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Increasing T-Cell Age Reduces Effector Activity but Preserves Proliferative Capacity in a Murine Allogeneic Major Histocompatibility Complex–Mismatched Bone Marrow Transplant Model

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ABSTRACT

Aging of T cells is characterized by a series of alterations in surface antigen expression and a concomitant decline in functional activity in many assays. We have extended this analysis by comparing the ability of T cells from mice of different ages to cause graft-versus-host disease (GVHD) by using a parent into F_1 model (C57BL/6 T cells into C57BL/6 × C3H host animals). Young (3-5 months), adult (12-14 months), or old (19-24 months) T cells were introduced into irradiated F_1 hosts. Animals that had undergone transplantation were assessed for clinical and pathologic evidence of GVHD and for survival. At a given T-cell dose (2×10^6 cells), there was a T-cell (donor) age-dependent decline in severity of GVHD, with all recipients of young T cells succumbing to lethal GVHD, 75% of recipients of adult T cells succumbing, and no deaths occurring among recipients of old T cells. In vivo CD4 T-cell expansion was greater for young than old T-cell groups after transplantation, whereas old CD8 cells showed enhanced in vivo expansion compared with young cells. Among CD4 and CD8 cells, the T-cell receptor repertoire, surface antigen expression on activated cells, and homing receptor function were similar for all ages after expansion in vivo. The progeny of old T cells reisolated after transplantation expressed type 1 cytokines (interferon-γ and tumor necrosis factor-α) at a lower frequency than young cells and had decreased cytolytic function against H-2^k-bearing target cells. This provides a partial explanation for the decreased GVHD. Carboxyfluorescein diacetate succinimidyl ester labeling of transplanted cells showed comparable rates of proliferation when comparing GVHD-competent (12 months) and GVHD-incompetent (19 months) T cells in both syngeneic and F_1 host animals. We suggest that the lack of effector activity demonstrated by old T cells in vivo is a reflection of a cell-autonomous defect downstream of signals required for antigen-driven proliferation.

KEY WORDS

Aging T cells • Graft-versus-host disease • Effector activity • Cytokines • T-cell expansion

INTRODUCTION

Aging is characterized by a time-dependent decline in the ability of an organism to respond to a wide variety of stressors. In the immune system, aging manifests as a decline in the ability to mount a response to novel pathogens or to mount a successful memory response to antigens encountered earlier in an organism's life span. The basis for the observed decline in immune function is complex and involves both sys-

temic deficits (such as the reduced production of naive T lymphocytes) and cell-autonomous defects (such as a reduced proliferative capacity or less robust activation of signal transduction machinery in response to antigens or cytokines).

Decreased proliferative capacity has been well documented for T-cell clones from humans, in which clonal exhaustion occurs after repeated rounds of proliferation [1], perhaps triggered by progressive telomere shortening [2]. In murine systems, telomere-dependent clonal exhaustion does not seem to occur: baseline telomere length is greater, and telomere loss with cell division either is not seen or is less dramatic because of the constitutive expression of telomerase activity in murine cells [3]. However, several studies have demonstrated decreased proliferative responses of T cells isolated from aged mice, and much of the characterization of decreased proliferation has focused on abnormalities of T-cell signaling (see review [4]). Many specific components in T-cell activation pathways have been shown to be abnormal after in vitro activation of older T cells from humans and rodents. From proximal (early) to distal (late), abnormalities have been noted in clustering of co-stimulatory molecules in lipid rafts [5], tyrosine phosphorylation of T-cell receptor (TCR) chains [6], kinase activity of Zap70 and Lck [7], mitogen-activated protein kinase activity [8], binding of adapter molecules [9], calcium flux [10], and subsequent nuclear factor of activated T cells translocation [11] and nuclear factor-κ B activation [12]. It is not known whether the multiple abnormalities documented are downstream readouts of the same proximal defect, represent multiple independent abnormalities of old cells, or are reflective of an underlying change in the physiology of old T cells. In addition, it is difficult to distinguish between a potentially senescent T cell and a memory T cell, because the surface antigenic characteristics of old T cells overlap with those of memory cells. Independent of surface phenotype, there is evidence that the functional capacity of T cells continues to decline with advancing age, because naive cells from young animals function better than naive cells from old animals in models that use TCR transgenic mice [5].

As aging progresses, the diversity of the resting lymphocyte pool also contracts, raising the possibility that age-related immunodeficiency is due to a lack of responding cells. This contraction stems from at least 2 phenomena—a decline in the thymic output of naive cells, and clonal expansions of T cells that increase in frequency with age, particularly in the $CD8^+$ compartment [13-15]. The diversity of $CD4^+$ populations also seems to decline with age [16].

We analyzed the alloimmune responses of T cells from young, adult, and old donors and compared their proliferation, cytolytic function, and cytokine secretion in vitro and in vivo. In vivo we tested the ability of T cells of different ages to cause graft-versus-host disease (GVHD) in the context of a complete major histocompatibility complex mismatch and found that old T cells were unable to cause disease. Paradoxically, we showed that old cells respond to mitogenic signals with equivalent or more rapid cell division kinetics in vivo, yet they are unable to serve as effectors for GVHD. In assessing this lack of effector function, we analyzed cell-surface phenotypes, TCR repertoire, homing activity, cytokine expression, and cytolytic capacity of in vivo-expanded cells. Proliferation seems to be antigen driven and distinct from homeostatic responses, because the kinetics of proliferation and the degree of expansion are limited when the same T cells are placed into syngeneic hosts. Our data suggest that the aging process in mice differentially inhibits the cytolytic and cytokine response of alloreactive T cells while maintaining their ability to proliferate in response to alloantigens.

MATERIALS AND METHODS

Animals

T-cell donors—young (3-5 months), adult (12-14 months), and old (19-24 months) female C57BL/6J mice—were obtained from the National Institute on Aging contract colony (Bethesda, MD). Transplant recipients—female C57BL/6J × C3H (C3FEB6F1) mice—were obtained from Jackson Laboratory (Bar Harbor, ME) or from Taconic (Germantown, NY). Bone marrow donors—female C57BL/6J CD45.1-expressing mice (B6.SJL-Ptprc^{a}Pep3^b/BoyJ)—were obtained from the National Cancer Institute (Bethesda, MD).

T-Cell Purification and Phenotyping

T cells were purified by negative selection from a cell suspension of lymph node and spleen. Cells from 3 animals for each age-specific group were resuspended in cold magnetic bead wash (phosphate-buffered saline [PBS], 0.5% bovine serum albumin, and ethylenediaminetetraacetic acid 2 mmol/L), and antibodies against B cells, granulocytes, and monocytes (fluorescein isothiocyanate [FITC]-labeled anti-B220, anti-Gr-1, and anti-Mac-1 [BD Pharmingen, San Diego, CA]) were added. Cells were then incubated with anti-FITC magnetic beads (Miltenyi, Auburn, CA), followed by purification on an LS+ column (Miltenyi). Preparations were tested for purity and surface antigen expression by fluorescence-activated cell sorting (FACS). FITC-labeled anti-CD4, anti-$CD43^{-0}$, and anti-CD62-L; allophycocyanin (APC)-labeled anti-CD44; phycoerythrin (PE)-labeled anti-CD8; CyChrome-labeled anti-CD8 and anti-CD4; and Fc block were all purchased from BD Pharmingen. A panel of FITC-labeled murine TCR Vβ-specific

antibodies was also obtained from BD Pharmingen. All FACS collection was performed on a FACStar Plus machine, and data analysis was performed with Cell Quest software (BD Biosciences, San Jose, CA).

