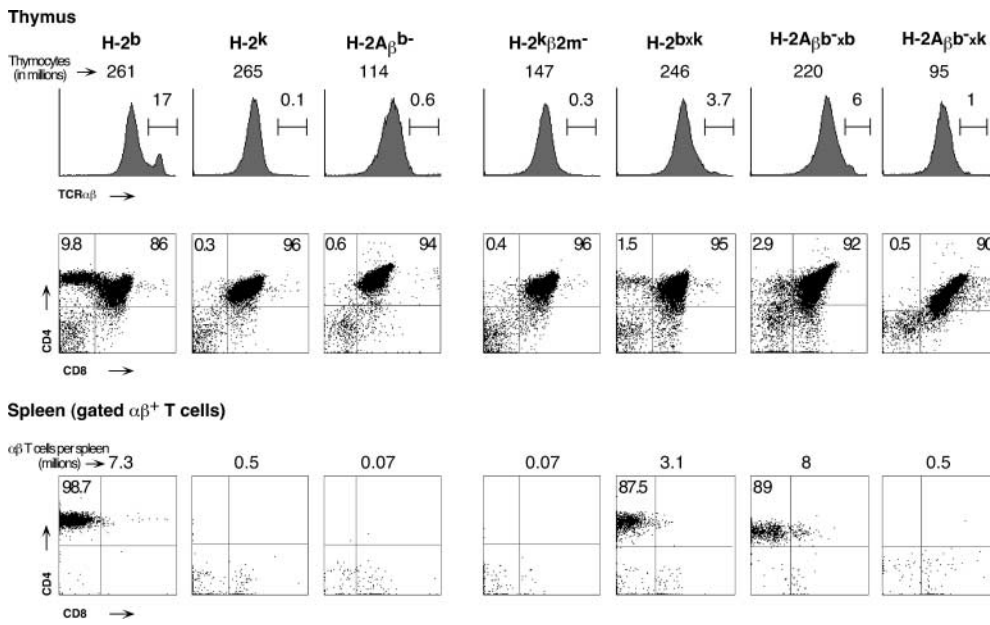
ive T cells in two ways. First, the enhanced survival in NK-less MHC-allogeneic and MHC⁻ hosts suggests that NK cell killing can cause striking losses in survival. Second, enhanced survival in NK-less syngeneic (H-2^b) hosts shows that NK cells can lead to loss of survival even under conditions where killing is unlikely to occur, suggesting that the NK cells may compete with T cells for appropriate peripheral niches.

Survival Is Not Due to an Unusual Cross-reactive Specificity of the Marilyn TCR for MHC Class I Molecules, or for Allogeneic or Hybrid MHC Class II Molecules. Although it would be highly unusual for a mainstream CD4⁺ T cell to be restricted by anything but classical MHC class II molecules, the ability of the Marilyn T cells to survive in the absence of MHC class II and expand in the presence of allogeneic H-2^k MHC molecules, might have been due to an extremely rare developmental affinity for molecules other than their A^b-restricting elements (43). To check for such recognition, we bred the Marilyn Tgs to mice carrying several different MHC haplotypes and looked at development in the thymus and the spleen (Fig. 3). We found that the Marilyn T cells exhibit the typical CD4 T helper cell pattern. In H-2^b mice, the thymuses and spleens were large and contained a normal number of mature CD4⁺ T cells expressing high levels of TCR. In contrast, there were no mature Marilyn T cells in H-2^k or H-2A^b⁻ mice. Together, these results show that development of the Marilyn T cells requires the expression of the classical A^b class II MHC molecule, and that neither the H-2^k class I or II molecules, nor any hybrid H-2^b Aα/Eβ molecules can substitute. The Marilyn CD4⁺ T cells did not develop in H-2^k mice that lacked β2m, showing that the lack of CD4⁺ T cells in H-2^k mice is not due to negative selection by the H-2^k class I molecules. They developed equally well in heterozygous F₁H-2^b/k and in hemizygous F₁H-2^b/A⁻ mice (containing one normal and one deleted Aβ^b allele). As seen with some

other TCR Tgs (44), MHC-heterozygous mice contained fewer mature T cells, as might be expected with a half dose of the appropriate selecting molecule, A^b. Even at these low levels of restricting elements, however, development was not diminished by the presence of H-2^k class I or II alleles, showing that H-2^k does not cause deletion of the developing Marilyn T cells. Finally, Marilyn did not develop in F₁H-2^k/A⁻ mice, showing that a half dose of A^k or E^k is not sufficient to allow for their development in the absence of A^b. Thus, the Marilyn CD4⁺ T cells exhibit the classic requirements for development, and their ability to survive in allogeneic or MHC class II⁻ hosts is consequently not due to an unexpected positive cross-reaction with inappropriate MHC or non-MHC molecules.

Initiation of "Homeostatic" Expansion in the Absence of MHC Molecules: Survival and Expansion of Naive CD4⁺ T Cells Varies According to the Context. Up to this point, we had measured only the absolute numbers of surviving transferred Marilyn T cells. It is known, however, that resting T cells proliferate when transferred in small numbers into empty hosts lacking other T cells (6, 9, 11, 13–27), and such space-induced division, thought to reflect homeostatic mechanisms that maintain appropriate T cell numbers, can influence the numbers of recovered cells. The transferred Marilyn T cells may thus have been dividing and dying in some hosts while remaining stable in others (45). Indeed, a close look at Fig. 1 B shows that the numbers of recovered Marilyn T cells expanded with time in RAG/γcKO hosts expressing MHC class II molecules, regardless of haplotype, but remained stable in hosts not expressing MHC molecules.

