Antigen-independent Changes in Naive CD4 T Cells with Aging

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Summary

In the elderly, a dramatic shift within the CD4+ T cell population occurs, with an increased proportion having a memory phenotype with markedly decreased responsiveness. To determine what aspects of the aged phenotype are dependent upon repeated contact with antigen in the environment, we examined CD4+ cells isolated from TCR Tg mice. There is good evidence that no cross-reacting antigens for the Tg TCR recognizing pigeon cytochrome c are found in the environment of the animal, so that alterations in the Tg CD4+ cells with aging are likely to be due to antigen-independent processes. We found that in aged animals, TCR transgene[pos] CD4+ cells, although decreased in number and antigen responsiveness, maintain a naive phenotype rather than acquiring a prototypical aged memory phenotype. In contrast, the population of transgene[lo-neg] CD4+ cells increase in proportion and express the aged phenotype. Consistent with their naive status, transgene^{pos} cells of aged individuals remain CD44^{lo} CD45RB^{hi}, secrete IL-2 and not IL-4 or IFN-γ upon antigenic stimulation, and require costimulation to proliferate to anti-CD3 stimulation. These findings suggest that the aging-associated shift to CD4 cells expressing the memory phenotype is dependent on antigenic stimulation. However, the decrease in antigen responsiveness of naive transgene^{pos} cells, as revealed by a lower secretion of IL-2 and IL-3 and a lower proliferative capacity, suggests that additional intrinsic changes occur with aging that do not depend on encounter with antigen.

Studies in humans and rodents established that the decline in protective immunity with advancing age is largely due to changes in the T cell compartment (1–19). Among the more dramatic changes that contribute to the diminished T cell function in aged individuals are a decline in the frequency of CD4+ T cells producing IL-2 (1) and decreased expression of IL-2 receptors (2). Data on the production of other cytokines by T cell populations stimulated in vitro support an age-related increase in IL-4 and IL-5 production (3–5), an increase in IL-10 production (6), and a decrease in IL-3 production (5, 7). This is coupled with a decrease in the early events of signal transduction (8–12) and an overall decrease in proliferation of CD4+ T cells in response to various kinds of TCR- and costimulus-mediated stimulation (13–17).

As animals age, there is an increase in the proportion of T cells expressing a memory cell phenotype, reflected by an increased expression of CD44 and decreased expression of CD45RB (3, 14–16, 18, 19). The T cells of the aged share some of the properties associated with memory cells, including their requirements for stimulation and patterns of cytokine production (20–25), but their decreased prolifera-

tion and overall lowered levels of cytokine production are more consistent with an accumulation of unresponsive cells, which also share the memory phenotype (26). Thus, unlike the antigen-stimulated memory cells generated in young animals, which proliferate vigorously and often produce high levels of IL-2 and other cytokines (22, 27), the memory cells found in aged animals are hyporesponsive (11, 12, 14). The age-related transition to a higher proportion of cells with a memory phenotype is seen in both the CD4 and CD8 populations (3, 14–16). The majority of these cells that accumulate with aging seem to be resting, not activated lymphocytes, based on their size, DNA profile, lack of activation markers (19, 28), and their requirement for further stimulation for cell cycle entry.

The naive and memory subsets of the CD4 and CD8 populations of aged individuals can be further divided based on their ability to extrude the fluorochrome, Rhodamine 123 (R123)[1] (29–31). The extrusion of R123 is dependent on P-glycoprotein, the 170 kD ATP-dependent plasma

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membrane pump encoded by the multiple drug resistance genes, which has been extensively studied in tumor cells (32, 33). The function of P-glycoprotein in normal cells is unknown. With age, CD4 T cells that stain dimly with R123 increase in number, and this change appears to be indicative of the activation state or developmental history of the T cell (30, 34). Indeed, activated cells, as well as cells within the memory subset of the CD4 population, are R123^{dim} (29–31). Furthermore, the R123 extruding fraction, i.e., R123^{dim} cells, from unimmunized mice has been shown to include preactivated cells capable of IL-2 and some IFN-γ production (30).

To investigate whether interaction with environmental antigens contributes to the aging-associated generation of memory phenotype T cells and whether naive T cells that persist in aged mice retain normal function, we have examined CD4 T cells isolated from mice transgenic (Tg) for a T cell receptor (TCR) that recognizes pigeon cytochrome c (PCC) in the context of I-E^k (i.e., AND Tg mice) (35). Previous studies of young mice indicate that the TCR Tg^{pos} CD4$^+$ cells are greater than 90% of naive phenotype and function, and that the naive cells respond vigorously to defined peptide antigen presented by appropriate antigen-presenting cells (APCs) (36). Importantly, few, if any, Tg^{pos} memory cells develop in these mice without intentional antigenic stimulation, suggesting that no cross-reacting antigens are found in the environment. This is not unexpected, since PCC differs from the murine homologue in only three positions in the fragment recognized by the Tg TCR (37). Indeed, the frequency of CD4$^+$ T cells in normal animals specific for PCC is less than 0.1% (38), and the response is of limited heterogeneity, being comprised almost exclusively of Vβ3Vα11 T cells (38, 39) supporting its oligoclonality. Thus, the chance for crossreaction is minimal. In contrast, the Tg^{lo-neg} cells in AND mice express endogenous receptors and have a memory phenotype (36, 40), presumably reflecting selection by environmental antigens. Therefore, it is expected that age-related changes in the TCR Tg^{pos} CD4 population would likely be due to the antigen-independent aspects of aging. We find that in aged mice, the CD4$^+$ T cells expressing nontransgenic α and β chain TCR increase in number and are predominantly of the memory phenotype, whereas the Tg^{pos} CD4$^-$ T cells retain a naive phenotype, have lower levels of R123, and become hyporesponsive. Thus, some age-associated deficiencies of CD4 T cell responses apparently develop independent of response to antigen.