Bone Marrow Transplantation

Three- to 4-month-old female C3FEB6F1 recipients were conditioned with 13 Gy of gamma irradiation in 2 equivalent doses separated by 4 hours on the day of transplantation. Mice were maintained on chlorinated water for 3 weeks. All groups received 5×10^6 T cell-depleted B6 CD45.1 bone marrow; GVHD groups received 2×10^6 B6 T cells from young, adult, or old donors via intravenous injection.

GVHD Scoring and Pathologic Assessment

Clinical assessment of GVHD was performed weekly by using a semiquantitative scale, as described previously [17]. Parameters assessed included weight change, activity, posture, skin integrity, and fur texture. Pathologic assessment of GVHD damage to liver and gut was performed on tissue specimens obtained 21 and 35 days after transplantation. Blinded samples were scored by 1 individual (C.L.) according to a semiquantitative scale that measures ongoing damage, cellular infiltration, and regenerative processes affecting the large bowel, small bowel, and liver, as previously described [18]. Thirteen parameters were scored to assess liver damage, whereas 15 parameters were assessed for the small bowel and 9 for the large bowel.

Intracellular Cytokine Assay

Spleen T cells were harvested 11 days after transplantation and plated in 24-well plates in the presence of irradiated allogeneic stimulator cells (C3H T-cell–depleted splenocytes). Cells were cultured for 16 hours, and brefeldin A (10 μg/mL) was added during the last 14 hours of culture before antibody staining. Samples were split for surface staining with anti-CD8/CD44/CD122 or anti-CD4/CD44/CD62-L (FITC, peridinin chlorophyll-a protein [PerCP], and APC) to define activated subsets, followed by fixation and permeabilization according to the manufacturer's instructions (Cytofix/Cytoperm reagents; BD Pharmingen) before staining with PE-conjugated antibodies specific for murine interleukin (IL)-2, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-4, or IL-10, followed by FACS analysis. Each data point was derived from pooled cells of 3 animals.

In Vitro Cytolysis Assay

Spleen T cells were harvested 11 days after transplantation and enriched by positive selection with anti-CD5 beads. Purified T cells were incubated with (10^4) chromium-51-labeled allogeneic (p210) or H-2^d (p815) tumor cells at an effector-target ratio of 50:1 down to 6.25:1 to determine specific cytolytic activity. Target cell labeling, incubations, harvesting, and data analysis were performed as previously described [19]. There was no specific lysis of syngeneic cells (0%-10%) at any effector-target ratio. Spontaneous release was 16% of total release. Each point represents the mean ± SD of 3 wells. Each sample represents pooled cells from 3 animals, as described previously for the cytokine assay.

CFSE Labeling and Proliferation Assessment

T cells (prepared as described previously) were incubated for 15 minutes in warm (37°C) PBS containing 10 μmol of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene OR) at a concentration of 10^7 cells per milliliter. Cells were then washed with cold PBS, spun down, resuspended in PBS, and kept on ice until intravenous injection into irradiated host animals as described previously. An aliquot of labeled cells was run on FACS to verify uniform labeling of CD4 and CD8 populations. For the experiment in Figure 6, animals received 5×10^6 labeled cells at the time of transplantation. Spleen and lymph node cell suspensions were stained with CyChrome-labeled anti-CD4 and PE-labeled anti-CD8 before FACS analysis and determination of CFSE profile.

Statistical Analysis

Kaplan-Meier curves were compared by using the log-rank test for significance; GVHD scores were compared by using 1-way analysis of variance (ANOVA) on data from weekly intervals. Differences between groups became significant ($P < .01$) by week 3 and remained so throughout experiment. When animals died, GVHD scores for the prior week were included in subsequent analyses. Cell counts after transplantation were compared by using 1-way ANOVA and the Tukey posttest. GraphPad software (San Diego, CA) was used for all statistical tests.

RESULTS

Comparison of Phenotype of Young, Adult, and Old T Cells

B6 T cells were purified from spleen and lymph node by using a depletion technique to remove B cells and myeloid cells; this yielded 85% to 95% T cells (sum of CD4 and CD8). In Figure 1, cells were divided into CD8 (Figure 1A) and CD4 (Figure 1B) populations and co-stained with $CD43^{-0}$, CD44, or CD62-L. The fraction of $CD44^{hi}$ cells increased with age in both T-cell subsets, a finding that was consistent throughout all of our experiments. High expression of CD44 has been described on both activated T cells and memory cells, and increased expression is