To determine the relative influence of cell division versus cell survival in the fate of transferred naive and memory CD4⁺ T cells, and the potential role of MHC molecules in these processes, we labeled the Marilyn T cells with the dye, CFSE, which dilutes by half at each cell division, and followed their division patterns with time. We also moni-
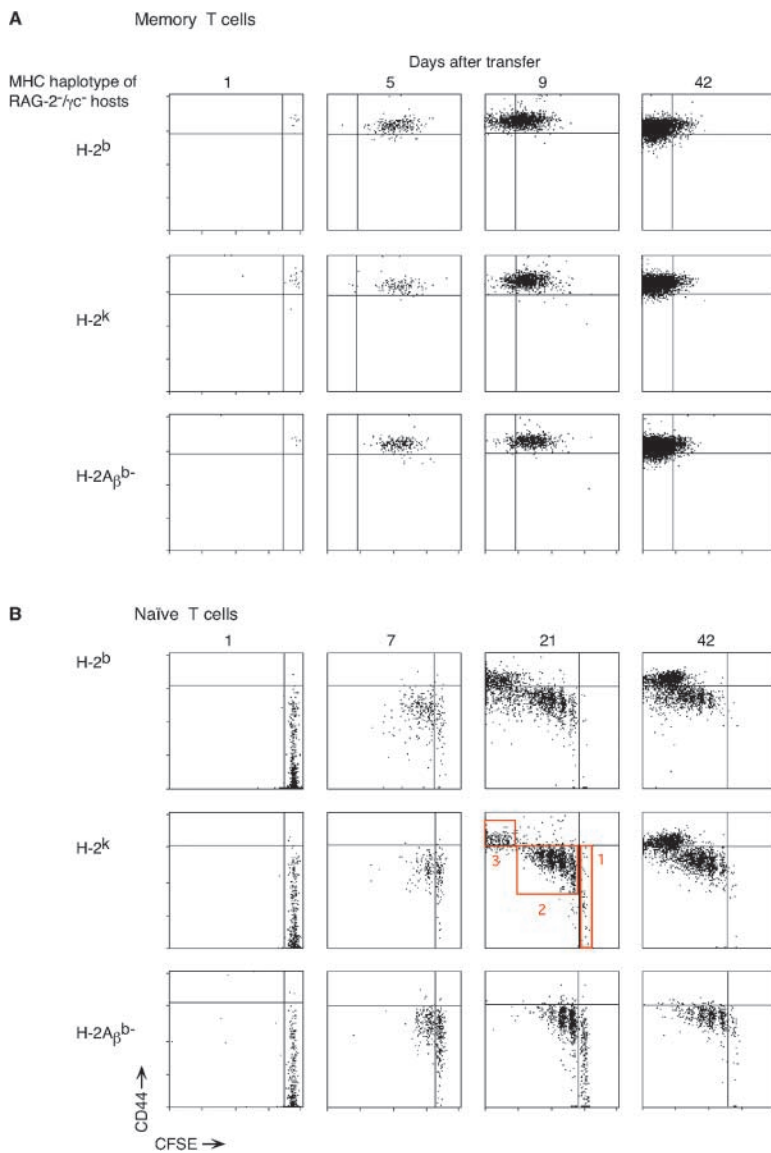
A   Memory T cells

Figure 4. Cell division and CD44 expression of memory (A) and naive (B) CD4^{+} T cells transferred into RAG-2 and γc KO hosts of different MHC types. 10^5 CFSE-labeled, FACS®-sorted, memory or naive Marilyn CD4^{+} T cells were injected into the indicated recipients and spleen cells from individual hosts were analyzed by flow cytometry at different times after transfer. The data were electronically gated for CD4^{+}/TCRβ^{+} cells, and the expression of CFSE and CD44 was examined. Quadrants to distinguish CD44^{hi} from CD44^{int} cells were set according to the pattern observed in normal B6 mice. Red boxes indicate the three subsets: 1, undivided CFSE^{hi}/CD44^{lo} cells; 2, CFSE^{int}/CD44^{int} cells; 3, CFSE^{lo}/CD44^{hi} cells.

tored the cell surface marker, CD44, which is expressed at low levels by naive T cells, increases after antigenic stimulation, and remains high on most memory T cells. We first looked at transferred memory CD4^{+} T cells, and Fig. 4 A shows that they behaved as a homogeneous population, expanding and setting up residence equally well in all hosts, regardless of MHC type. Thus, the "homeostatic" division of memory cells in empty hosts, like their survival, is not dependent on the presence of MHC molecules.

Naive cells behaved differently from memory cells (Fig. 4 B) and could be classed into three stages on the basis of cell division and CD44 expression. Some cells did not divide at all for up to 6 wk (stage 1), other cells divided somewhat (though more slowly than the memory T cells), but did not transit to the CD44^{hi} state (stage 2), and in MHC^{+} hosts, others continued to divide such that they lost enough of the CFSE dye to appear negative, and up-regulated CD44 (stage 3). Because of their extensive prolifera-

tion, cells in this latter group comprised 70–81% of the recovered cells and were therefore the main arbiters of any differences seen in total cell recovery. An analysis of these three stages in nine separate experiments (Fig. 5) showed that the number of cells in each stage varied according to the MHC context, the presence or the absence of memory T cells, and the maturational state of the naive CD4^{+} cells (lymph node vs. thymus origin).

