Homeostasis-stimulated Proliferation Drives Naive T Cells to Differentiate Directly into Memory T Cells

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Abstract

The developmental requirements for immunological memory, a central feature of adaptive immune responses, is largely obscure. We show that as naive CD8 T cells undergo homeostasis-driven proliferation in lymphopenic mice in the absence of overt antigenic stimulation, they progressively acquire phenotypic and functional characteristics of antigen-induced memory CD8 T cells. Thus, the homeostasis-induced memory CD8 T cells express typical memory cell markers, lyse target cells directly in vitro and in vivo, respond to lower doses of antigen than naive cells, and secrete interferon γ faster upon restimulation. Like antigen-induced memory T cell differentiation, the homeostasis-driven process requires T cell proliferation and, initially, the presence of appropriate restricting major histocompatibility complexes, but it differs by occurring without effector cell formation and without requiring interleukin 2 or costimulation via CD28. These findings define repetitive cell division plus T cell receptor ligation as the basic requirements for naive to memory T cell differentiation.

Key words: memory T cells • developmental requirements • TCR ligation • proliferation • homeostasis

Introduction

Naive T cells can be stimulated through their TCRs by cognate antigens to proliferate in normal mice (1). They also undergo homeostasis-driven proliferation in lymphopenic mice in the absence of overt antigen stimulation. Although administration of exogenous antigen is not needed, homeostasis-driven T cell proliferation requires the presence of appropriate restricting MHCs (2–6), indicating that the engagement of TCR by endogenous peptide–MHC complexes is required. Antigen-stimulated T cell proliferation results in their expression of CD44 and differentiation into memory T cells. Proliferating T cells in lymphopenic individuals also display CD44 (3, 7–9) and largely on this basis these cells are often ambiguously termed "activated/memory" cells. However, whether the resultant T cells following homeostasis-driven proliferation are true memory cells, i.e., are able to respond with enhanced intensity and speed to reencounter with the same antigen, has not been determined. Neither is the role of proliferation, whether induced by antigen or by homeostasis, clear in memory T cell development.

We recently described a system for generating large numbers of memory CD8 T cells (10). In this system, naive CD8 T cells from 2C TCR transgenic mice on the recombination activating gene (RAG)[1]-$1^{-/-}$ background (termed 2C/RAG mice) were transferred into syngeneic RAG-$1^{-/-}$ recipients lacking their own lymphocytes. The recipients were then immunized with a potent antigenic peptide. 1 mo or more after immunization, the surviving 2C cells expressed the cell surface markers and functional properties of memory CD8 T cells. Unexpectedly, however, we subsequently noted that transferred naive 2C T cells also developed into memory CD8 T cells even if the recipients were not immunized. The pursuit of this observation, presented here, shows that (a) homeostasis-mediated proliferation of naive T cells results in their differentiation into functional memory T cells, (b) this differentiation is dependent on T cell proliferation and initially, the presence of appropriate MHCs, and (c) this differentiation pathway from naive to

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[1]*Abbreviations used in this paper:* B6, C57BL/6; CFSE, carboxyfluorescein diacetate-succinimidyl ester; GFP, green fluorescent protein; RAG, recombination activating gene.

memory cells occurs without forming effector cells and without requiring IL-2 or costimulation via CD28.

Materials and Methods

Mice. The 2C TCR recognizes SIYRYYGL peptide in association with MHC class I K^b molecules (11). 2C TCR transgenic mice (12) were all on the RAG-1–deficient ($RAG\text{-}1^{-/-}$ [13]) background and have been backcrossed onto C57BL/6 (B6) background for seven generations. $RAG\text{-}1^{-/-}$ mice, backcrossed with B6 mice for 13 generations, were used between 3 and 10 wk of age as adoptive transfer recipients. Naive 2C and B6 mice and B6 mice deficient in either IL-2 or CD28 (from The Jackson Laboratory) were used at 3–7 wk of age as donors. B6 mice expressing the green fluorescent protein (GFP) transgene (B6/GFP) were from Drs. M. Okabe and M. Yokoyama (Research Institute for Microbial Diseases, Osaka University, Suita, Japan; reference 14).

Adoptive Transfer. Different numbers of naive T cells from lymph nodes of 2C or B6 mice or FACS®-purified $CD8^+CD44^-$ cells from these mice were injected intravenously into nonirradiated syngeneic ($H\text{-}2^b$) $RAG\text{-}1^{-/-}$ recipients or syngeneic normal B6 recipients. 3 wk or more after the transfer, CD8 T cells from the nonimmunized recipients were analyzed. Antigen-induced memory 2C cells were generated by subcutaneously injecting some recipients of the 2C cells with 50 μg of the SIYRYYGL peptide in complete Freund's adjuvant and analyzing them 4 mo or more later. For analysis of cell division, lymph node cells from naive 2C or B6 mice were labeled with carboxyfluorescein diacetate-succinimidyl ester (CFSE) and then transferred into nonirradiated $RAG\text{-}1^{-/-}$ recipients. In addition, 2C cells and B6 T cells were activated in vitro with 10 nM SIYRYYGL peptide or plate-bound anti-CD3ε plus anti-CD28 antibodies (10 μg/ml each), respectively, for 3 d before transfer into normal B6 recipients.