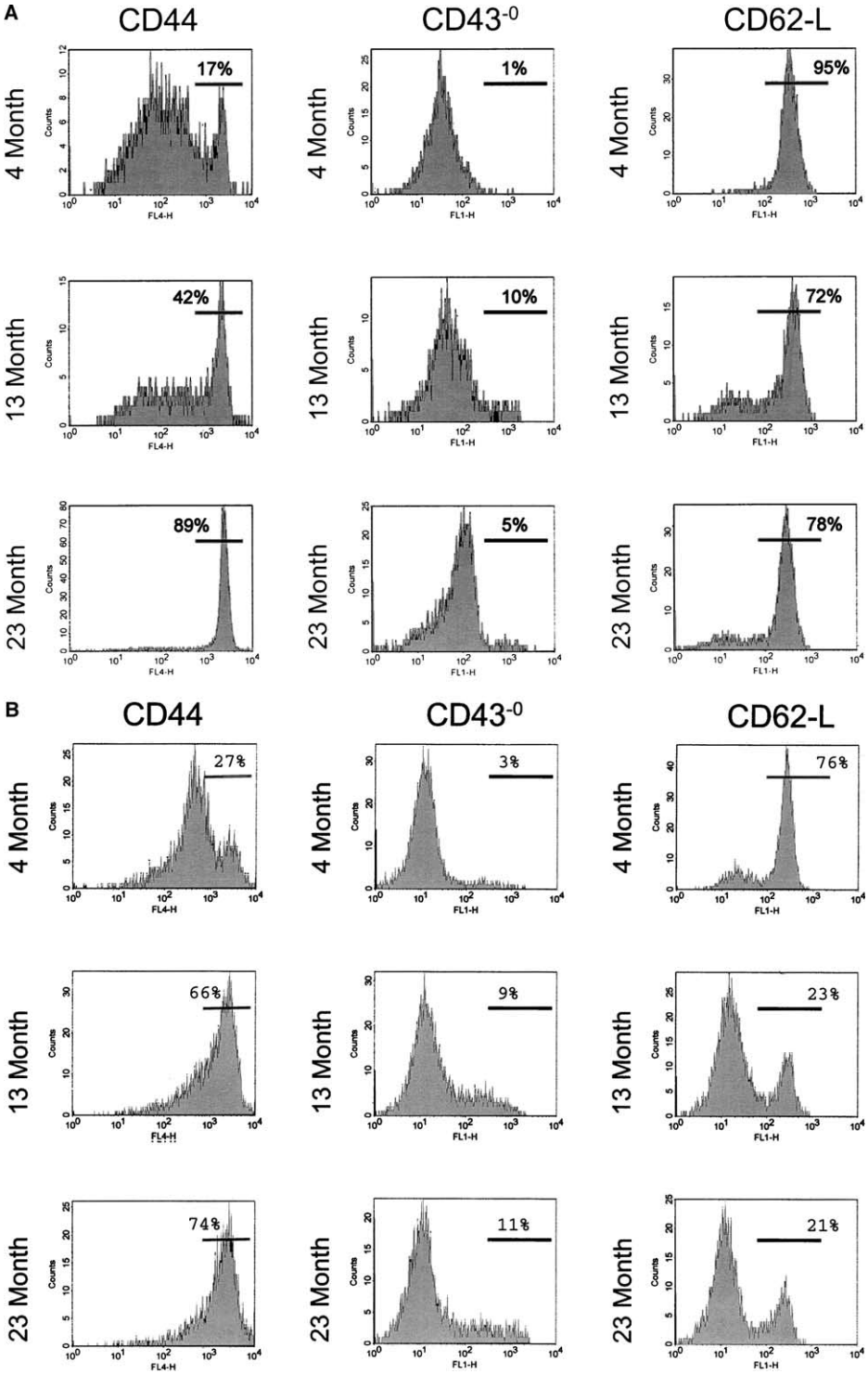

Figure 1. Surface antigen expression on $CD8^+$ and $CD4^+$ T cells before transplantation: expression of CD44, $CD43^{-0}$ (1B11), and CD62-L was compared on T cells isolated from young (4 months), adult (13 months), and old (23 months) C57BL/6J mice. A, $CD8^+$ cells showed an age-dependent increase in CD44 and $CD43^{-0}$ expression. CD62-L expression showed little change with age on $CD8^+$ cells. B, $CD4^+$ cells also showed an age-dependent increase in CD44 expression. $CD43^{-0}$ remained low in most cells of each age group, although some higher-expressing cells appeared in the adult and old populations. CD62-L expression was high on most young T cells, but this pattern reversed in adult cells, of which most expressed low levels of CD62-L, a pattern that persisted in the old group. Data presented are representative of 5 experiments. T cells from spleen and lymph nodes of 3 animals were pooled for each age group. The line represents the position of the gate used to derive percentages on each plot.

characteristic of T cells from older animals, presumably reflecting an increased fraction of memory cells. CD62-L expression was higher in young (naive) T-cell populations but decreased with age, particularly in the CD4 subset. By 12 months of age, the phenotypic pattern for CD62-L had been established, and little change was observed with increasing age. $CD43^{-0}$ is an activation-associated isoform of CD43 that is recognized by the 1B11 antibody [20]. $CD43^{-0}$ expression gradually increased on CD8 cells with age (the entire population moved to the right), and a subset of CD4 cells showed increased expression with increasing age. Recognition of $CD43^{-0}$ by the antibody 1B11 can be used as a surrogate marker for cutaneous lymphocyte antigen (CLA) in the mouse, because there is no known antibody that recognizes murine CLA. In humans, skin homing by T cells is associated with E-selectin binding by modified P-selectin glycoprotein ligand-1, which is human CLA [21]. Efficient P-selectin glycoprotein ligand-1-mediated E-selectin binding is dependent on core 2 b1-6N-acetylglucosaminyltransferase [22], which is also required to produce the CD43 epitope recognized by 1B11. In addition to the surface antigens in Figure 1, T cells were also analyzed for expression of the chemokine receptor, CCR-7 and α_4 and β_7 integrins before and after transplantation. No difference in expression of these surface antigens was noted as a function of T-cell age.

In Vivo Activity of Young versus Old T Cells in a GVHD Model System: Survival

To analyze the immune response to alloantigens in vivo, we compared the effector function of T cells from young (3-5 months), adult (12-14 months), and old (20-24 months) animals upon transplantation into irradiated major histocompatibility complex-mismatched host animals ($H\text{-}2^b$ into $H\text{-}2^{b/k}$). C3FEB6F1 host animals were irradiated and underwent transplantation with purified B6 T cells along with T cell-depleted bone marrow from Ly5.1 congenic B6 donors (to discriminate between T cells from B6 donors and T cells derived from marrow precursors). Animals were followed up weekly for the GVHD clinical score (see Materials and Methods), and survival was monitored. The survival of animals that received bone marrow alone (no GVHD control) and those that received bone marrow plus 2×10^6 purified B6 T cells of the indicated age is shown in Figure 2A. Recipients of the oldest T cells showed no evidence of GVHD and survived transplantation as well as animals that received only T cell-depleted bone marrow. The curves shown are representative of 4 experiments and show significant differences in survival between recipients of young and old T cells, as well as of adult and old T cells.

GVHD Clinical Score and GVHD-Specific Pathology

To provide a clinical correlate for the survival data, weekly assessment for GVHD of animals who had undergone transplantation was performed with an established index (see Materials and Methods). Consistent with the survival data, by 3 weeks after transplantation, recipients of old T cells had significantly lower GVHD clinical scores than recipients of young or adult T cells throughout the period of observation; the scores were equivalent to those of the marrow control group (Figure 2B). Histopathologic changes in GVHD target organs were compared at 3 weeks after transplantation by examination of liver and bowel with an established scoring system (see Materials and Methods). In contrast to the clinical assessment and survival data, recipients of old (22 months) T cells showed evidence of pathologic change when compared with control animals that received only T cell-depleted marrow. Differences in histopathology scores between groups that received T cells were not statistically significant (Figure 2C).

Comparison of T-Cell Expansion In Vivo

On day +11 after the infusion of 10^6 B6 T cells into irradiated C3FEB6F1 hosts, the spleen and lymph nodes of recipients of old T cells were larger, as verified by total cell counts (Table 1). Donor T-cell counts in the spleen were not significantly different among young, adult, and old animals. A total of 8 to 10×10^6 donor T cells were recovered, on average, from each spleen. In lymph nodes, the total cell count and donor T-cell count were significantly different when comparing adult and old ($P < .05$) and young and old ($P < .001$) T-cell groups; the older cells generated more progeny (1-way ANOVA). Combining the donor T-cell counts from spleen and lymph nodes (8 nodes per animal), we can document a minimum expansion of 12- to 14-fold over the input number of donor T cells. Table 1 shows the ratio of CD4 to CD8 cells before transplantation and on day +11 in spleen and lymph nodes from recipients of the different ages of T cells. The final columns in Table 1 show the expansion of donor CD4 and CD8 cells recovered for each of the input T-cell ages. For all ages, CD8 cell expansion was greater than CD4 expansion. The oldest T cells showed greater CD8 expansion than young cells (34-fold versus 24-fold). Conversely, young CD4 cells underwent greater expansion (5.6-fold) than old CD4 cells (4.1-fold). These expansion figures represent minimum boundaries, because they were calculated on the basis of T cells recovered from spleen and lymph nodes and are not an estimate of total body T cells.