Materials and Methods

Animals. H-2$^{k/k}$ or H-2$^{b/b}$ TCR-$\alpha\beta$ (i.e., AND) Tg mice were bred from a C57BL/6 × SJL founder, provided by Dr. S. Hedrick (University of California, San Diego), by successive backcrosses onto the B10.Br or C57BL/6 background, respectively. Unless indicated, the mice used in these studies were on the H-2$^{k/k}$ background. The mice were housed at either the animal facilities at the University of California at San Diego (La Jolla, CA) or at the Scripps Research Institute, (La Jolla, CA) until their

use at 2–4 mo (young) and 15–26 mo old (aged). Mice with evidence of gross pathology were excluded from the study.

Cell Isolations. The isolation of spleen cells enriched for CD4 cells has been described previously (36, 41). In brief, the cells were passed through a nylon wool column and the nonadherent cells were stained with cy-chrome anti-CD4, phycoerythrin anti-Vα11, biotin anti-Vβ3, and Texas red streptavidin. The CD4$^-$Vβ3$^+$ Vα11$^-$ cells and the CD4$^+$Vβ3$^-$Vα11$^-$ cells were sorted using either the FACStar Plus or the FACSVantage (Becton Dickinson). The sorted populations contained less than 1.5% cells falling outside the sort gates.

Immunofluorescent Staining. The following antibodies and fluorescent reagents were used: Cy-chrome anti-CD4 (clone RM4-5; PharMingen, La Jolla, CA), FITC anti-CD44 (clone 1M7; PharMingen, La Jolla, CA), FITC anti-CD45RB (clone 23G2; PharMingen), FITC anti-CD3ε (clone 145-2C11; PharMingen), phycoerythrin anti-Vα11 (clone RR8-1; PharMingen), biotin anti-Vβ3 (clone KJ25; PharMingen), phycoerythrin anti-CD25(IL-2 receptor, α chain; clone 3C7; PharMingen), phycoerythrin anti-CD69 (clone H1.2F3; PharMingen), Texas red streptavidin (Biomeda, Foster City, CA), and R123 (Molecular Probes, Inc., Eugene, OR). For the analysis of CD3, CD44, CD45RB, and CD62L expression on CD4 Tg^{pos} cells, the cells enriched for CD4 were stained with the appropriate antibodies and analyzed on a FACStar® Plus flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed with Cell Quest software.

To examine R123 staining, spleen cells were incubated with 12.5 μM R123 for 10 min at 37°C, washed three times with cold PBS, incubated an additional 30 min at 37°C, washed, and then incubated with antibodies to CD4, Vα11, and Vβ3 as described above.

Cell Culture. Cells were cultured in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with penicillin (200 μg/ml), streptomycin (200 μg/ml), glutamine (4 mM), 2-mercaptoethanol (50 μM), Hepes (10 mM), and 10% FCS (Hyclone, Logan, UT). DCEK-ICAM, a fibroblast cell line that expresses B7-1 constitutively, and is stably transfected with ICAM and class II MHC (I-E^k) molecules, was used as APCs (42). These cells do not express other costimulatory molecules, such as LFA-1, CD48, and heat stable antigen, and do not secrete detectable levels of cytokines, such as IL-2, IL-4, IFN-γ, and TNF-α (25). DCEK-ICAM/B7 cells are excellent APCs for stimulating both naive and other CD4 T cells (25).

Assays to detect DNA synthesis, an indicator of proliferation, were performed in microcultures. For antigen-specific stimulation, varying numbers of CD4$^+$ cells (1.25–5 × 10^5/ml for 0.2 ml microcultures) were incubated along with mitomycin c-treated (100 μg/ml for 30 min at 37°C) DCEK-ICAM/B7 APCs (2.5 × 10^5/ml) with or without 5 μM PCC fragment 88–104 (PCCF). For anti-CD3 stimulation, varying numbers of T cells were cultured with or without 7.2 ng/ml anti-CD28 antibody (prepared from ascites generated in pristane-primed nude mice with 37.51 cells [provided by Dr. J. Allison, University of California, Berkeley (43)]) in microwells precoated with 10 μg/ml anti-CD3 antibody (2C11). The microcultures were pulsed the last 16 h of the 3 day culture with 1 μCi [^{3}H]TdR (6.7 Ci/mmol; ICN, Irvine, CA), harvested and counted. The cultures were incubated 24 h before removing the culture supernatant for the assessment of cytokine production.

Effector cells were generated in bulk culture from sorted CD4$^+$Vβ3$^+$Vα11$^+$ T cells. These cells were incubated at 1.5 × 10^5/ml with twice as many mitomycin c-treated DCEK-ICAM/ B7, 5 μM PCCF, and IL-2 (20 U/ml). After 4 d of culture, the

cells were washed and either stained with fluorochrome conjugated antibodies for phenotyping or were cultured an additional 24 h with antigen and APCs for determining cytokine production profiles.

Cytokine Detection. Culture supernatants were collected after 24 h of culture with PCCF and DCEK-ICAM/B7 and were assayed for the presence of cytokines. IL-2 and IL-3 were detected by bioassays as previously described (27). The data were quantitated from standard curves using recombinant cytokines and are expressed as units per milliliter where 50% of the maximum response represents 1 U of activity. Units per ml were calculated from the reciprocal of the dilution that gives half-maximal activity, divided by the culture volume. IL-2 was detected by measuring proliferation of the NK3 cell line that responds to both IL-2 and IL-4. Anti-IL-4 monoclonal antibody (11B11) was added to the assay to block any IL-4-induced proliferation. IL-3 was detected by proliferation of the 32Dc15 cell line (44) that does not respond to other cytokines under our experimental conditions. The lower limit of assay detection for both IL-2 and IL-3 was 5 U/ml. 1 U of IL-2 determined by this assay corresponds to 14 pg protein.