Antibodies, Intracellular IFN-γ Staining, and Flow Cytometry. Antibodies to CD8, CD25, CD69, CD44, CD62L (L-selectin), Ly-6C, and IL-2Rβ were purchased as conjugates from BD PharMingen. Anti-CD11A (LFA-1) antibody was conjugated with FITC. Clonotypic antibody 1B2, specific for the 2C TCR, was conjugated to biotin. Cells were stained in the presence of 3 μg/ml anti-FcR antibody in PBS containing 0.1% bovine serum albumin and 0.1% NaN_3 and analyzed on a FACSCalibur™, collecting 10,000–10,000,000 live cells per sample. To detect intracellular IFN-γ, cells were incubated in the presence or absence of immobilized anti-CD3ε antibody for 3 h. Then, brefeldin A was added and the cultures were incubated for another 5 h. The cells were then surface stained with antibody to CD8 before being fixed and stained for intracellular IFN-γ.

Cytolytic Assays. Cells from lymph nodes and spleens were incubated with a cocktail of biotin-labeled antibodies to FcR, CD4, Mac-1, NK1.1, and B220, followed with streptavidin-labeled microbeads (Miltenyi Biotec), using 2 beads/cell, and purified on a SuperMACS cell sorter. Magnetically purified memory (85%) and naive (95%) CD8 T cells were used in CTL assays. ^{51}Cr-labeled T2-K^b cells were used as target cells in a 6-h CTL assay because low E/T ratios were used (1:1 or 2.5:1). Except for sextuplet wells to determine spontaneous and maximum ^{51}Cr release, all samples were assayed in triplicate. Specific lysis was calculated as: [(experimental counts − spontaneous counts)/(total counts − spontaneous counts)] × 100.

Tumor Rejection. EL4 thymoma cells were transfected with an hsp65-P1 vector expressing mycobacterial heat shock protein 65 fused with a P1 peptide containing SIYRYYGL epitope (15). Transfectants were screened for their ability to serve as good targets for 2C CTL clones in cytolytic assays and a positive transfectant, called EL4-SYRGL, was used for implantation. B6 mice were implanted with 3×10^6 EL4 tumor cells on one flank and 3×10^6 EL4-SYRGL tumor cells on the opposite flank. 2 d later, mice were adoptively transferred with a graded number (1×10^4, 1×10^5, or 1×10^6) of naive 2C cells, or memory 2C cells from immunized mice, or memory 2C cells from nonimmunized mice. Tumor sizes were measured with a caliper at days 7, 10, and 16 after implantation.

Results

Spontaneous Memory Cell Differentiation. In contrast to freshly isolated naive 2C T cells, the persisting 2C T cells in nonimmunized recipients expressed the same elevated levels of CD44, Ly-6C, IL-2Rβ, and LFA-1 as memory 2C T cells from the immunized recipients (Fig. 1 a). They did not express IFN-γ constitutively but could be induced to express this cytokine within 8 h of stimulation with anti-CD3ε antibody (Fig. 1 b), and after 24 h stimulation almost all of these cells (>96%) were positive for IFN-γ (data not shown). Memory cells from both nonimmunized and immunized recipients showed a similar dose–response profile in TCR downmodulation and CD69 expression, requiring ~30-fold lower peptide concentration than naive cells to downmodulate the TCR level by 50% or to induce CD69 expression by ~80% of the cells (Fig. 2 b). They also lysed target cells ex vivo in a peptide- and TCR-dependent manner (Fig. 2 a). In addition, memory cells from immunized and nonimmunized recipients rejected or suppressed the growth of EL4 tumor cells that expressed the SIYRYYGL epitope but not the parental tumor cells (control), whereas naive cells failed to suppress the growth of both types of tumor cells (Fig. 2 c). Thus, in these assays, memory 2C T cells arising in $RAG\text{-}1^{-/-}$ recipients were either the same or very similar phenotypically and functionally, whether or not the recipients were immunized with the exogenous antigenic peptide.

To determine if the spontaneous differentiation of naive into memory CD8 T cells in lymphopenic recipients occurs with T cells expressing diverse TCRs, we transferred total lymph node cells from normal B6 mice, containing CD8 and CD4 T cells and B cells, into syngeneic $RAG\text{-}1^{-/-}$ recipients. As was seen with 2C cells, the transferred naive B6 CD8 T cells also differentiated into memory T cells in 30 d. Thus, they expressed high levels of CD44, Ly-6C, IL-2Rβ, and LFA-1 (Fig. 1 a), produced IFN-γ within 8 h of anti-CD3ε stimulation (Fig. 1 b), and lysed target cells directly ex vivo in a ConA–based CTL assay (Fig. 2 a). Hence, the spontaneous differentiation of memory CD8 T cells after adoptive transfer of naive cells into syngeneic $RAG\text{-}1^{-/-}$ recipients is not unique to cells expressing a particular TCR: it is a general property associated with the adoptive transfer into lymphopenic recipients.