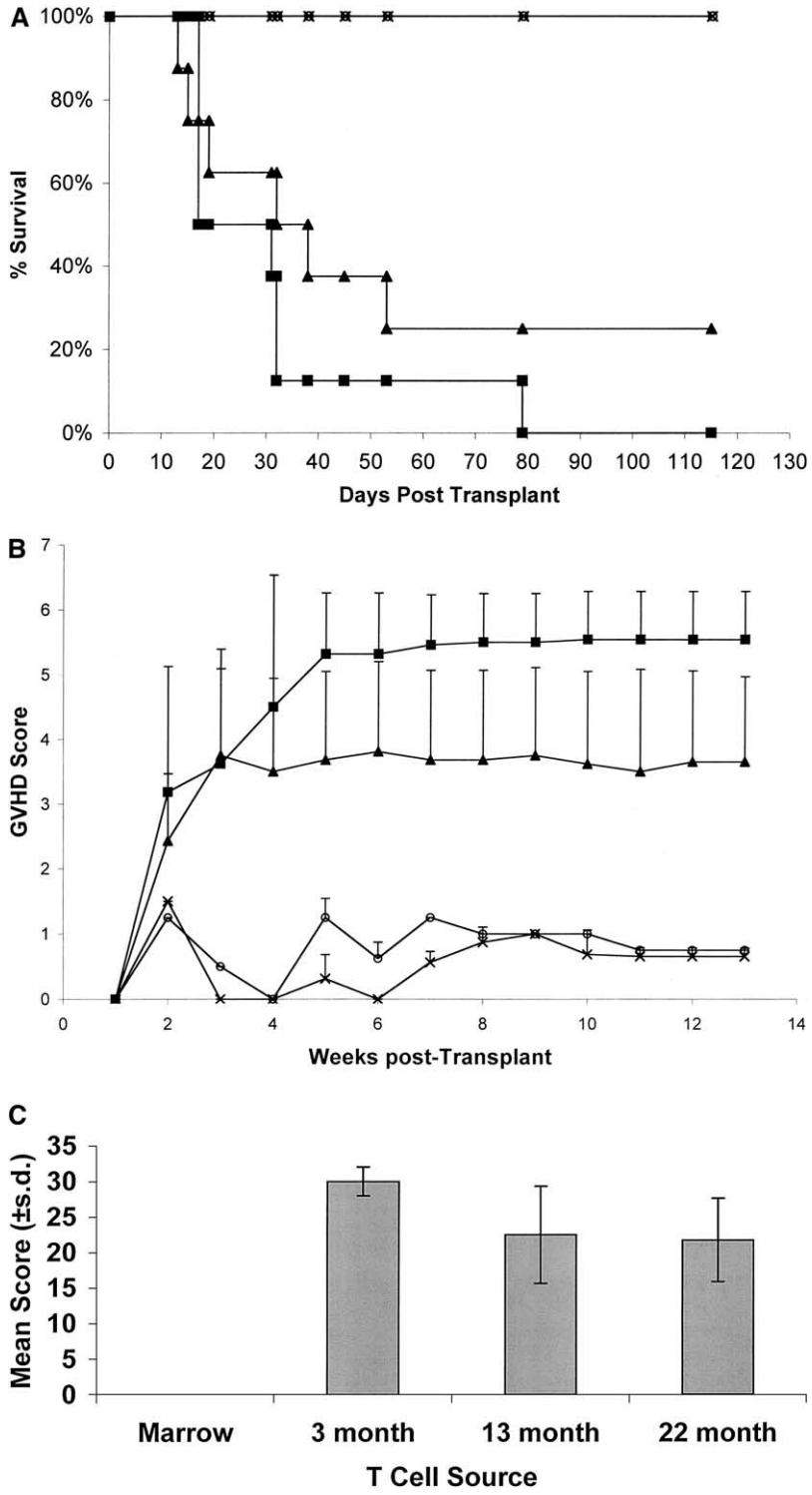

Figure 2. In vivo activity of young, adult, and old T cells in allogeneic bone marrow transplantation. A, Comparative survival of recipients of 2×10^6 T cells from 3-month-old (■), 12-month-old (▲), or 24-month-old (○) donors or bone marrow alone (×). All animals received 5×10^6 T cell-depleted marrow cells from B6 CD45.1 congenic mice. Animals that received marrow alone (n = 4) all survived, as did animals that received old T cells (n = 8). All animals that received young T cells (n = 8) died of GVHD, whereas 75% of animals (n = 8) that received adult T cells died of GVHD. This curve is representative of results from 5 separate transplantations. Differences between young and old ($P < .01$) and adult and old ($P < .05$) groups were significant (log-rank test). B, GVHD index (clinical score): weekly assessment of clinical GVHD was performed by using a scoring system composed of weight change, posture, activity, grooming, and skin condition, as described in Materials and Methods. GVHD was severe in recipients of young T cells (■) and intermediate in recipients of adult T cells (▲). Animals that received no GVHD-inducing T cells (×) and animals that received old T cells (○) had similarly low clinical scores. GVHD scores were compared by using 1-way ANOVA on data from weekly intervals. Differences between groups (young versus old and adult versus old) became significant ($P < .01$) by week 3 and remained so throughout the experiment. C, Histopathology index from mice killed at day +21. No significant differences were noted between experimental groups (n = 4 animals per group).

Table 1. *Donor T-Cell Posttransplantation Expansion*

T-Cell Donor Age	Before Transplantation	Spleen (Day +11)	Lymph Nodes (Day +11)	Minimum Estimate-Fold Expansion	
Young (5 mo)					
CD4/CD8 ratio	1:0.7	1:3	1:2.7	Donor (B6)	
Total cells (average)		48.4 ± 7.1 ($\times 10^6$)	3.9 ± 0.3 ($\times 10^6$)	CD4	CD8
No. T cells (average)		9.9 ± 3.9 ($\times 10^6$)	3.4 ± 0.3 ($\times 10^6$)	5.6	24
Adult (14 mo)					
CD4/CD8 ratio	1:0.7	1:4.6	1:5.1	Donor (B6)	
Total cells (average)		53.3 ± 15.8($\times 10^6$)	5.2 ± 1.3*($\times 10^6$)	CD4	CD8
No. T cells (average)		8.4 ± 2.0 ($\times 10^6$)	4.5 ± 1.1*($\times 10^6$)	3.9	25.8
Old (21 mo)					
CD4/CD8 ratio	1:0.5	1:4.3	1:3.8	Donor (B6)	
Total cells (average)		62.9 ± 14.4($\times 10^6$)	7.0 ± 1.3†($\times 10^6$)	CD4	CD8
No. T cells (average)		8.4 ± 1.9 ($\times 10^6$)	6.0 ± 0.9†($\times 10^6$)	4.1	34