Fig. 5 A, a compilation of four separate experiments in which naive peripheral lymph node CD4^{+} T cells were transferred into empty hosts, illustrates the numbers of cells recovered in each of the three stages. First, in all hosts, regardless of MHC type, the number of undivided T cells (stage 1) declined steadily, with a calculated half-life of ~1 wk. ~2% of the cells remained in this undivided state at 6 wk, in both MHC class II^{+} and MHC class II^{−} hosts. Thus, the sheer survival of truly resting naive CD4^{+} T cells seems to be independent of MHC recognition.
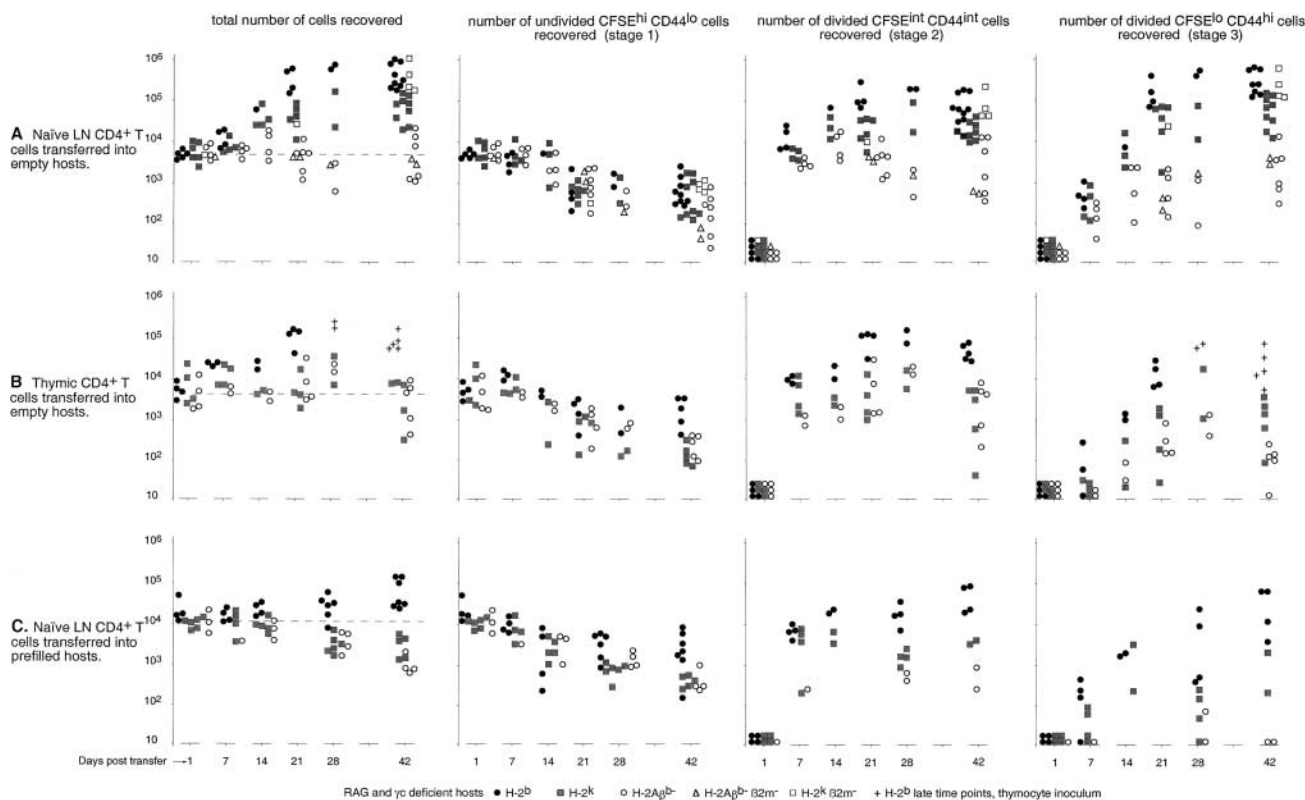
Figure 5. Survival, expansion, and transition to the CD44^{hi} state of naive T cells in different hosts. 10[5] CFSE-labeled mature CD4^{+} thymocytes (B) or lymph node CD4^{+} T cells (A and C) were injected into either empty hosts (A and B), or hosts prefilled with memory cells (C), of the indicated MHC types. The left-most panel represents the total number of cells recovered and the three right-hand panels show the number of cells in the different subsets as defined in Fig. 4 B. The data represent individual recipients in nine independent experiments (four, two, and three experiments each in A, B, and C, respectively). Due to loss of samples there are only two experiments represented for subsets 2 and 3 in C. At days 21 and 42, the data for H-2[b] recipients in B are represented as small crosses. These numbers are imprecise because the recovered cell populations contain some cells generated by development in the host thymus. Being CFSE[−], these cells are impossible to distinguish from any transferred cells that have undergone many divisions.

Second, the initiation of space-induced "homeostatic" cell division was also MHC independent. A proportion of the naive cells initiated "homeostatic" expansion (stage 2), undergoing several divisions in the presence or in the complete absence of MHC class II molecules.