IFN-γ (27) and IL-4 were detected by ELISA. Both IL-4 and IFN-γ were quantitated by comparison with standard curves generated using recombinant cytokines and are expressed as ng per ml for IL-4 or units per ml for IFN-γ. The IL-4 ELISA employs the 11B11 antibody for capture and biotinylated BVD6-24G2 (PharMingen) for detection. The lower limits of assay detection for IFN-γ and IL-4 were 10 U/ml and 40 U/ml, respectively. 1 U of IL-4 and IFN-γ determined by these assays corresponds to 0.7 pg and 0.1 ng protein, respectively.

Results

The Phenotype of T Cells in Aged TCR Tg Mice. To investigate whether aged AND mice retained naive Tg^{pos} cells or whether the CD4 population shifted to a Tg^{neg} or more memory phenotype, we examined the expression of the Tg TCR on CD4$^+$ cells of young and aged mice. No striking differences were observed in the number of CD4$^+$ T cells isolated from the spleens of young (2–4 mo; $n = 8$) or aged (15–26 mo; $n = 8$) TCR Tg mice (data not shown). Gating on the CD4$^+$ population, the expression of the Tg TCR chains Vα11 and Vβ3 were examined by flow cytometry (Fig. 1). The proportion of CD4$^+$ T cells expressing both Vα11 and Vβ3 progressively decreased with age, while that of Tg^{lo-neg} cells increased, but a large proportion of CD4 T cells remained Tg^{pos}. An example of Vβ3/Vα11 expression in gated CD4 T cells from one experiment is shown in Fig. 1 *A* and the relative expression as a function of age in all animals is shown in Fig. 1 *B*. In young mice, the percentage of Tg^{pos} T cells averaged over 80% of the total CD4$^+$ T cells, while in mice over 20 mo of age, Tg^{pos} T cells represented less than 50% of the CD4$^+$ T cell population. Many CD4^1 T cells in aged mice expressed intermediate levels of Vβ3 and/or Vα11 (Fig. 1 *A*). As shown in Fig. 1 *C*, although the expression of Vβ3 or Vα11 declined with increasing age, the level of CD3 expression remained high on cells of both young and aged mice (mean fluorescence intensity of CD3: Vβ3 − [144 versus 72 young], [140 versus 88 aged]; Vα11 - [144 versus 71 young], [142 versus 80 aged]), suggesting that the Tg$^{lo\ neg}$ CD4$^+$ T cells
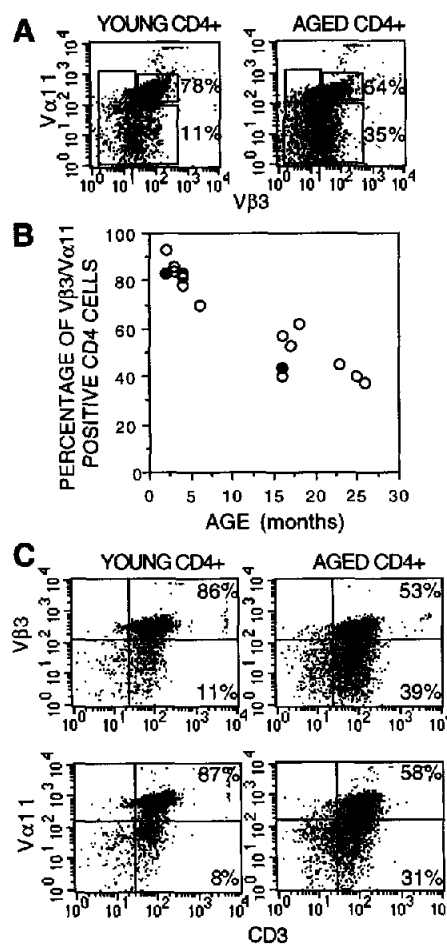

Figure 1. The expression of Vα11 and Vβ3 on CD4$^+$ spleen cells isolated from AND Tg mice. Gating on the CD4$^+$ cells, the levels of Vα11 and Vβ3 expression of a 4-mo-old (*left*) and a 26-mo-old (*right*) mouse are shown in *A*, and the percentage of cells expressing both Vα11 and Vβ3 for each mouse is shown in *B* (AND Tg mice on the H-2$^{s/k}$ background [*open symbols*] and H-2$^{b/k}$ background [*closed symbols*]). The expression of CD3 versus Vβ3 and CD3 versus Vα11 on CD4$^+$ spleen cells isolated from AND Tg mice is shown in *C*. Gating on the CD4$^+$ cells, the levels of CD3 and Vβ3 or Vα11 expression of a 4-mo-old (*left*) and a 19-mo-old (*right*) mouse are shown

express endogenous encoded TCR α- and β-chains (22, 40, 45) in addition to various levels of Tg-encoded Vα and Vβ. Indeed, Balomenos et al. (40) have recently demonstrated an increase in the coexpression of endogenous and Tg Vβ3 TCR in aged AND mice.