Cells were harvested from spleen and lymph nodes of C3FEB6F1 hosts on day +11 after transplantation with 10^6 B6 T cells (6 animals per group). The total cell count and donor T-cell count were significantly greater in lymph nodes of old versus adult (*$P < .05$) and old versus young (†$P < .001$) groups, whereas differences in spleen cell counts were not significant. CD4/CD8 ratios before transplantation and for each group after transplantation (average value from 6 animals) in cells isolated from spleen or lymph nodes are presented. An estimate of the minimum expansion of CD4 and CD8 cells was calculated by dividing the absolute number of donor CD4 and CD8 cells recovered from spleen and lymph nodes by the number of input CD4 or CD8 cells injected at the time of transplantation. The 3 age groups were compared by one-way ANOVA with the Tukey posttest.

T-Cell Phenotype and Function after In Vivo Expansion

Figure 3 shows expression of CD44, $CD43^{-0}$, and CD62-L determined 8 days after transplantation. For $CD8^+$ cells (Figure 3A), there was a convergence of T-cell surface antigen expression, regardless of the age of the responding cells, toward an activated phenotype characterized by high expression of $CD43^{-0}$ and CD44 and low expression of CD62-L. For $CD4^+$ cells (Figure 3B), phenotypes also converge toward high expression of CD44, high expression of $CD43^{-0}$, and low expression of CD62-L for young and adult responding cells. Among the old responding T cells, most cells assumed the same activated phenotype, although the percentage of activated cells was lower for each marker: 63% $CD44^{hi}$ positive old cells versus 83% positive young cells; 58% $CD43^{-0}$ positive old cells versus 81% positive young cells; and 42% CD62-L positive old cells versus 21% positive young cells. This suggests a subpopulation of old cells that are phenotypically less activated than their younger counterparts.

TCR Repertoire: Vβ Expression before and after Transplantation

Another possible explanation for the lack of GVHD effector activity in older cell populations is a lack of alloreactive cells due to the loss of TCR repertoire diversity. We assessed TCR Vβ expression by using a panel of antibodies against 15 Vβ families in conjunction with staining for CD4 and CD8. Figure 4 (CD4 before and after transplantation [Figure 4A]; CD8 before and after transplantation [Figure 4B]) shows Vβ expression profiles before and after transplantation (day +11) for T cells from young, adult, and old animals. Some Vβ families that showed expansion (or contraction) on transplantation in animals that got GVHD (young or adult) also expanded (or contracted) to a similar degree in animals that did not get GVHD (old). We looked for conservation of a pattern of response by measuring the direction of change in expression for each Vβ (increase, decrease, or no change) before and after transplantation and compared this change for each T-cell age group. When comparing young and adult T cells, changes were concordant (in the same direction) for 11 of the 15 Vβ groups tested (for both CD4 and CD8 cells), whereas changes were concordant for only 6 (CD4 cells) and 7 (CD8 cells) out of 15 Vβ groups tested when comparing young and old T cells. Although suggestive, these data are not significantly different from results expected by chance ($P = .07$ with a χ^2 test).

T-Cell Effector Function: Cytokine Expression and Cytolytic Activity of In Vivo-Expanded Cells

We next characterized the effector function of old, adult, and young T cells recovered after in vivo expansion. Day +11 activated splenic T cells were harvested and tested for intracellular cytokine expression (Figure 5A). Fewer activated $CD4^+$ T cells from old (24 months) mice expressed the GVHD-inducing Tc_1 cytokines IFN-γ and TNF-α compared with young cells (5 months) or adult cells (14 months). In contrast, a similar fraction of activated $CD4^+$ cells expressed IL-2, independent of age. In activated $CD8^+$ populations, a similar pattern was observed; the fraction of IFN-γ-positive cells was lower in the old group,