At the time of transfer, the majority of CD8 T cells from both the 2C transgenic and normal B6 donors were naive as indicated by the absence or only low levels of cell surface

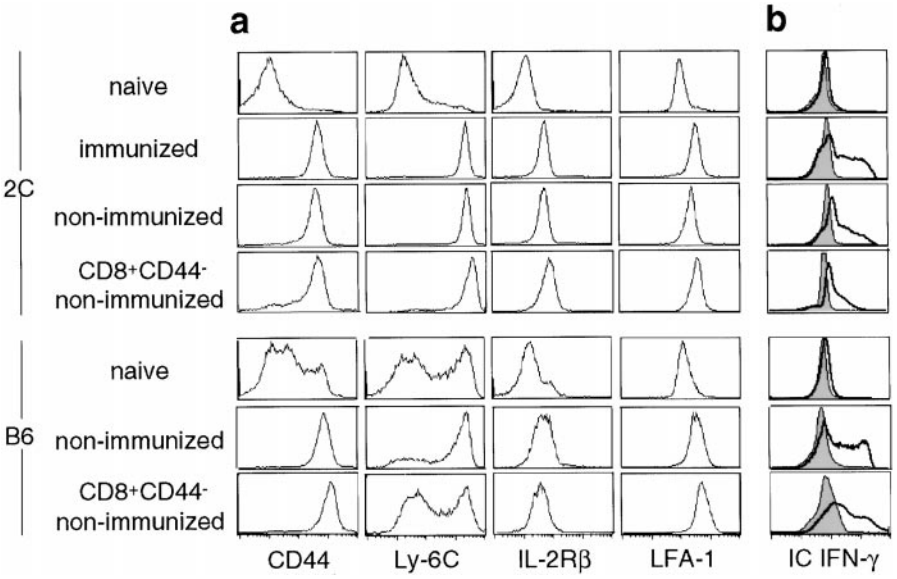

Figure 1. Naive CD8 T cells spontaneously differentiate into memory cells after transfer into RAG-$1^{-/-}$ recipients. Total lymph node cells or $CD8^+CD44^-$ cells from 2C/RAG or B6 mice were adoptively transferred into syngeneic RAG-$1^{-/-}$ recipients. After 3 wk or more, CD8 T cells from these nonimmunized recipients were analyzed for memory cell phenotype and function (non-immunized). For comparison, antigen-induced memory cells were generated by immunizing some recipients of 2C cells with SIYRYYGL peptide in CFA and studying them at least 4 mo later (immunized). Naive 2C and B6 T cells were from 2C/RAG and normal B6 donors, respectively. (a) Persisting CD8 T cells in nonimmunized recipients acquire a cell surface phenotype characteristic of memory T cells. Lymph node cells from RAG-$1^{-/-}$ recipients or from naive donors were analyzed by flow cytometry using antibodies to TCR and CD8 plus antibodies to CD44, Ly-6C, IL-2Rβ, or LFA-1. The expression of CD44, Ly-6C, IL-2Rβ, and LFA-1 on TCR^+CD8^+ T cells is shown as histograms. (b) CD8 T cells from nonimmunized recipients are rapidly induced to express IFN-γ. Intracellular (IC) IFN-γ expression by $CD8^+$ T cells is shown as histograms: bold outline, with anti-CD3ε stimulation (8 h); filled, without stimulation.

CD44 and Ly-6C, minimal IFN-γ expression, and negligible levels of cytolytic activity (Figs. 1 and 2). Nevertheless, the spontaneously derived memory T cells in the RAG-$1^{-/-}$ recipients could have resulted from a preferential expansion of a few memory T cells (7) in the transferred cell population. To exclude this possibility, $CD8^+CD44^-$ cells were purified from 2C/RAG mice (99.9% $CD44^-$) and B6 mice (99.8% $CD44^-$) and then transferred into RAG-$1^{-/-}$ recipients. Within 3 wk of transfer, the purified naive cells had acquired the characteristic surface phenotype of memory cells and could be rapidly induced to express IFN-γ (Fig. 1, a and b). Moreover, when equal numbers of naive or memory 2C cells were added to a fixed number of lymph node cells from naive B6 mice and the mixtures were transferred into RAG-$1^{-/-}$ recipients, after 60 d the ratio of 2C cells (distinguished by a clonotypic antibody to the 2C TCR) to B6 CD8 T cells was the same whether the 2C cells added were naive or memory phenotype (data not shown). These findings show that the memory CD8 T cells arising in nonimmunized RAG-$1^{-/-}$ recipients are not the result of selective expansion of a few memory T cells present in the transferred inoculum, but indeed are derived from transferred naive cells in the absence of exogenous antigen.

Progressive Acquisition of Memory Phenotype. To examine the kinetics of the spontaneous memory CD8 T cell differentiation, 1×10^5 naive 2C cells were transferred into RAG-$1^{-/-}$ recipients and the number of surviving 2C cells and their CD44 expression were monitored at different times. As shown in Fig. 3 a, the number of 2C T cells in the spleen and lymph nodes and their levels of CD44 expression increased progressively over time. 40 d after transfer, more T cells were recovered from spleen and lymph nodes than had been initially transferred, indicating that the transferred T cells had proliferated in the recipients. To demonstrate the proliferation more directly, cells were labeled with CFSE and then transferred into syngeneic recipients. When a cell divides, the intensity of CFSE fluorescence decreases by about half and therefore provides an accurate count of the number of cell divisions (16). When 2.5×10^5 CFSE-labeled cells were transferred, within 5 d >90% of the cells divided and the median number of divisions was 2.5 (Fig. 2 b). In contrast, when 40 times more cells (1.1×10^7) were transferred, fewer cells (~65%) divided and the median number of divisions was 1 during the same period. Furthermore, when 2.5×10^5 cells were transferred into syngeneic B6 recipients having normal levels of T cells, <20% of the cells divided. Similarly, most of the newly transferred CFSE-labeled naive 2C cells did not divide in RAG-$1^{-/-}$ recipients that had previously received naive 2C cells and been immunized with peptide ("filled" with memory cells). Thus, the extent of proliferation was greater when fewer cells were transferred and depended on the recipients' status as "empty" or "filled," characteristics of homeostasis-mediated proliferation.