To evaluate the phenotype of the aged CD4$^+$ T cells, we focused on Tg^{pos} and Tg^{lo-neg} cells, which in all probability represent those T cells which have never encountered antigen versus those which are most likely antigen selected, respectively. Results of a typical experiment are shown in Fig. 2. In aged conventional animals, a phenotypic shift occurs in the total CD4$^+$ T cell population from a predominantly naive population to a predominantly memory population, as determined by the levels of expression of CD44 and CD45RB (3, 14–16, 18, 19). Gating on the total transgene-expressing or transgene-nonexpress-
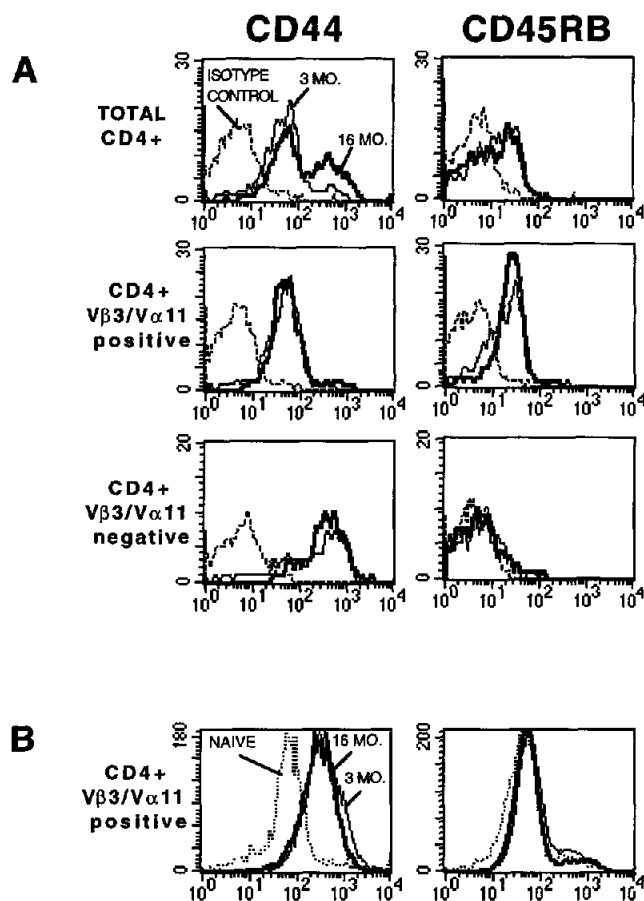
CD44   **CD45RB**

A

TOTAL
CD4+

ISOTYPE CONTROL   3 MO.
16 MO.

10^0 10^1 10^2 10^3 10^4 10^0 10^1 10^2 10^3 10^4

CD4+
Vβ3/Vα11
positive

10^0 10^1 10^2 10^3 10^4 10^0 10^1 10^2 10^3 10^4

CD4+
Vβ3/Vα11
negative

10^0 10^1 10^2 10^3 10^4 10^0 10^1 10^2 10^3 10^4

B

CD4+
Vβ3/Vα11
positive

NAIVE   16 MO.   3 MO.

10^0 10^1 10^2 10^3 10^4 10^0 10^1 10^2 10^3 10^4

Figure 2. The expression of CD44 and CD45RB on CD4+ T cells from aged AND Tg mice The expression of CD44 and CD45RB on total CD4+ T cells (first row), Vβ3+Vα11+ CD4+ T cells (second row), and Vβ3−Vα11− CD4+ T cells (third row) from young and aged AND Tg mice are shown in A. The relative expression of the CD molecules on CD4+ T cells from a 3-mo-old mouse (solid line) versus a 16-mo-old mouse (bold line) are shown. Although results with cells from the young were similar, only antibody isotype controls are shown with cells from the aged (dotted line). Gating of CD4+Vβ3+Vα11+ versus CD4+Vβ3−Vα11− are shown in Fig. 1. Shown are representative data from one experiment with one mouse from each age group with a minimum of five experiments performed. The phenotype of effectors generated in vitro from naive transgene-expressing T cells from 3-mo-old (solid line) versus 16-mo-old (bold line) AND Tg mice are shown in B. FACS-sorted Vβ3Vα11 positive CD4+ T cells were cultured at 1.5 × 10⁵/ml with twice as many mitomycin c-treated DCEK-ICAM/B7, 5 μM PCCF, and IL-2 (20 U/ml). After 4 d, the cells were washed and stained with fluorochrome conjugated antibodies to CD4, Vβ3, Vα11, CD44, and CD45RB. The relative levels of expression of CD44 (left) and CD45RB (right) are shown. Control cells freshly isolated from a young AND Tg mouse were also stained and included for comparison (dotted line). Essentially all the effector cells recovered from culture were Vβ3Vα11 positive CD4+ T cells. Shown are representative data from one experiment with two experiments performed

ing CD4+ spleen cells from mice at varying ages, the relative levels of expression of CD44 and CD45RB was analyzed (Fig. 2 A). As we reported earlier (36), in young AND mice the Tgpos CD4+ cells expressed a relatively naive phenotype, i.e., CD44lo CD45RBhi, as compared with the Tglo−neg CD4+ cells, which were CD44hi CD45RBlo.

However, unlike previous findings from conventional aged mice (3), the transgene expressing CD4+ cells from aged individuals retained a CD profile consistent with that of naive cells. Importantly, when CD4+ T cells from aged Tg mice were stimulated with antigen and APCs in vitro, they were able to switch to an activated phenotype (Fig. 2 B). Moreover, like young mice, the Tglo−neg CD4+ cells from young and aged donors had a memory-like phenotype. These findings were similar in all age groups studied. Therefore, the slight shift to a memory phenotype among the whole CD4+ population in the aged Tg mice is the result of an increased representation of the more memory-like Tglo−neg cells, while Tgpos cells retain a naive phenotype consistent with lack of antigen exposure. This was exemplified by the increased proportion of CD44hi cells in the total CD4 T cell population. Owing to the heterogeneity and lower levels of fluorescence intensity of CD45RB expression, the age-associated shift toward an increased representation of a memory phenotype was not apparent except once the total CD4 cells were separated into Tgpos and Tgneg populations.