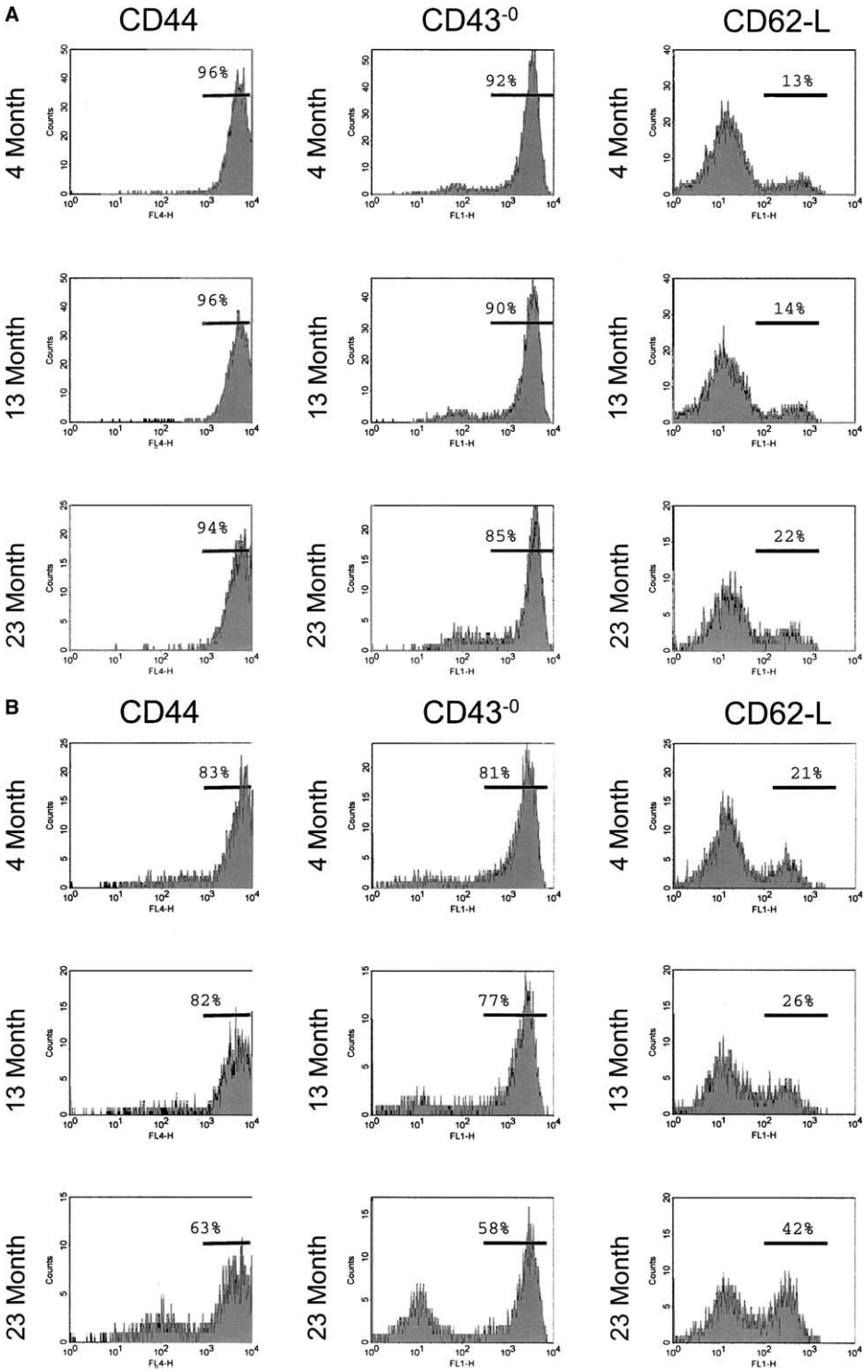

Figure 3. Surface antigen expression on CD8 and CD4 T cells after transplantation: 8 days after transplantation, T cells were reisolated from spleen and lymph nodes of 3 animals that received the indicated ages of T-cell inoculum (from the same experiment as shown in Figure 1). A, CD8 T cells were reanalyzed for the expression of CD44, $CD43^{-0}$, and CD62-L. Cells from each age group showed an activated phenotype characterized by a high expression of CD44, high expression of $CD43^{-0}$, and low expression of CD62-L. B, CD4 T cells from young and adult groups also displayed an activated phenotype characterized by high expression of CD44, high expression of $CD43^{-0}$, and low expression of CD62-L. However, among T cells in the 23-month-old group, a subset of cells expressed less CD44, less $CD43^{-0}$, and more CD62-L. Data in this figure are representative of cells reisolated from 3 separate transplant experiments between days +7 and +14. Each sample represents pooled cells from 3 animals. The line represents the position of the gate used to derive percentages on each plot.

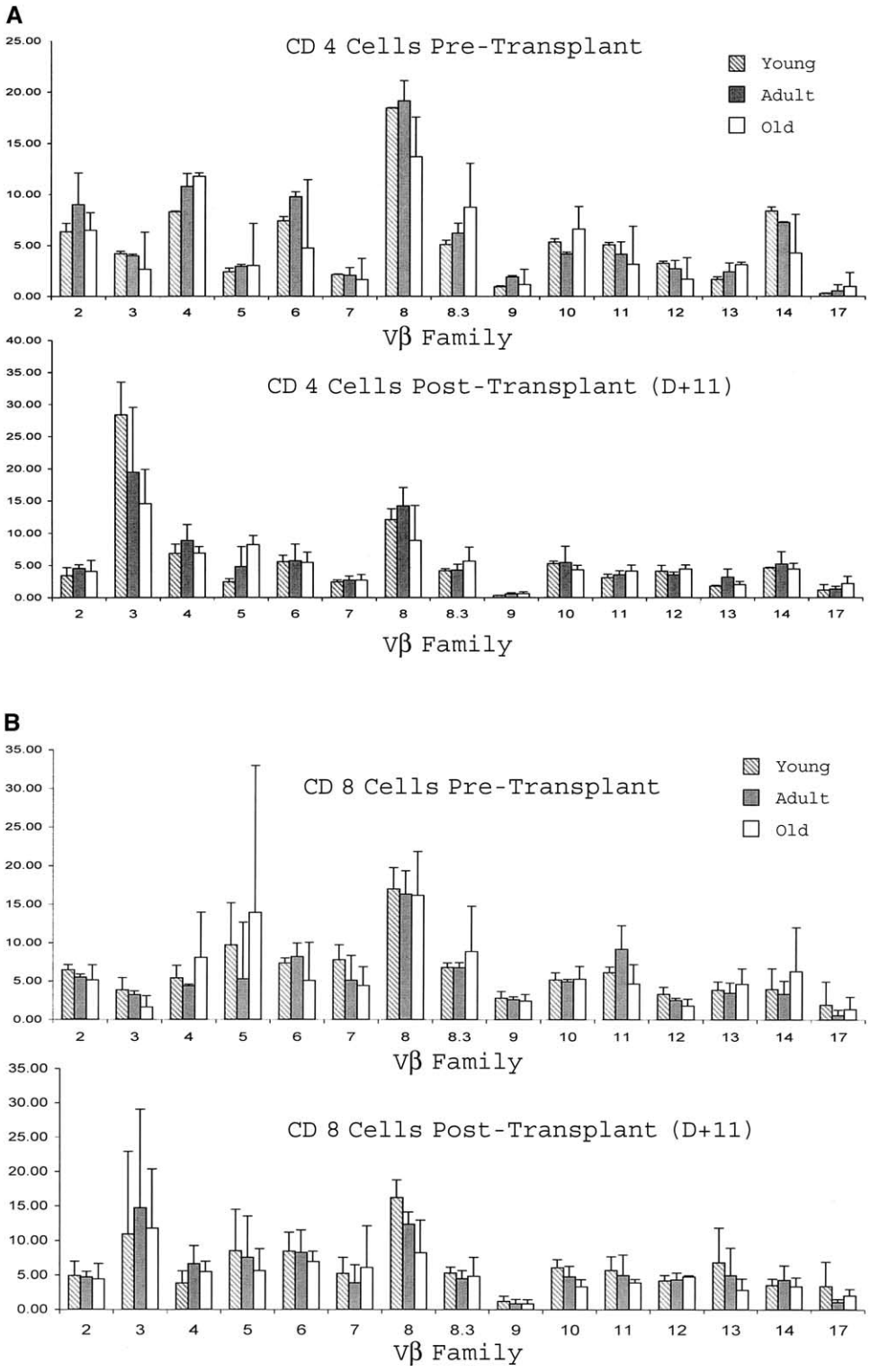

Figure 4. TCR Vβ expression profiles in young, adult, and aged mice before and after transplantation. A, CD4 cells before and after transplantation. B, CD8 cells before and after transplantation. T cells isolated from spleen and lymph nodes of animals of the indicated age were stained with antibodies against CD4 (CyChrome), CD8 (phycoerythrin), and a panel of FITC-labeled TCR Vβ-specific antibodies that recognize Vβ 2, 3, 4, 5, 6, 7, 8.1, and 8.2 (labeled 8 in the figure), 8.3, 9, 10, 11, 12, 13, 14, and 17. Data are presented as the mean percentage (±SD) of cells (CD4 or CD8) positive for the specific Vβ from 3 separate experiments. In vivo-expanded cells were isolated on day +11.