Third, at ~2 wk, MHC-dependent differences began to appear. T cell numbers in MHC class II[−] hosts remained fairly stable after this time, whereas cells in MHC^{+} hosts continued to expand and up-regulate their CD44 levels (stage 3). By day 42, CD44^{hi} cells averaged ~1,500 in the MHC class II^{−} hosts, compared with 80,000 in H-2[k] (a 53-fold difference) and 200,000 in H-2[b] (2.5-fold, or approximately one cell division). Thus, the Marilyn CD4^{+} T cells were able to survive (stage 1) and initiate space-induced expansion (stage 2), dividing several times in the absence of MHC class I or II molecules. However, there seems to be a transition point to CD44^{hi} and continued proliferation (stage 3), which naive T cells cannot traverse in an environment missing MHC molecules (see also Fig. 4 B). This transition did not require the correct restriction element, as both H-2[b] and H-2[k] hosts provided a sufficiently permissive environment. There was a small (2.5-fold) difference in expansion between cells transferred into H-2[b] and H-2[k] hosts,

but this difference disappeared in H-2[k] hosts that lacked β2m (Fig. 5 A, open squares), suggesting that the lower survival in H-2[k] might reflect the activity of a small number of residual NK cells in the γc^{−} hosts (Fig. 2; reference 46).

Thymocytes Do Not Efficiently Transit to the CD44^{hi} State. The finding that naive T cells displayed several different behaviors could be related to some heterogeneity of the inoculum. Although RAG2.KO Marilyn CD4^{+} T cells seem to be a homogeneous population of naive cells when tested by FACS® or by functional tests (unpublished data and 31), we considered the possibility that our inoculum might be made up of a mixture of new thymic emigrants and naive T cells that had already passed a critical maturation step in the periphery. To test this possibility we analyzed the survival and expansion of newly matured CD4^{+} T cells from the thymus after transfer to MHC-syngeneic, MHC-allogeneic, or MHC-deficient hosts. We found (Fig. 5 B) that thymocytes entered stages 1 and 2 similarly to naive lymph node cells, but far fewer thymocytes divided more than five divisions and transited to the CD44^{hi} state (stage 3). The transition to stage 3 was 10-fold lower in H-2[b], 20-fold lower in H-2A[b−], and 40-fold lower in H-2[k] recipients, compared with naive peripheral cells. Thus, the varied be-
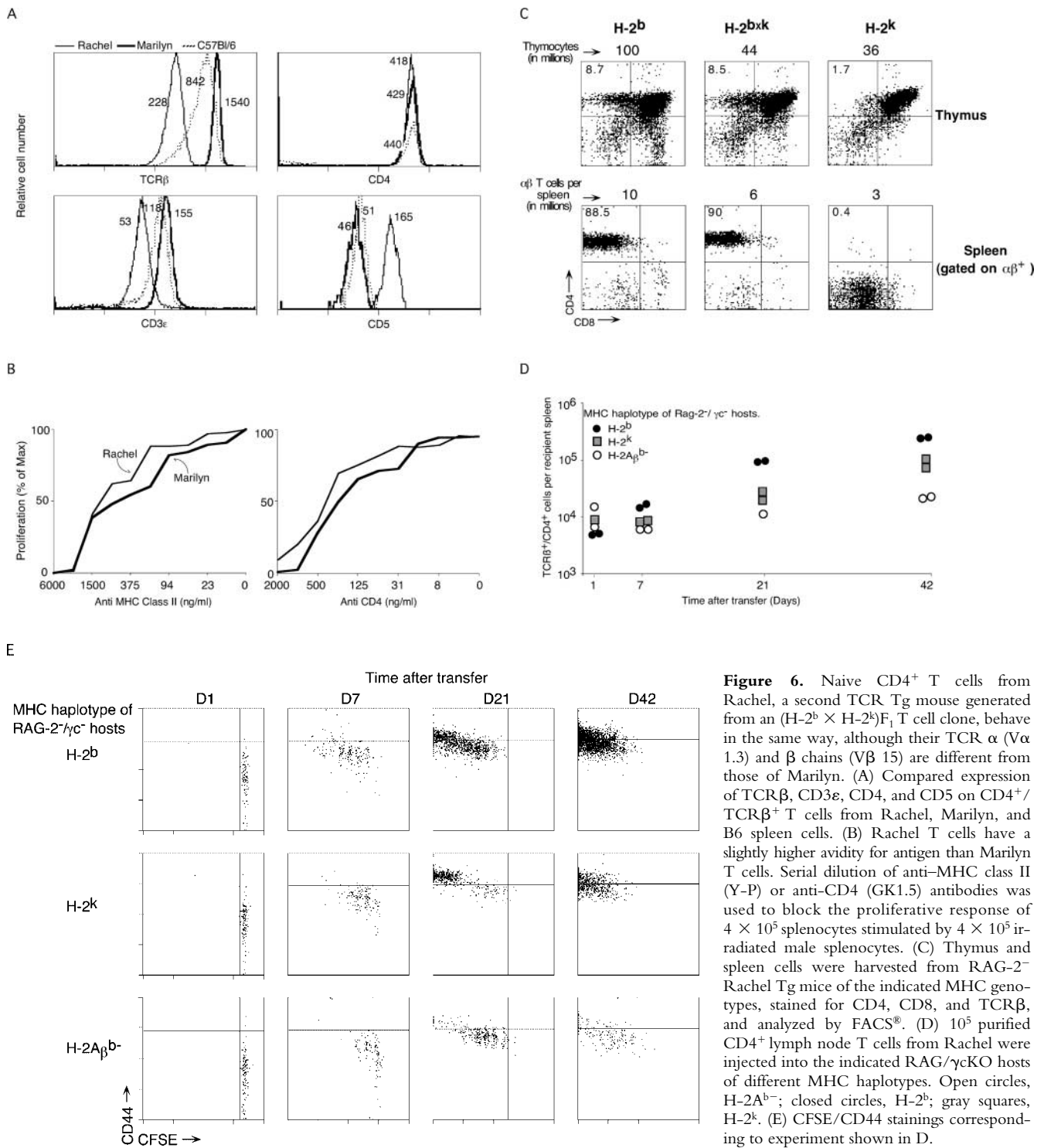
Figure 6. Naive CD4^{+} T cells from Rachel, a second TCR Tg mouse generated from an (H-2[b] × H-2[k])F₁ T cell clone, behave in the same way, although their TCR α (Vα 1.3) and β chains (Vβ 15) are different from those of Marilyn. (A) Compared expression of TCRβ, CD3ε, CD4, and CD5 on CD4^{+}/TCRβ^{+} T cells from Rachel, Marilyn, and B6 spleen cells. (B) Rachel T cells have a slightly higher avidity for antigen than Marilyn T cells. Serial dilution of anti–MHC class II (Y-P) or anti-CD4 (GK1.5) antibodies was used to block the proliferative response of 4×10^5 splenocytes stimulated by 4×10^5 irradiated male splenocytes. (C) Thymus and spleen cells were harvested from RAG-2^{−} Rachel Tg mice of the indicated MHC genotypes, stained for CD4, CD8, and TCRβ, and analyzed by FACS®. (D) 10^5 purified CD4^{+} lymph node T cells from Rachel were injected into the indicated RAG/γcKO hosts of different MHC haplotypes. Open circles, H-2A$_\beta$[b−]; closed circles, H-2[b]; gray squares, H-2[k]. (E) CFSE/CD44 stainings corresponding to experiment shown in D.