The Functional Phenotype of Aged Tgpos CD4 T Cells. To evaluate whether the Tgpos CD4+ population expressed a naive phenotype by functional criteria, we examined their ability to produce a panel of cytokines. Naive cells have been shown to produce IL-2 as the major cytokine following 24 h. of antigen exposure, whereas long-term memory cells (or activated cells) produce high levels of IL-2 in addition to significant levels of IL-4 and IL-5 or IFN-γ (20–23). To obtain pure Tgpos CD4 T cells, we sorted Vβ3+Vα11+ CD4+ cells from the spleens of young and old mice, and stimulated these cells for 24 h. with antigen (PCCF) and APC (DCEK-ICAM/B7). As shown in a representative experiment in Fig. 3 (A, B, and E), stimulation of cells from young TCR Tg mice induced production of high levels of IL-2, and moderate levels of IL-3 but no detectable IL-4 or IFN-γ. Similar patterns of cytokine production were obtained with cells from old mice; however, the levels of IL-2 and IL-3 secretion were significantly reduced. Thus, the Tgpos cells of aged mice behaved like naive T cells and did not make IL-4 or IFN-γ, but they differed from Tgpos cells of young mice in that less IL-2 and IL-3 were produced by the equivalent number of cells. Importantly, effector cells generated from sorted Tgpos CD4+ T cells of aged Tg mice were capable of IL-4 and IFN-γ secretion upon antigenic exposure (Fig. 3 F). To substantiate the significance of the reduction of IL-2/IL-3 production, Fig. 3 C and D show the units of cytokine in cultures of cells of young and aged animals in three experiments in which sorted Tgpos CD4+ T cells were compared. In the aged, IL-2 production was reduced by 50% or more and IL-3 production was reduced by 25% or more. This suggests that even though aged Tgpos CD4 cells retain a naive phenotype they have become hyporesponsive.

The use of antibodies recognizing the TCR to isolate pure populations of transgene-expressing cells did not itself induce cytokine production, since similar cultures of sorted
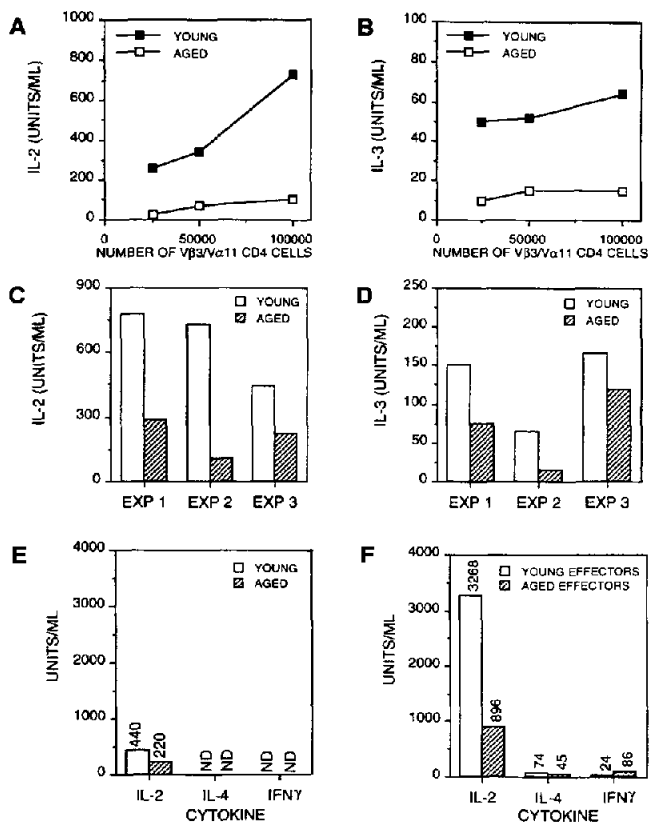
Figure 3. Cytokine production by sorted Vβ3Vα11 positive CD4+ cells from 2-mo-old (closed symbols and solid bars) and 16-mo-old (open symbols and hatched bars) AND Tg mice. Cytokine production by freshly isolated cells and by effector cells (that were generated as described in Fig. 2 B) are shown in A–E and F, respectively. The Vβ3+ Vα11+ CD4+ cells were sorted from preparations of freshly isolated spleen cells or from in vitro generated effector cells. Varying numbers of these cells were cultured with 5 μM PCCF and 1 × 10^5 DCEK-ICAM/B7 cells for 24 h. Supernatants were tested for IL-2, IL-3, IL-4, and IFN-γ as described in Materials and Methods. The lower limit of assay detection for both IL-2 and IL-3 was 5 U/ml. No IL-4 (detection limit, 40 U/ml) nor IFN-γ (detection limit, 10 U/ml) secretion was detected in cultures of freshly isolated cells (ND, not detectable). Shown are representative data from one experiment with three experiments (panels A, B, and E) or two experiments (F) performed In panels C, D, E and F of this figure are the data from individual experiments in which the levels of cytokine secreted by 1 × 10^5 sorted Vβ3Vα11 positive CD4+ cells are shown.

Figure 4. Proliferation by sorted Tg^pos and Tg^neg CD4+ T cells from young versus aged AND Tg mice in response to insoluble anti-CD3 antibody. In A, sorted Vβ3Vα11 positive CD4+ T cells from 2-mo- (squares) versus 16 mo-old (circles) AND Tg mice were cultured at varying numbers in the presence of plate-bound anti-CD3 antibody (2C11) plus (open symbols) or minus (closed symbols) 7.2 ng/ml of soluble anti-CD28 antibody. The microcultures were pulsed with [³H]TdR the last 16 h of the 3 d culture, harvested, and counted. Shown are representative data (mean ± SD) from one experiment with three experiments performed. In B, 75,000 sorted Vβ3Vα11 positive or negative CD4+ T cells from 2-mo- (open bars) versus 17-mo-old (solid bars) AND Tg mice were cultured as described above. Shown are representative data (mean ± SD) from one experiment with two experiments performed

cells in the absence of antigen did not produce any cytokines. Furthermore, the transgene expressing CD4 cells were not activated. In the absence of antigenic stimulation, the cells isolated from both old and young mice were found to remain small and resting as judged by their forward scatter profile as well as their lack of expression of determinants found on activated T cells, such as IL-2 receptor, transferrin receptor, and CD69 (data not shown). Finally the levels of cytokine production seen in these experiments are comparable to those seen in many previous experiments with unsorted cells, which had never been stained with antibody (21, 22, 36).