To evaluate the dependence of memory T cell development on the extent of proliferation, different numbers of lymph node cells from B6 mice were transferred into syngeneic RAG-$1^{-/-}$ recipients. 52 d later, surviving CD8 T cells in the recipient's lymph nodes were assayed for representative phenotypic and functional hallmarks of memory CD8 T cells: CD44 expression and rapid induction of IFN-γ after anti-CD3ε stimulation. As shown in Fig. 3 c, when increasing numbers of cells were transferred (1×10^5, 1×10^6, 1×10^7, and 2.5×10^7), progressively smaller proportions of persisting T cells expressed IFN-γ or became $CD44^{high}$. Thus, the spontaneous transition of naive to memory CD8 T cells appears to be directly linked to the extent of homeostasis-mediated proliferation.

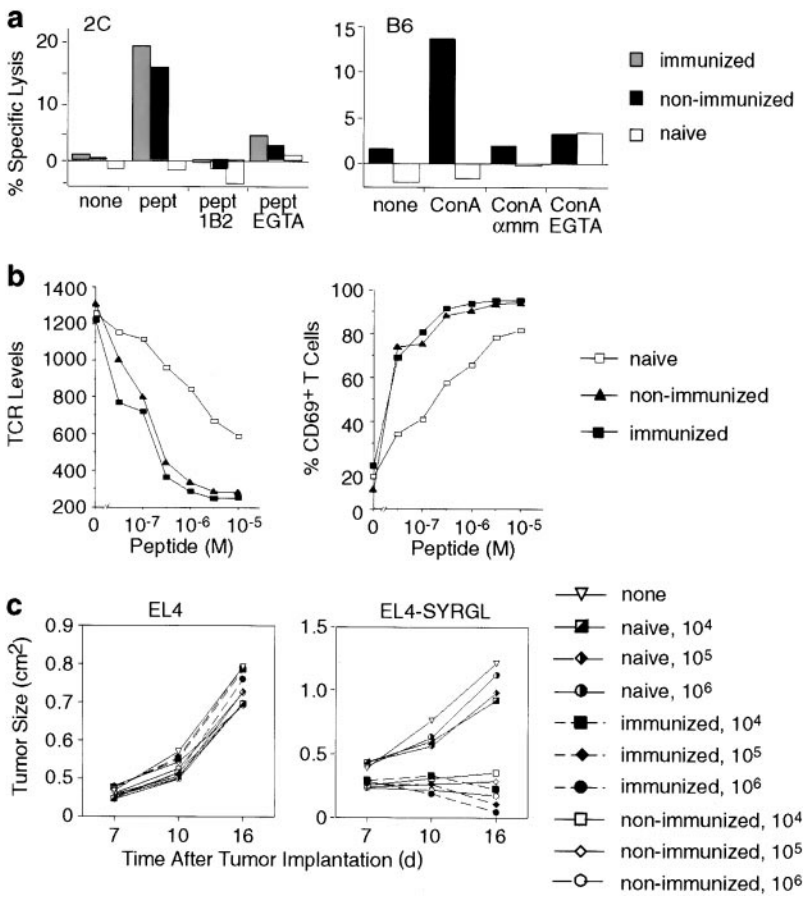

Figure 2. Functional comparison of naive and memory 2C T cells. Naive and memory 2C cells from either immunized or nonimmunized recipients were generated as in the legend to Fig. 1. (a) CD8 T cells from nonimmunized recipients are directly cytolytic. Purified 2C T cells from RAG-$1^{-/-}$ recipients or naive donors were added at an E/T ratio of 2.5:1 in the presence of 1 nM SIYRYYGL peptide (pept). Target cell lysis depended on the presence of the peptide and was blocked by antibody (1B2) to the 2C TCR (30 μg/ml) or by EGTA (4 mM). The cytolytic activity of purified $CD8^+$ cells from B6 recipients and donors was assayed by ConA-mediated killing of target cells at an E/T ratio of 1:1. Target cell lysis required the presence of ConA (5 μg/ml) and was inhibited by α-methylmannoside (αmm, 5%) or EGTA. (b) CD8 T cells from nonimmunized recipients downmodulate TCR and upregulate CD69 in response to lower concentrations of antigenic peptide than naive cells. Lymph node cells from naive 2C/RAG mice and immunized and nonimmunized RAG-$1^{-/-}$ recipients of 2C cells were incubated with irradiated B6 splenocytes in the presence of different concentrations of SIYRYYGL peptide for 4 h. Cells were analyzed for the levels of 2C TCR, CD69, and CD8. Fluorescence intensity (geometric mean) of TCR staining of $CD8^+$ cells and percentages of TCR^+CD8^+ T cells that are $CD69^+$ are shown as a function of peptide concentrations. (c) CD8 T cells from nonimmunized recipients are more effective than naive cells in tumor rejection. B6 mice were implanted with 3×10^6 EL4 tumor cells on one side and 3×10^6 EL4 tumor cells expressing the SIYRYYGL epitope (EL4-SYRGL) on the other side. 2 d later, mice were either not transferred with any 2C cells or were transferred with indicated numbers of naive 2C cells, memory 2C cells from immunized mice, or memory 2C cells from nonimmunized mice. Tumor sizes (in cm^2) generated from transferred EL4 or EL4-SYRGL tumor cells are shown at different days after implantation. When 10^4 and 10^5 T cells were transferred, memory 2C cells from immunized recipients were significantly more effective than those from nonimmunized recipients in rejecting EL4-SYRGL tumor at day 23 (not shown).