havior of peripheral lymph node cells may partially be explained by the presence of newly emigrated thymocytes, many of which seem not to have reached the maturational stage allowing them to transit to the CD44^{hi} state, the one aspect of behavior in which MHC signals might be critical.

Naive CD4^{+} T Cell Survival in Hosts Containing Memory T Cells. Although naive T cells normally share the peripheral space with memory T cells, it has been suggested that the two populations are independently regulated (47). To study naive CD4^{+} T cell survival in the presence of memory T cells, we "prefilled" a set of RAG/γc double KO hosts with memory Marilyn cells and allowed them to rest for 3–6 wk to permit the memory T cells to expand (Fig. 1) and set up residence. We then injected these hosts with 10^5 CFSE-labeled naive Marilyn cells. Fig. 5 C shows that the presence of a stable population of memory CD4^{+} T

cells had a striking effect on the behavior of the newly transferred naive T cells, dampening their ability to expand and transit to the CD44^{hi} state. The naive cells expanded more slowly, reaching total numbers at 6 wk that were fourfold (in H-2^b) to 40-fold (in H-2^k) lower than those in empty hosts. The difference seemed to be almost exclusively caused by reduced transit to stage 3 (CFSE^{lo}/CD44^{hi}), as the survival in stages 1 and 2 were nearly similar to those in empty hosts. The number of cells moving to stage 3 was ~15–90-fold lower in prefilled versus empty H-2^b and H-2^k hosts, respectively. Thus, at least in an adoptive transfer system, naive cells do not seem to be regulated independently of memory cells.

Naive Rachel CD4$^+$ T Cells, Which Use a Different TCR, Behave Like Marilyn T Cells. To test whether the Marilyn T cells might be exceptional in some way, we repeated the experiments with a second TCR Tg mouse (Rachel) that we also generated from an (H-2^{b}xH-2^k)F$_1$ T cell clone to avoid any reactivity against either haplotype. This TCR (Vα1.3 and Vβ15) is also specific for H-Y/A^b and, when crossed to RAG.KO, the Rachel mice harbor only naive (CD44^{int}/CD62L^{hi}) CD4$^+$ T cells (not depicted). In some respects, Rachel is rather different from Marilyn. Fig. 6 A shows that Rachel T cells express lower levels of TCR and CD3 (seven- and threefold, respectively), and fourfold higher CD5 levels than do Marilyn T cells. Yet, in spite of the lower levels of TCR, Rachel seems to have a somewhat higher avidity for the H-Y/A^b complex, as seen in blocking experiments with anti–MHC class II or anti-CD4 antibody (Fig. 6 B). Breeding experiments similar to those displayed in Fig. 3 for Marilyn showed that Rachel thymocytes behave as expected (Fig. 6 C). They are selected in thymuses expressing A^b and are neither positively nor negatively selected by H-2^k.