Requirements for Costimulation in the Aged Tg^pos CD4 T Cells. To assess immunologic responsiveness with aging,
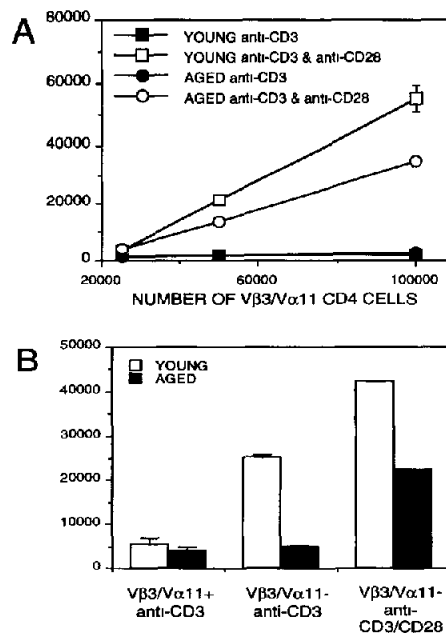
most studies have relied upon stimulation with polyclonal activators such as antibody to CD3. Although this stimulation is thought to mimic antigen-specific responses through the TCR, treatment with anti-CD3 without added APCs or costimulation has been shown to stimulate cells preferentially in the memory pool (24). In fact, naive cells are completely dependent on costimulation (25), while memory cells can respond somewhat in the complete absence of costimulation (22, 46). To assess further the naive status of the Tg^pos CD4+ population, we determined proliferation of the sorted Tg^pos cells from young and old mice in response to immobilized anti-CD3 antibody with or without costimulation provided by anti-CD28 (Fig. 4 A). Consistent with previous analyses of naive cells, Tg^pos cells did not proliferate to anti-CD3 antibody alone, although upon addition of anti-CD28 antibody, the sorted Vβ3+Vα11+ CD4+ cells responded vigorously. With the optimum stimulus provided by anti-CD3 plus anti-CD28, the aged Tg^pos cells nonetheless proliferated significantly less than the young Tg^pos cells. This provides further support that the Tg^pos cells are naive and suggests that even with optimal stimulation they are hyporesponsive. Moreover, the increased proliferation by the Tg^neg cells versus Tg^pos cells from the young mice in re-

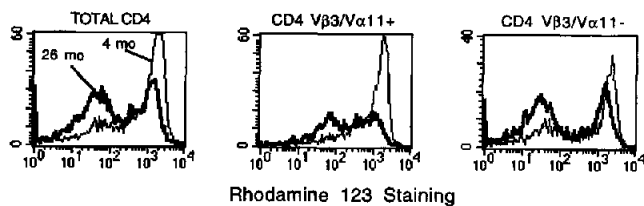**Figure 5.** R123 staining within the whole CD4 population (*left*), Vβ3Vα11 positive CD4 population (*middle*), and Vβ3Vα11 negative CD4 population (*right*) in 4-mo-old (*solid line*) and 26-mo-old (*bold line*) AND Tg mice. Cells were incubated with R123 and conjugated antibodies to CD4, Vβ3, and Vα11 as described in Materials and Methods. Gating on CD4+ cells (*left*), Vβ3Vα11 positive CD4 cells (*middle*), or Vβ3Vα11 negative CD4 cells (*right*), the relative fluorescence of R123 was analyzed. Shown are representative data from one experiment with three experiments performed.

sponse to anti-CD3 alone further supports the decreased dependence upon costimulatory molecules by memory cells (Fig. 4 B). As shown by others (17) and as demonstrated here (Fig. 4 B), the cells expressing the memory phenotype (i.e., Tg^{neg} cells) from the aged mice were less responsive to stimulation by anti-CD3 alone or with anti-CD28 antibody.

R123 Staining of Aged Tg^{pos} CD4 T Cells. Among the many changes observed with aging is an increase in the percentage of cells that stain dimly with the fluorescent dye, R123 (29–31). The uptake and extrusion of R123 has been correlated with P-glycoprotein activity (29–31). Whether continued antigenic stimulation or other elements in the aged environment are responsible for these shifts in R123 staining is not clear. Therefore, we examined R123 staining patterns in the unseparated and sorted Tg^{pos} and Tg^{lo-neg} CD4+ populations from aged mice. Our findings in the unseparated population were similar to those reported by others using cells from conventional mice and humans (29–31), in that the CD4+ cells from aged TCR Tg mice displayed bimodal staining with R123 (Fig. 5). In the total CD4 population the R123^{dim} population was increased in aged mice. Importantly, the presence of the dimly staining subset was evident in both the CD4+ Vβ3Vα11 negative and positive populations from aged mice (although to a lesser extent in the Tg^{pos} population), suggesting that this change may also be independent of conversion to memory phenotype and thus due to an antigen-independent aspect of aging.