The direct coupling of IFN-γ expression with cell proliferation was shown by transferring CFSE-labeled lymph node cells from 2C or B6 donors into RAG-$1^{-/-}$ recipients and assaying for IFN-γ and CD44 expression as a function of the number of cell divisions. 9 d after transfer, lymph node cells from the recipients were incubated in vitro in the presence or absence of anti-CD3ε antibody for 8 h and then assayed for intracellular IFN-γ expression. Very few CD8 T cells from recipients of either 2C or B6 donors expressed IFN-γ in the absence of anti-CD3ε stimulation, but many of them expressed IFN-γ within 8 h of stimulation (Fig. 4 a). The proportion of IFN-γ–positive cells increased linearly in relation to the number of cell divisions, indicating that a relatively constant proportion of the dividing cells acquires the capacity for rapid induction of IFN-γ expression at each cell division.

Direct Differentiation of Naive to Memory T Cells. Lymph node cells from the nonimmunized RAG-$1^{-/-}$ recipients were also analyzed directly for CD44 and CD25 expression. By day 9 after transfer, most of the transferred T cells underwent two to six cell divisions and CD44 was upregulated progressively in a manner similar to IFN-γ (Fig. 4 b). In contrast, the T cell activation markers CD69 and CD25 (the IL-2 receptor α chain), which are known to be induced on antigen-activated effector T cells, were not detected on transferred CD8 T cells after any number of cell divisions (Fig. 4 c, and data not shown). Similarly, CD69 and CD25 expression were not detected on T cells on days 12 and 15 when they have undergone nine or more divisions (data not shown). Together with data showing that proliferating T cells in nonimmunized RAG-$1^{-/-}$ recipients did not express IFN-γ unless stimulated by anti-CD3ε, these findings suggest that effector cells are not formed when homeostasis drives naive T cells to differentiate into memory T cells.

To examine more closely whether effector cells can be generated and detected in our transfer system, 2 d after the transfer of CFSE-labeled 2C T cells, RAG-$1^{-/-}$ recipients were immunized with a potent agonist peptide specific for the 2C TCR. 3 d later, 2C cells from lymph nodes of the immunized recipients were assayed directly for IFN-γ, CD44, and CD25 expression. Most of these antigen-stimulated cells behaved as effector cells: 80% expressed IFN-γ constitutively and 20% were positive for CD25 (Fig. 4, d and e). Cell division after antigen stimulation also occurred much faster than homeostasis-mediated cell division, e.g., approximately eight divisions (peak frequency) in 3 d compared with four divisions in 9 d, respectively. These results

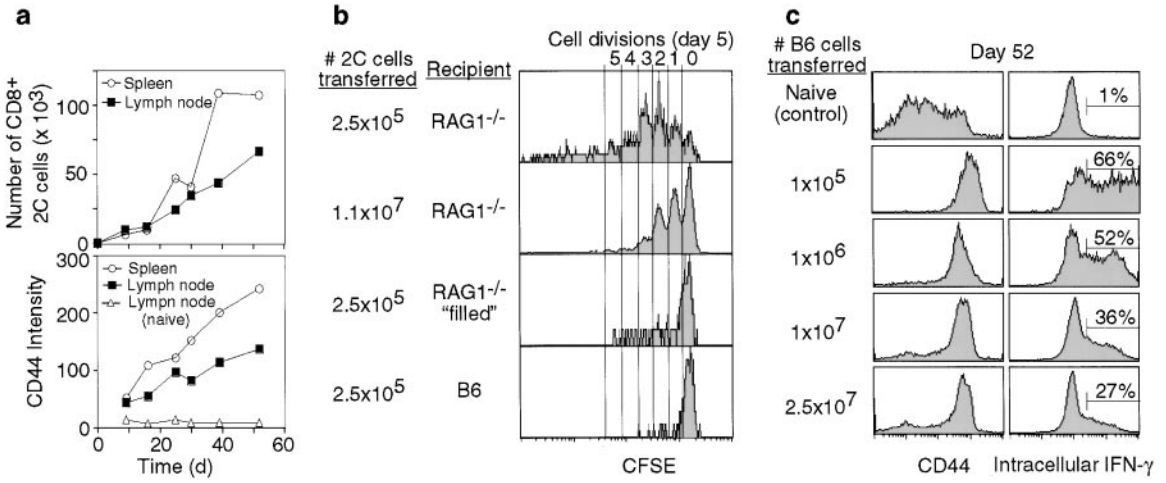

Figure 3. Naive CD8 T cells undergo homeostasis-mediated proliferation in RAG-$1^{-/-}$ recipients. (a) Transferred T cells undergo proliferation in RAG-$1^{-/-}$ recipients in the absence of apparent antigen stimulation. The number of surviving T cells (TCR^+CD8^+) in the lymph nodes and spleen and the intensity of CD44 expression (geometric mean) on these T cells are shown as a function of time. Lymph node (naive) refers to 2C T cells from naive donor mice. (b) Proliferation is mediated by homeostatic mechanisms. Different numbers of CFSE-labeled naive 2C T cells were adoptively transferred into syngeneic normal B6 recipients, RAG-$1^{-/-}$ recipients, or RAG-$1^{-/-}$ recipients that had received naive 2C cells and peptide immunization 4 mo before the transfer of CFSE-labeled cells ("filled"). The intensity of CFSE on 2C cells from different recipients is shown. (c) Memory T cell differentiation is linked to the extent of cell division. The levels of CD44 and intracellular IFN-γ expression after anti-CD3ε stimulation (8 h) are shown on $CD8^+$ T cells. Naive (control) indicates B6 donor cells.

further support the notion that naive CD8 T cells can directly differentiate into memory cells during homeostasis-driven proliferation without first becoming activated effector cells whereas effector cells are prominent in the course of antigen-induced memory T cell differentiation (17, 18).