Purified naive Rachel CD4$^+$ T cells behaved similarly to Marilyn cells when transferred into hosts of different MHC haplotypes. They disappeared in allogeneic RAG$^{-/-}$ hosts (unpublished data), but, in RAG$^{-/-}$ hosts also lacking NK cells (γc$^{-/-}$), they survived in the absence of MHC class II molecules and actually expanded in hosts expressing H-2^k (Fig. 6 D). CFSE staining (Fig. 6 E) showed that Rachel T cells passed through the same three stages as described above for Marilyn, and that the requirements for transition from one stage to another seemed to be the same. Neither survival, nor the first few space-induced divisions required the presence of MHC class II molecules. The minimal number (5,200) of stage 3 (CFSE^{lo}/CD44^{hi}) cells observed in A^{b-} hosts shows that, like Marilyn T cells, the Rachel T cells did need the presence of MHC class II to transit to the CD44^{hi}/CFSE$^-$ stage and did not need their own restricting element (A^b), as they were able to undergo this transition in both H-2^b (150,000 stage 3 cells recovered at day 42) and H-2^k (45,000) hosts.

Thus, the MHC independence of naive CD4$^+$ T cell survival, and the lack of allele specificity in the expansion phase, is not the special property of a single T cell clone, but seems to be a general phenomenon as long as the cells are not alloreactive and are not rejected.

Discussion

Four main conclusions arise from our results. First, the survival of naive T cells and the role of MHC molecules in that survival is more complex than previously thought. MHC molecules are involved in some aspects of T cell subsistence but not in others. Naive CD4$^+$ T cells can exhibit several kinds of behaviors when transferred into adoptive hosts. Some survive for weeks without dividing, and this survival is not dependent on MHC recognition. A second group divides slowly five or six times and this too seems to be MHC independent. A third group continues to divide and, after five or six divisions, up-regulates CD44. This last transition is the only process that seems to require the presence of MHC molecules. Second, there is no allele specificity in those aspects of expansion that appear MHC dependent. For example, naive Marilyn or Rachel T cells do not need to recognize any particular allele of MHC to make the CD44 transition. The mere presence of an MHC class II molecule is enough. Third, rejection or competition, either by CTL or by NK cells, may account for the survival failures seen in previous studies where T cells were transferred into MHC-allogeneic or MHC-deficient hosts (6, 9, 11, 14–19, 21–23, 26, 27). Fourth, in contrast to a previous suggestion (48), the numbers of peripheral naive T cells are not regulated independently of other lymphocyte populations. Their expansion is clearly inhibited in the presence of memory T cells, and perhaps also by NK cells. As discussed below, these results bring together many of the conflicting results of previous studies.

Survival, in the Absence of MHC Molecules, Has Been Seen Before. Once we separate sheer survival of nondividing cells (stage 1) from space-induced expansion (stages 2 and 3), it is clear that many previous studies have shown that CD4$^+$ T cells can survive and even undergo a few divisions (stage 2) in the absence of MHC recognition (9, 11, 12, 28), although some authors did not seem to notice (14, 15, 19, 23–25, 27). Why then has this not been universally detected? There are several reasons. First, many investigators measured only the total number of recovered cells. As the cells that transit to CD44^{hi} (stage 3) expand to far greater numbers than cells in the other two stages, they make up the majority of recovered cells. Because the transition to the CD44^{hi} state seems to be MHC dependent, previous studies that measured only the total number of recovered cells, or only overall cell division, came to the conclusion that all of the survival aspects of naive T cells were MHC dependent (6, 13, 15–18, 23, 24, 26, 27, 29, 49). This is not the case. Survival and the first few divisions of space-induced division are both MHC independent. Only the CD44 transition seems to depend on the presence of MHC molecules, and even this requirement does not extend to a need for a specific MHC allele.

Rejection and/or Competition, Rather Than Lack of MHC Signaling, Can Cause Naive T Cell Loss. A second reason that MHC-independent survival has not always been seen is that cells transferred into allogeneic hosts can be rejected by host CTL or NK cells. Naive Marilyn T cells, for exam-

ple, survived in allogeneic or MHC class II⁻ recipients only in RAG/γc double KO hosts that lacked both CTL and NK cells, and they actually expanded in H-2^k hosts that were additionally deficient for β2m, a deficiency that has been shown to further reduce NK cell activity (50). Although NK cell–mediated rejection may seem surprising when the donor and host differ only at MHC class II loci, the commonly used MHC haplotypes actually have other differences. The haplotype of strain 129 (designated H-2^{bc}; reference 40) differs from H-2^b (B6 mice) in a chromosomal region known to code for NK target molecules (41, 42). MHC mutants generated in 129 embryonic stem cells will therefore carry these NK target differences, despite extensive backcrossing to B6. Thus, NK-mediated rejection, or competition, was the most likely cause of the short survival times seen in previous studies (6, 15, 17–19, 21, 27) in which H-2^b T cells were transferred into MHC mutant recipients (DM, TAP or A^{b-}). One previous study that attempted to block NK cell killing, in a fully allogeneic situation where NK reactivity would be very high, had no controls for antibody efficacy (17). In our hands, anti-NK antibody treatment was totally ineffective across fully allogeneic barriers (Fig. 2). Thus, without controls for antibody efficacy (17), only the genetically NK-deficient animals allow us to be certain that rejection is not the cause of lack of survival.