Antigen-induced Responsiveness of Naive Tg^{pos} CD4 Cells from Aged versus Young Mice. Based on our earlier finding of decreased IL-2 production, it was not surprising to find that antigen-induced proliferation was also somewhat decreased with age, even over a range of T cell numbers and in repeated experiments (Fig. 6). Importantly, the reduction in proliferation and cytokine production by the Tg^{pos} cells from aged mice was evident over a range of PCCF concentrations (0.1–30 μM) (Fig. 7). Moreover, proliferation by the Tg^{pos} cells from the aged mice remained lower in 3- and 4-d cultures (data not shown).
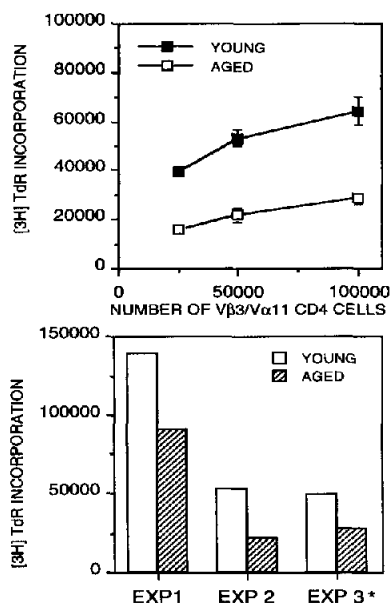

Figure 6. Proliferative activity of Vβ3Vα11 positive CD4 cells from 2-mo-old (*closed symbols* and *solid bars*) versus 16-mo-old (*open symbols* and *hatched bars*) AND Tg mice in the presence of 5 μM PCCF and 1 × 10^5 mitomycin c-treated DCEK-ICAM/B7 APCs. Triplicate cultures were pulsed with [³H]TdR the last 16 h of the 3 d culture, harvested, and counted. Shown in the upper panel are representative data (mean ± SD) from one experiment with three experiments performed. In the lower panel of this figure are the data from individual experiments in which the levels of proliferation (cpm) by 1 × 10^5 sorted Vβ3Vα11 positive CD4+ cells are shown. *Aged and young AND Tg mice on the H-2$^{b/b}$ background were used in experiment 3.

Discussion

In these studies, we have discovered several interesting aspects of the alteration in CD4 T cells associated with aging. First, we have presented data strongly suggesting that the shift of the CD4 T cell population to a more memory phenotype is antigen driven and does not happen if antigen is not available. Second, we have shown that cells that retain a naive phenotype and have most likely never encountered antigen undergo changes associated with aging, including the relative loss of IL-2 and IL-3 production, reduced proliferative capacity, and an increase in the number of R123^{dim} cells. This suggests that factors unrelated to antigen exposure are responsible for the hyporesponsiveness of aged CD4 T cells.

In this study, we addressed the issues of whether, in the absence of contact with cognate antigen, CD4+ T cells of aged mice retain normal antigen responsiveness, and CD4+ T cells acquire the memory cell phenotype associated with aging. To do so, we analyzed the phenotype and responsiveness of CD4+ T cells in aged versus young AND Tg mice. The CD4+ T cells with lower Tg expression but normal CD3 levels, which have been reported to express endogenous TCR, increased with age (Fig. 1) and exhibited the memory phenotype as defined by higher levels of expression of CD44 and lower levels of expression of CD45RB (Fig. 2). This is similar to the findings in aged
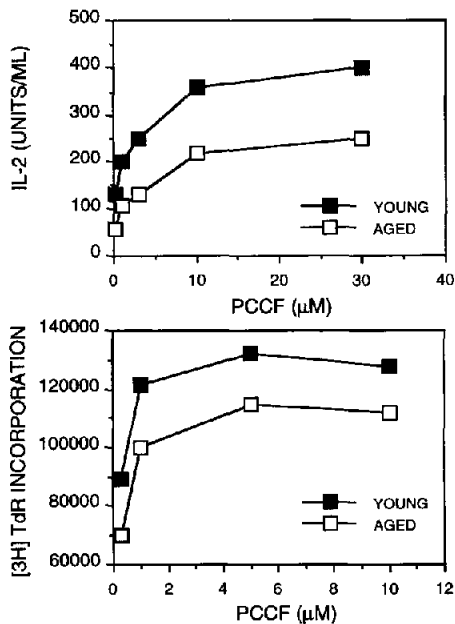
Figure 7. The effect of PCCF concentration on IL-2 production and proliferative activity of Vβ3Vα11 positive CD4 cells from 2-mo-old (*closed symbols*) versus 16-mo-old (*open symbols*) AND Tg mice in the presence of 1 × 10⁵ mitomycin c-treated DCEK-ICAM/B7 APCs.

conventional and Tg mice (40), wherein a greater proportion of CD4⁺ T cells express the CD profile typical of memory and effector cells. Because environmental antigens are likely capable of stimulating only the T cells in the AND Tg mice that express alternate TCR, and since memory cells have longer lifespans than naive cells (21), the shift to an increased representation of CD44ʰⁱ CD45RBˡᵒ CD4⁺ cells, the aged phenotype, is most likely the result of an antigen-driven process. We suggested that exposure to environmental antigen eventually results in an increased representation of antigen-experienced cells that express a memory phenotype.

The finding that the Tgᵖᵒˢ CD4⁺ T cells in aged AND Tg mice remain naive supports the antigen dependence of the acquisition of the memory phenotype in aged animals. The naive status of the Tgᵖᵒˢ CD4⁺ T cells was confirmed by phenotypic and functional parameters. The CD44ˡᵒ CD45RBʰⁱ profile expressed by the Tgᵖᵒˢ cells in aged animals is unique to naive cells (Fig. 2) (22). In confirmation of this, we have found that the cells lack expression of the activation markers, CD69, IL-2 receptor, and CD71, and do not have increased size as judged by forward scatter (data not shown). In addition, upon antigenic stimulation the Tgᵖᵒˢ CD4 population of aged mice produce IL-2 but no detectable IL-4 nor IFN-γ (Fig. 3). This pattern of cytokine production is also restricted to naive CD4 T cells. Finally, the absence of proliferation to insoluble anti-CD3 antibody in the absence of costimulation is also diagnostic for naive CD4 T cells (Fig. 4). As reported with the transgene expressing CD4 cells from young mice (22, 47), the naive transgene-expressing CD4 cells from aged mice were