Requirement for Proliferation for Memory T Cell Differentiation. Although our findings clearly show that the spontaneous transition from naive to memory CD8 T cells progressively occurs in parallel with cell proliferation, it was not clear that cell division was actually essential. To address

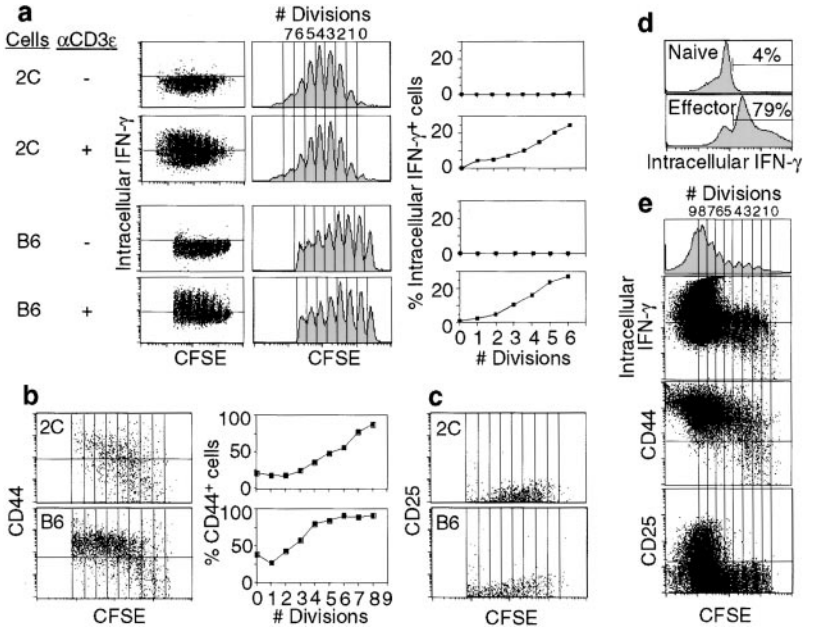

Figure 4. Transferred T cells differentiate progressively into memory cells without becoming activated effector cells. (a) Transferred T cells progressively acquire the capacity for rapid induction of IFN-γ expression. Lymph node cells from 2C or B6 donors were labeled with CFSE and transferred into RAG-$1^{-/-}$ recipients for 9 d. Then, lymph node cells from the recipients were cultured in the presence or absence of anti-CD3ε antibody for 8 h and assayed for intracellular IFN-γ expression. The expression of IFN-γ by $CD8^+$ T cells is shown as a function of CFSE intensity (left). The percentage of IFN-γ^+ cells within each cell division cohort (middle) is shown as a function of the number of cell divisions (right). IFN-γ^+ cells are those above the horizontal line (left). Similar to CD8 cells, CD4 T cells from B6 lymph node also proliferated in the RAG-$1^{-/-}$ recipients and acquired high levels of CD44 and rapid IFN-γ expression after anti-CD3ε stimulation (data not shown). (b and c) Progressive differentiation of memory cell phenotype occurs in the absence of activated effector cell formation. (b) CD44 expression versus CFSE intensity is shown for $CD8^+$ cells by two-dimensional dot plots (left). The percentage of $CD44^+$ T cells is also shown as a function of the number of cell divisions (right). $CD44^+$ cells are those above the horizontal line (left). (c) CD25 expression versus CFSE intensity is shown for $CD8^+$ cells. (d and e) Transferred T cells differentiate into activated effector cells after immunization. 2 d after the transfer of CFSE-labeled 2C T cells, RAG-$1^{-/-}$ recipients were immunized with SIYRYYGL peptide in complete Freund's adjuvant. 3 d later, 2C cells from lymph nodes of the immunized recipients were assayed directly for IFN-γ, CD25, and CD44. (d) The expression of IFN-γ by $CD8^+$ 2C T cells from immunized recipients (effector) is compared with that of naive 2C T cells. (e) The expression of IFN-γ, CD25, and CD44 by $CD8^+$ effector T cells is shown as a function of CFSE intensity.