NK cells can also prevent the survival of T cells by competing for appropriate niches. Figs. 1 and 2 show that Marilyn T cells survive and expand better in NK-deficient hosts, even when there is no MHC difference, suggesting that the mere presence of NK populations can dampen the space-induced proliferation of naive T cells, even under conditions where NK-mediated killing would be unlikely. Perhaps the γc-deficient hosts have a greater profusion of available cytokines, such as IL-7 and IL-15, because of a reduced competition by other γc receptor-bearing cells. In support of this interpretation, transferred naive CD4 T cells proliferated faster (more cells were recovered), in RAG-γc–deficient mice than in RAG-γc$^+$ mice, even in syngeneic H-2^b hosts, where NK-mediated killing would be unlikely (Fig. 1, A and B, and Fig. S1, A and B, available at http://www.jem.org/cgi/content/full/jem.20030963/DC1). However, this cannot be the entire story, as differences in MHC (where NK killing would be possible) resulted in major differences in survival, which could not be accounted for by mere competition. Most likely both processes are in play. In support of this is the finding that the transferred CD4 T cells actually disappeared much faster in allogeneic and MHC-deficient hosts than they did when their TCR was turned off by genetic means (7, 8), suggesting that active killing was likely at play in the transfers.

TCR and/or Lck Dependence Is Not the Same As MHC Dependence. A third reason that MHC independence of T cell survival has sometimes been missed is that authors who created mutations in TCR, in TCR-related signaling molecules, or in the diversity of MHC–peptide complexes, often assumed that the consequent lack of survival (or expansion) was due to a loss of MHC/self-peptide recognition.

Seddon et al. (11), for example, showed that space-induced division and the transition to the CD44^{hi} state are dependent on p56lck, though sheer survival is not, and Polic et al. (7) and Labrecque et al. (8) found that naive cells disappear slowly, with a half-life of ∼46 and 27 d, respectively, if they lose surface expression of TCR (7, 8). Because of the previous data showing that naive T cells disappeared in MHC-deficient environments, these groups concluded that the need for TCR/lck signaling most likely reflected a requirement for TCR–MHC interactions. However, there are reasons to suggest otherwise. First, the loss of surface TCR also causes the surface loss of the TCRζ chain, which is not unique to TCR but also mediates signaling via CD2 and Thy-1 (51, 52), whose functions are not yet known (though peripheral T cells in CD2.KO mice display decreased antigen responses; 53). Second, p56lck mostly associates with the CD4 molecule, which also seems to have a signaling link to the ζ chain. When the CD4 molecules of transferred T cells cannot bind to host MHC class II molecules (because the MHC molecules have mutated CD4 binding sites), the basal phosphorylation of the ζ chain decreases (10, 54). Thus CD4, CD2, and/or Thy-1 might be the important molecules signaling T cell survival, rather than TCR–MHC interactions.

A close look at Figs. 1 B and 5 shows that both Marilyn and Rachel T cells have a slight proliferative advantage in H-2^b over H-2^k hosts, hinting at the involvement of their TCR specificity. However, because A^b, A^k, and E^k differ at the binding sites for CD4 (55, 56), the small differences in proliferation might be due to a difference in CD4 binding, rather than a difference in TCR recognition.

MHC Dependence Is Not the Same As Allele Specificity. There have been some cases in which transferred T cells proliferated rapidly in the presence of hybrid class II A/E molecules and not in their absence. For example, a recent study (12) showed that a small number of B6 CD4$^+$ T cells responds and expands rapidly when transferred into CD3ε$^-$A^{b-} hosts, but not in hosts that express no MHC class II molecules. Although the authors suggested (following the current trend) that CD4$^+$ T cell survival requires MHC class II molecules, our interpretation is different. In a polyclonal population of B6 CD4$^+$ T cells, a few can respond to peptides from strain 129 minor H antigens, complexed with the hybrid MHC class II molecules. Such expansion therefore may have little to do with the question of T cell survival, but more to do with a GvH response. In support of this view are data from the same study showing that AND TCR Tg T cells (which are specific for pigeon cytochrome c, and not 129 minor antigens) do not undergo this rapid cell division in such lymphopenic hosts, and (from our study) neither do Marilyn and Rachel TCR Tg T cells, which are specific for H-Y. Any polyclonal T cell inoculum will also contain T cells responsive to environmental antigens. Survival and expansion in these cases will have less to do with homeostatic mechanisms than with normal immune responses.

Therefore, we used a clonal population to an antigen (H-Y) that has no known cross-reactive environmental an-

tigens. Further, to control for alloreactivity to the hosts, we cloned the Marilyn and Rachel TCRs from (H-2^k × H-2^b)F$_1$ T cell clones, tolerant of both parental MHC haplotypes, and tested them both as clones (unpublished data) and as TCR Tg mice (Figs. 3 and 6; reference 32) for reactivity to H-2^k and to an extensive battery of other MHC and minor antigens. In all tests, we found that these TCRs react only to H-Y/A^b and (for Marilyn) to Mls^a (as expected for a Vβ6 TCR; reference 57). Thus, unlike other T cells that have been used for survival studies, Marilyn and Rachel can be transferred to allogeneic (H-2^k) hosts without risk of inadvertent positive or negative cross-reactions.

The monoclonality of these T cells also makes it unlikely that they survive in A^{b-} mice because of high affinity cross-reactions for low levels of hybrid Aα^b/Eβ^b class II molecules (28). To maintain the notion that survival and expansion require allele-specific MHC recognition, Marilyn's TCR would have to cross-react on H-2^k molecules as well as hybrid A/E molecules. First, this would require that the Marilyn TCR have four different specificities, three of them for different MHC molecules (H-Y/A^b, Mls^a, A^b/E^b hybrids, and either A^k or E^k). This is a lot to ask of one TCR, and would call into question the whole concept of allele specificity. Third, Rachel behaves grossly the same way as Marilyn, even though she expresses a different pair of TCR V genes and divides a little faster. Third, our tests showed that the affinity of the Marilyn and Rachel TCRs for such putative complexes are not even sufficiently high to allow development in H-2^k or A^{b-} thymuses (Fig. 3).