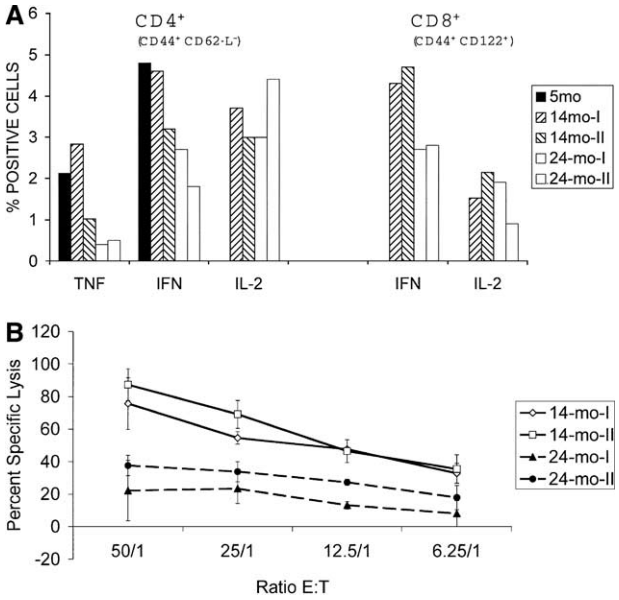

Figure 5. Effector function of transplanted T cells. A, Intracellular cytokine expression in activated CD4 and CD8 T cells: T cells (B6) were isolated and pooled on day +11 from spleens of mice that had undergone transplantation (C3FEB6F1). Cytokine expression was determined by FACS after overnight incubation in the presence of irradiated stimulator cells (C3H B cells) and brefeldin A. A higher fraction of activated CD4 cells (CD62-L negative and CD44 high) from young (5 months) and adult (14 months) animals expressed TNF-α and IFN-γ than did old CD4 cells. IL-2 expression was similar for all groups. Activated CD8 cells (CD44 high and $CD122^+$) from 14-month-old animals expressed more IFN-γ than old CD8 cells, whereas IL-2 expression was again similar. Each data point is a single FACS analysis that used pooled cells from 3 donor animals. B, Cytolytic activity of freshly isolated progeny of adult (14 months) and old (24 months) T cells isolated at day +11 after transplantation was assessed by incubating purified T cells with chromium-51-labeled allogeneic target cells (p210 leukemia line; $H\text{-}2^k$) for 4 hours, followed by determination of specific chromium release. Adult T cells efficiently lysed allogeneic target cells over a range of effector-target ratios, whereas old cells had little activity at any concentration. Each data point represents the mean ± SD of triplicate determinations; each curve was made with pooled cells from 3 animals.

whereas IL-2 expression was similar. Expression of IL-4 and IL-10 was low in all groups, as was TNF-α expression in $CD8^+$ cells of all ages (data not shown).

Effector activity was also assessed in day +11 T cells by measurement of cytolytic activity against an alloantigen-bearing tumor cell line (Figure 5B). Although adult T cells demonstrated effective and specific cytolysis, there was little activity in the old T-cell population. As with the cytokine data, these results help to provide a partial explanation for the lack of GVHD morbidity and mortality in recipients of old T cells. There is no indication of a differential role for Fas/Fas ligand in cytotoxicity in this system, because expression of Fas was comparable on T cells from each age group, whereas expression of Fas ligand was not detectable above background on T cells from any age group (data not shown).

CFSE Comparison of Homeostatic versus Alloantigen-Driven Proliferation in Young and Old T Cells

Homeostatic expansion of both CD4 and CD8 T cells has been well documented in host animals lacking T cells [23,24], and homeostatic proliferation is thought to play a role in the maintenance of T-cell numbers during aging, when production of naive T cells is greatly reduced. It is possible to discriminate between homeostatic proliferation and antigen-driven responses by comparing the kinetics and degree of expansion of a given cell population when transferred into syngeneic versus antigen-mismatched hosts [25]. Figure 6 compares the proliferative responses of 12-month-old (GVHD-inducing) and 19-month-old (non-GVHD-inducing) T cells in syngeneic hosts (in which homeostatic expansion is predominant) with proliferative responses of the same T cells in allogeneic hosts (in which homeostatic and antigen-driven signals are both present) 64 hours after transplantation. Expansion of CD8 T cells in both age groups was comparable under both homeostatic and antigen-driven conditions, although 19-month-old T cells proliferated slightly faster (Figure 6A). Little CD4 cell proliferation was seen upon adoptive transfer to syngeneic hosts; in allogeneic hosts, a similar fraction of rapidly dividing CD4 cells was seen in both groups (Figure 6B).

DISCUSSION

T cells from old mice have poor effector function both in vitro and in vivo when measured by a variety of assays—including cytokine expression in activated cells, ability to lyse alloantigen-bearing target cells, and ability to cause GVHD—when compared with T cells from younger animals. Surprisingly, this lack of effector function is not secondary to a failure to proliferate (in vivo) but occurs despite vigorous proliferative responses, as measured by recoverable cell counts in vivo. In T-cell ablation studies, residual or transplanted T cells can undergo homeostatic proliferation, in which a peripheral antigen-independent expansion occurs in response to a T-cell deficit. Rates of proliferation of cells undergoing homeostatic expansion are slower than for those undergoing antigen-driven expansion, and cells express lower levels of activation antigens [25]. In our model, we have the potential for both homeostatic and antigen-driven proliferation of transplanted T cells. Parallel transplantation of identical young, adult, and old T-cell populations into irradiated congenic and allogeneic hosts demonstrates more rapid T-cell expansion in