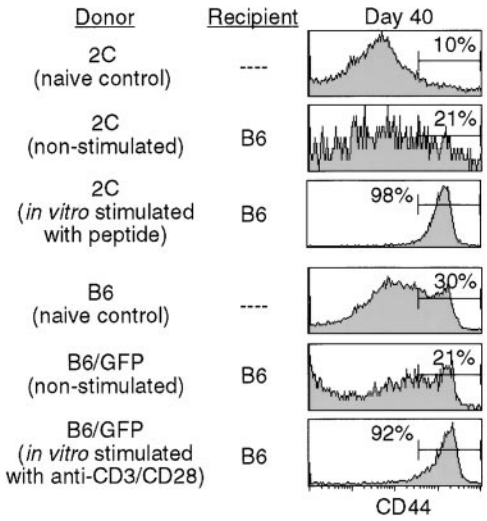

Figure 5. Proliferation is required for memory CD8 T cell differentiation. Lymph node cells from 2C donors or B6/GFP donors were either directly transferred into syngeneic B6 recipients (non-stimulated), or were activated in vitro with an antigenic peptide (10 nM SIYRYYGL for 2C cells) or with anti-CD3ε plus anti-CD28 (10 μg/ml each for B6/GFP), allowed to proliferate for 3 d, and then transferred into normal B6 recipients. 40 d after transfer, CD8 T cells from lymph nodes were analyzed for CD44 expression, gating on $CD8^+2C^+$ (for 2C) and $CD8^+GFP^+$ (for B6 T cells) cells.

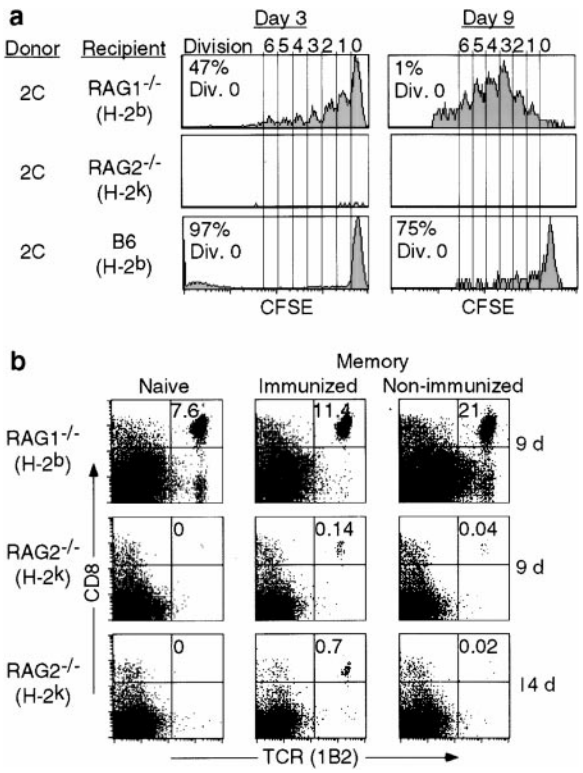

Figure 6. Requirements for homeostasis-driven proliferation. (a) Homeostasis-driven proliferation requires the presence of both "space" and the correct MHC. Proliferation of $CD8^+TCR^+$ cells in various recipients is shown as histograms of CFSE profiles. RAG-$2^{-/-}$ (H-2^k) recipients were treated with anti-Ly49G2 (200 μg intraperitoneally) to deplete NK cells 1 d before the transfer and another 100 μg on the day of transfer. H-2^k was not recognized by 2C cells as shown by mixed lymphocyte reaction (data not shown). (b) Comparison of survival of naive and memory 2C T cells in recipients with "incorrect" MHCs. An equal number of naive and memory 2C T cells (1×10^6) was transferred into either syngeneic (H-2^b) RAG-$1^{-/-}$ recipients or RAG-$2^{-/-}$ recipients having an incorrect MHC class I (H-2^k). 9 and 14 d after the transfer, splenocytes and lymph node cells were assayed for CD8 and TCR (1B2) expression by flow cytometry. Numbers indicate the percentages of $CD8^+TCR^+$ cells in lymph node. A similar result was also obtained in the spleen (not shown). The memory 2C T cells were generated as described in Materials and Methods and RAG-$2^{-/-}$ (H-2^k) recipients were treated with anti-Ly49G2 antibody as above.

this issue, we transferred lymph node cells from 2C donors or B6 donors that expressed the GFP transgene (B6/GFP) into syngeneic normal B6 recipients. The transferred T cells did not appreciably proliferate in the normal recipients (Fig. 3) and 40 d later, the CD44 expression profile of the surviving T cells from both 2C and B6/GFP donors resembled that of the respective naive donor cells (Fig. 5). If, however, 2C or B6 CD8 T cells were activated through their TCR and allowed to proliferate for 3 d in vitro before being transferred into normal B6 recipients, the surviving cells showed higher levels of CD44 expression, typical of memory T cells. Thus, the mere survival of transferred T cells in the recipients is not sufficient for their differentiation into memory T cells. Proliferation is required.

Requirements for Homeostasis-driven Proliferation. To examine the requirements for the homeostasis-driven T cell proliferation in our adoptive transfer system, CFSE-labeled naive 2C cells were transferred into RAG-$1^{-/-}$ recipients having the correct (or syngeneic) MHC haplotype (H-2^b) into H-2^k RAG-$2^{-/-}$ recipients lacking the correct MHC, or into normal (i.e., nonlymphopenic) B6 recipients having the correct MHC (H-2^b). CFSE intensity, as measured by flow cytometry, revealed that the transferred T cells proliferated vigorously in the H-2^b RAG-$1^{-/-}$ recipients but divided only minimally in the H-2^b B6 recipients (Fig. 6 a), confirming that the presence of "space" is required for the proliferation. However, as early as 3 d after transfer, few surviving 2C cells were detected in H-2^k RAG-$2^{-/-}$ recipients and by day 9, these cells could not be detected. The failure of the naive cells to survive was probably not due to destruction by NK cells because the recipients were pretreated with anti–Ly-49G2 mAb to deplete H-2^b–specific NK cells. In addition, some of the transferred memory 2C cells from immunized recipients survived and remained $CD44^{high}$ 9 and 14 d after transfer into H-2^k RAG-$2^{-/-}$ recipients (Fig. 6 b). Although some of the transferred memory 2C cells from nonimmunized recipients were also detected at day 9 in H-2^k RAG-$2^{-/-}$ recipients, they were not detectable at day 14, indicating that there may be a difference in the requirement for MHC for the survival of memory cells from immunized and nonimmunized recipients. Nevertheless, our findings are consistent with various recent reports that not only the presence of "space" but also the presence of the correct MHC is required for homeostasis-driven proliferation of naive T cells (2–6).