A referee suggested that perhaps the T cells might have an affinity for hybrid and allogeneic MHC molecules that is too weak to allow for positive selection, but enough to allow for peripheral survival. This seems very unlikely to us, and would not explain the data. If such a low affinity would be enough to induce expansion in the periphery, then it could not be said to be specific, as such weak cross-reactions should occur randomly and often, predicting that the T cells would be able to see almost any MHC allele. Thus, the data showing that T cells (even random populations of naive T cells) do not survive in allogeneic hosts still remains to be explained, and the difference between γc$^+$ and γc$^-$ hosts would not be predicted.

In short, recognition of the host by the transferred T cells and recognition of the transferred T cells by the host, can lead to quite misleading results regarding the MHC dependence of naive T cell survival and expansion. In our study, where neither type of recognition occurs (for example, Marilyn or Rachel T cells into NK cell–deficient hosts that were also deficient for MHC class II, or deficient for MHC class I and allogeneic for class II), the naive T cells survived and began the process of space-induced expansion without any need to recognize an MHC molecule.

Memory Cells Inhibit Expansion of Naive Cells but Not Vice Versa. It has been suggested that expansion in empty hosts reflects a normal mechanism by which naive T cells maintain homeostatic levels, and that the number of naive and memory cells is independently regulated (48). We disagree with both of these views. First, Fig. 5 shows that the expansion of transferred naive cells was inhibited when memory cells were present. Cell division occurred later, at a less rapid rate, and fewer cells transited to the CD44^{hi} state. Thus, the existence of a memory cell population can inhibit expansion and CD44 up-regulation of naive cells. Second, in normal mice that have been thymectomized, the number of naive CD44^{lo} T cells wanes slowly over a period of several months, and the ability to respond to new antigens also slowly disappears (58), suggesting that the naive cells are dying, rather than becoming memory cells. Neither naive nor memory cells expand to fill the empty space (58). Thus, the "homeostatic" expansion exhibited by naive cells transferred into empty hosts is not characteristic of normal animals that become depleted of naive T cells after thymectomy.

A Two-Niche Model. We do not know if the transferred naive T cells behave in three different ways because there are three different subsets of T cells or three different niches into which they might land. Nor, because of the complexity of the interactions involved, can we be certain which behaviors are artifacts of adoptive transfer and which are normal mechanisms. Our best guess at the moment, however, starts with the idea that resting naive T cells have the ability to move into two different niches, one that is normally occupied by naive cells and a second that is normally occupied by memory cells (and perhaps also by NK cells). As they emigrate from the thymus, they transit to the first niche, which requires γc-dependent cytokines (31) but not p56lck (24) or MHC recognition (this study). They can remain in this state for several months, but if they do not encounter a stimulus that moves them to the memory niche, they eventually die (58).

If memory cells are present, naive cells are inhibited (or outcompeted) from moving into the memory niche, unless they receive an immunogenic stimulus. In the absence of memory cells, however, the naive cells can pass to the memory niche. Although the first few cell divisions occur independently of MHC molecules (at least in empty hosts; Fig. 5 A), successful transition requires MHC as well as signaling through TCR (or ζ; references 7 and 8) and p56lck (24), and results in the up-regulation of CD44. However, the recognition of MHC does not seem to be allele specific, leaving open the possibility that the stimulus passes via CD4 molecules, either alone or in combination with a non-TCR molecule that signals through the TCRζ chain, such as CD2 or Thy-1. Once the T cells have fulfilled the requirements to enter the CD44^{hi} niche, they no longer require MHC molecules to remain in it (in empty hosts, memory cells expand and survive without MHC or TCR; Fig. 1 and references 7, 59, and 60).

In the absence of memory cells, naive T cell transit to the memory compartment is not automatic. This is illustrated by several TCR Tgs, which, if crossed to RAG KO mice to prevent expression of endogenous TCRs, contain only naive T cell populations until deliberately immunized with the appropriate antigen (31, 61–63). We do not presently know why naive cells are propelled to expand into the memory compartment upon transfer into empty hosts, and not in their empty original hosts. Perhaps the very act

of isolating and transferring them removes inhibitory signals that are normally operative (64). Or perhaps they compete with memory cells (12) for cytokines necessary for the transition (such as IL-7; reference 11) that would be more readily available in γc-deficient hosts. Finally, differences in the behavior of thymocytes and lymph node cells suggest that naive T cells may not be ready to make this transition immediately after emigration from the thymus, but that they may need first to transit through the first niche.

To conclude, when confounding factors such as alloreactivity or rejection/competition by CTL or NK cells are removed, the picture that emerges suggests that T cells do not need MHC molecules to survive in the periphery, to initiate space-induced division, or to remain in a particular niche once they have entered, whereas they do need MHC molecules to transit to a CD44^{hi} state and to undergo extensive proliferation. In some cases, the MHC molecules signal via the antigen-specific portions of the TCR (e.g., to initiate productive immune responses), whereas in other cases they may signal through other molecules such as CD4, CD2, and/or Thy-1 (e.g., to induce the transition to CD44^{hi} in empty hosts).

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