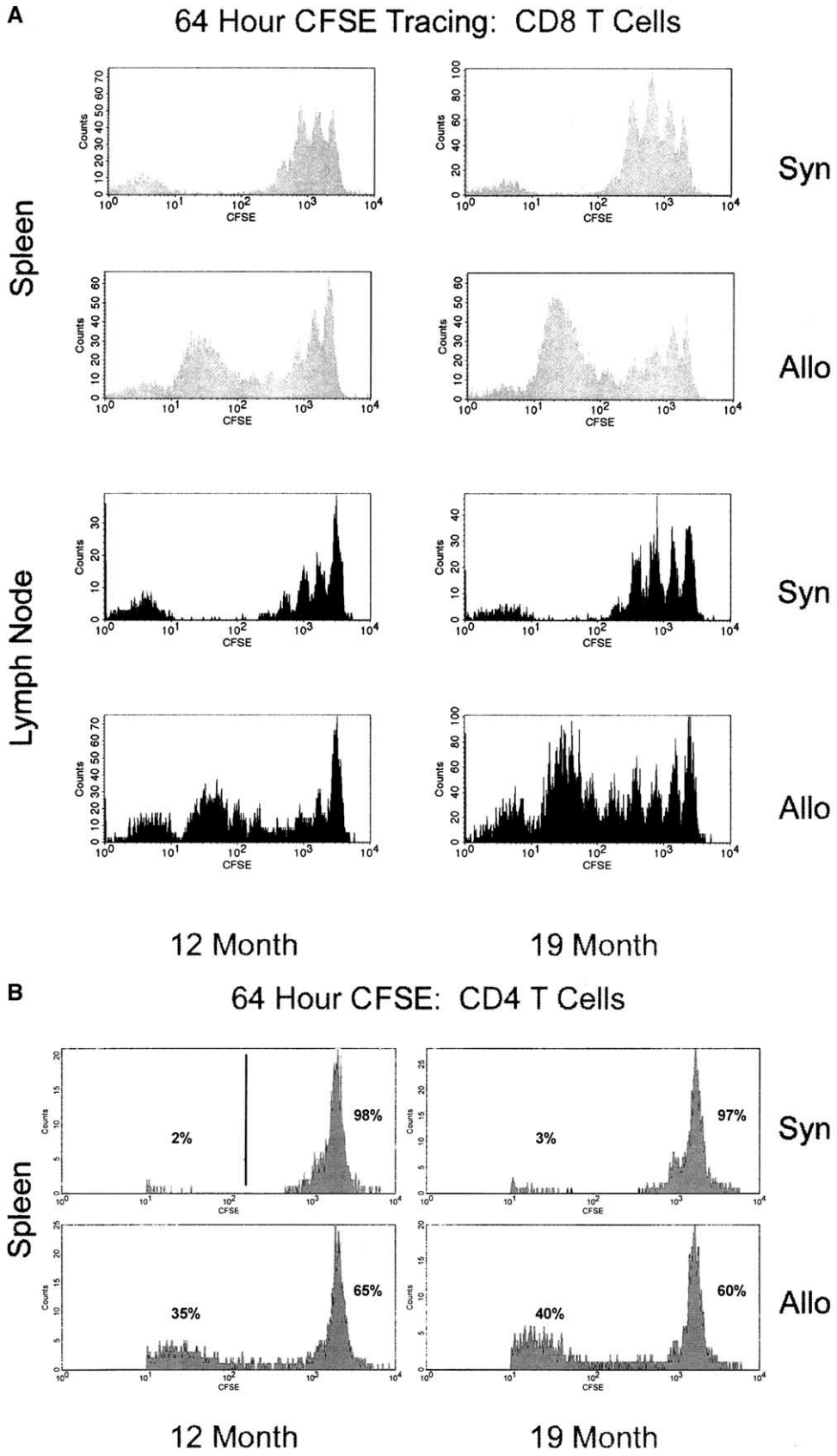

Figure 6. Proliferation of transplanted cell in syngeneic and allogeneic hosts. A, Proliferation of CD8 T cells. CFSE-labeled T cells (CD4 and CD8) were injected into irradiated C3FEB6F1 (Allo) or CD45.1 congenic (Syn) mice and harvested 2.5 days after transplantation. Sufficient CD8 cells were obtained from both spleen and lymph nodes to compare the proliferative responses of adult (12 months) and old (19 months) cells. B, Proliferation of CD4 T cells. Labeled T cells recovered from spleen showed little proliferation in syngeneic hosts, whereas a peak representing rapidly dividing cells was present in both the adult and old T cell groups in C3FEB6F1 (Allo) host animals. Similar results were obtained at 48 hours after transplantation, but by 96 hours after transplantation, the CFSE signal on rapidly dividing cells was lost (data not shown). Percentages reflect the fraction of CFSE-positive CD4 cells in a rapidly dividing population (left) or slowly dividing population (right).

allogeneic host animals, suggesting that we are primarily observing an antigen-driven process (Figure 6).

The differences that we observed in effector function could be due to the loss of a critical subset of cells with age (eg, a hole in the repertoire) or to a more subtle cellular alteration that preserves T-cell activation and proliferation, but not function. We have compared several phenotypic characteristics that have been used to differentiate young and old T cells. After transplantation (day +7 to day +14), there is a convergence in expression of these markers on cells of all ages. The predominant phenotype includes high expression of CD44 (on CD4 and CD8 cells), low expression of CD62-L (especially on CD8 cells), and high expression of $CD43^{-0}$ on both CD4 and CD8 cells. A subpopulation of old CD4 cells diverges from this predominant phenotype (Figure 3B), expressing lower levels of CD44 and $CD43^{-0}$ and higher levels of CD62-L; this is suggestive of less robust activation (or termination of response) of some old CD4 cells.

Differences in the ability of young and old cells to migrate into target tissues do not seem to play an important role in the lack of effector activity of older cells, because early steps in lymphocyte homing (in vitro tethering and rolling) are not different between groups (data not shown). Defects in later stages of tissue homing are also not a likely explanation for our results, because pathologic analysis of liver and gut specimens (day +21) revealed similar degrees of lymphocyte infiltration regardless of T-cell age.

The potential of reduced T-cell diversity in older populations as an explanation for our results was assessed by using a panel of TCR Vβ-specific antibodies (Figure 4). Although there were differences between groups in Vβ expression both before and after transplantation, T-cell pools from young, adult, and old donors demonstrated similar patterns and kinetics of proliferation in response to the environment (stimulation) encountered during transplantation, thus making it unlikely that an absence of alloreactive cells in the old T-cell population explains their failure to cause GVHD. Specific differences in Vβ expression between groups, such as a higher percentage of Vβ-3-positive young CD4 cells, could still play a role in the observed differences in GVHD activity, and higher-resolution mapping of TCR diversity could reveal a lack of a specific and critical subpopulation in older T-cell preparations.

In this parent into F_1 transplant model, although both CD4 and CD8 cells can serve as effectors of GVHD [19], CD4 cells seem to play the predominant role as producers of GVHD-inducing cytokines. Intracellular staining for IFN-γ and TNF-α from activated CD4 T cells isolated after transplantation revealed that a higher fraction of in vivo-expanded young/adult cells expressed these effector cytokines than did old T cells, whereas IL-2 expression was similar for all groups. The cytolytic activity of recovered in vivo-expanded T cells demonstrated specific activity of adult T cells, with less activity of old T cells against an $H\text{-}2^k$-bearing allogeneic cell line (p210). This was consistent with a lack of in vivo GVHD.

Our in vivo studies demonstrate that old (>19 months) T cells are capable of both homeostatic and antigen-driven proliferation upon adoptive transfer at rates comparable to those of transferred young (3-5 months) and adult (12-14 months) cells. Despite comparable proliferation, the progeny of old T cells have reduced effector function in vivo (as measured by the ability to induce GVHD) and in vitro.

Our results, which focus exclusively on the role T-cell donor age plays in GVHD induction, do not agree with a recent study that found increasing donor age to be a significant risk factor for GVHD in clinical transplantation [26]. In this study, all animals received a common T cell-depleted marrow inoculum from young B6 CD45.1 congenic animals plus purified T cells from different ages of donor B6 mice—thus, all reconstituting cells, with the exception of T cells, were derived from a young donor. In prior work, we have shown that marrow-derived APCs from older mice are more effective at inducing GVHD [27]. Thus, a possible explanation for the discrepancy between our findings and clinical experience is that GVHD in recipients of older marrow is related to enhanced APC function of older cells, which may compensate for decreased T-cell function.

In summary, our results suggest that in addition to previously described in vitro defects in the activation of old T cells, there are distinct functional defects in vivo downstream of successful activation and proliferation. It remains to be determined whether this is due to cell-autonomous changes resulting from aging, such as increasing mitochondrial dysfunction described in other tissues [28], or whether it is in some way related to selective pressures during aging for T cells capable of self-maintenance.

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