IL-2 and CD28 Are Not Required for Homeostasis-driven Memory T Cell Differentiation. Costimulatory CD28 molecules and cytokine IL-2 are important for antigen-induced

T cell proliferation. To determine their role in homeostasis-mediated proliferation, lymph node cells from normal B6 mice and B6 mice deficient in either CD28 or IL-2 production were labeled with CFSE and then transferred into syngeneic RAG-$1^{-/-}$ mice. 9 d later, CFSE intensity of $CD8^+TCR^+$ cells was analyzed and no significant difference was detected between wild-type and mutant mice (data not shown). Lymph nodes cells from mutant and wild-type mice were also transferred directly into RAG-$1^{-/-}$ recipients and 40 d later, cells were analyzed for the expression of various memory cell markers. No difference, including CD44 expression on $CD8^+TCR^+$ cells, was detected (data not shown). Thus, unlike antigen-induced T cell proliferation, CD28 and IL-2 are not required for homeostasis-mediated T cell proliferation and memory cell differentiation.

Discussion

Normally, in immunocompetent individuals having normal levels of lymphocytes, naive CD8 T cells can be stimulated through their TCR by exogenous antigen to proliferate and differentiate into effector and memory cells. The findings presented here demonstrate that in lymphopenic individuals, naive CD8 T cells can also be stimulated through their TCR by endogenous peptide–MHC complexes to proliferate and differentiate into memory T cells. Antigen-stimulated and homeostasis-driven memory T cell differentiation share many but not all requirements. Just as TCR ligation is critical for antigen-induced differentiation, homeostasis-driven T cell proliferation in our adoptive transfer system also requires the engagement of TCR, but with endogenous peptide–MHC complexes (Fig. 5; references 2–6). Because persistence of memory T cells does not depend on the presence of appropriate restricting MHC (2, 18, 19), TCR ligation is probably only necessary for the initiation of memory T cell differentiation.

Besides TCR ligation, we show that homeostasis-driven memory T cell differentiation is directly coupled to cell proliferation. Consistent with the requirement for proliferation, CD8 T cells bearing TCR-specific for the H-Y male antigen do not proliferate in syngeneic lymphopenic females (3, 4, 8, 20) and do not differentiate into memory cells (17). Since lymphocyte proliferation is an invariable response to antigen stimulation, proliferation is also likely to be required for antigen-induced memory T cell differentiation (17, 21). Unlike antigen-induced memory T cell differentiation, however, homeostasis-driven memory cell differentiation does not require (a) formation of activated effector cells, (b) administration of exogenous antigen, or (c) costimulatory CD28 molecules and cytokine IL-2. Thus, the two basic requirements for both antigen-induced and homeostasis-driven differentiation of naive to memory T cells are TCR ligation and repetitive cell division.

Why is proliferation so essential for memory T cell differentiation? One of the hallmarks distinguishing naive from memory CD8 T cells is the speed with which IFN-γ expression is induced after TCR ligation. In naive T cells, activation of IFN-γ expression is much slower because the IFN-γ locus has first to be relieved of epigenetic repression (22–24). That the IFN-γ gene is poised for rapid expression in memory cells suggests that the chromatin structure of this locus is remodeled during the transition from naive to memory cells (25–27). The requirement for proliferation for memory T cell differentiation is in accord with evidence that DNA replication facilitates chromatin remodeling (28, 29). Taken together, these diverse observations suggest that while signals from TCR ligation act selectively on the regulation of genes whose expression characterizes memory T cells, DNA replication (proliferation) facilitates the chromatin remodeling that allows these gene alterations to persist. The resulting epigenetic changes could then account for the stability of the memory T cells' phenotype over periods approaching an animal's lifetime.

Several different pathways have been proposed for memory T cell development (17, 30, 31). Our findings demonstrate that during homeostasis-driven memory T cell differentiation, naive CD8 T cells can directly differentiate into memory cells without first becoming activated effector cells (Fig. 3). Consistent with this finding, a recent study in which memory CD8 T cells were monitored in vivo after viral infection suggests that a small fraction of antigen-activated T cells appears to commit to becoming memory T cells too soon to have arisen from effector cell precursors (32). The development of memory and effector T cells thus parallels the distinct developmental pathways by which memory B cells and antibody-secreting (effector) B cells develop (30).

Finally, although homeostasis-driven memory cell differentiation probably does not occur in immunocompetent individuals, it seems to be involved in some pathologic conditions and may explain why lymphopenic individuals, including AIDS patients and bone marrow transplant recipients, are at greater risk of developing some autoimmune diseases (33–36). Autoreactive memory cells, generated via homeostasis-driven proliferation in these individuals, would require relatively little antigen for reactivation and are thus more likely than naive cells to promote the development of autoimmune diseases.

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