capable of differentiation to effector cells upon antigen stimulation. This was evidenced by their changes in CD profile as well as in patterns of cytokine secretion (Figs. 2 and 3). These findings validate our initial assumption that no environmental antigen engages the Tg TCR recognizing PCC. Furthermore, these findings suggest that the acquisition of a memory phenotype in the aged is not a consequence of bystander activity or the impact of an aged environment, since the predicted alterations in the phenotype of transgene expressing CD4 cells of the aged were not evident. However, this in no way rules out the possibility that bystander activity has a role in the expansion and/or persistence of memory cells (48). Because experiments were done on FACS®-sorted Tgᵖᵒˢ CD4⁺ T cell populations of extremely high purity (i.e., >98%), which do not respond to anti-CD3, it is safe to conclude that any of the changes that are observed are indeed in the CD4 T cells themselves and not a consequence of contaminating cells. Although one cannot rule out the possibility that variables inherent to the TCR Tg model may influence the phenotype and function of the Tgᵖᵒˢ CD4 T cells, our findings would suggest that when stimulated with antigen in vivo, the Tgᵖᵒˢ CD4 T cells were equivalent to the Tgⁿᵉᵍ CD4 T cells in their ability to switch to a memory phenotype. This is supported by several experiments: (*a*) Upon adoptive transfer and antigenic stimulation, the naive Tgᵖᵒˢ CD4 T cells differentiate to memory cells based on phenotype and function (47); and (*b*) Immunization of AND Tg mice with the superantigen/staphylococcal enterotoxin A (SEA) induces the switch of the majority of Tgᵖᵒˢ cells to a memory cell phenotype (Schuenke, K., and S.L. Swain, unpublished observation).

P-glycoprotein-mediated extrusion of the fluorescent dye R123 further defines age-dependent subsets within the naive and memory cell subpopulations of CD4 cells (29–31). P-glycoprotein activity is responsible for multidrug resistance in tumor cell lines and human tumors in vivo (32, 33), but several normal cells including lymphocytes display functional activity associated with P-glycoprotein expression (29–31). The function of P-glycoprotein in normal cells is not yet clear, but its demonstrated ability to serve as a chloride ion channel, ATP channel, a membrane ATPase, and a transport pump, suggest that it may play a role in signal transduction processes, or in protection from toxic agents (32, 33, 49). Our findings are similar to those reported by others for T cells from conventional animals (29–31), in that the CD4⁺ T cells from aged TCR Tg mice also displayed bimodal staining with R123 (Fig. 5). Importantly, the R123 extruding subset was evident in both the Tgˡᵒ ⁿᵉᵍ (i.e., memory phenotype) and Tgᵖᵒˢ (i.e., naive phenotype) populations of the aged, perhaps indicating fundamental underlying variations in the activation state or developmental history of the T cell as suggested by Witkowski et al. (34).

Superimposed upon the switch to memory cell predominance are age-related functional changes within the naive and memory T cell populations, including changes in patterns of protein phosphorylation (11) and changes in IL-2 production and IL-2 responsiveness by memory cells (12,

14). Of particular importance in our studies was that T cells, even of naive phenotype, displayed a decrease in ability to respond to specific antigen. Functional changes in the naive transgene expressing cells from the aged include markedly lower levels of IL-2 and IL-3 secretion seen with great consistency and decreased proliferation in response to antigenic stimulation (Figs. 3 and 6). Moreover, the decline in proliferative capacity does not appear to be due to delayed kinetics (unpublished observation) or suboptimal concentrations of antigen (Fig. 7). Although reduced proliferation may partly be a consequence of decreased IL-2 production, other alterations such as those in signal transduction through the TCR may contribute to the hyporesponsiveness observed in naive cells. Moreover, a decrease in IL-2 receptor expression as reported by others (2) may foster the decline in antigen-induced proliferation. Further studies will be needed to determine whether naive aged CD4 T cells have additional functional deficiencies. The alterations in the naive Tg^{pos} cells in cytokine production, proliferation, and expression of P-glycoprotein are presumably the consequence of the antigen-independent aspects of aging. At this time, it is unclear whether functional changes in naive transgene-expressing cells may be primarily concentrated in the R123^{dim} as opposed to the R123^{bright} subset.

An important implication of the observation that naive cells from the aged do not respond to antigen as well as their young counterparts is that specific immunization targeted at the naive population in aged individuals may not be effective. Moreover, the age-dependent accumulation of memory phenotype cells (as exemplified by the increase in the endogenous TCR encoded population) further suggests that the TCR repertoire is reshaped during aging by the history of exposure to antigen, leading to the predominance of antigen-engaged clonotypes; thus, potentially leading to constrictions of the overall repertoire and possible deficiencies in the responses to rarely encountered antigens (40). These findings suggest that the basis for the decline in responsiveness by the elderly to new antigens may be twofold, i.e., not only are there fewer naive phenotype cells in the elderly but this is compounded by a decline in antigen-specific function of these cells. It is possible that the intrinsic decline in naive T cell function may become more pro-

nounced if the function of APCs from the elderly is also altered, as suggested by others (13, 50).

Another implication of these studies is that the shift in cytokine profile associated with aging could be accounted for by the phenotypic shift from a population dominated by naive cells to one composed primarily of memory phenotype cells. Since the number of naive cells and IL-2 secretion by these cells are decreased, the overall production of IL-2 would be decreased in the aged; concordantly, memory cells, known to make some IL-4 and IFN-γ, would represent a higher fraction of the T cell population of the aged.

These studies have not yet addressed the changes that occur in the "memory" phenotype population with aging, except to document the increase in the P-glycoprotein (R123^{dim}) population and a decrease in proliferation in response to anti-CD3 with or without anti-CD28 costimulation. A relative comparison of antigen-induced responses by young and aged memory T cells is planned and should help to establish whether the aged memory cells are also, as expected, hyporesponsive.

In these studies, the contribution to the findings by newly generated T cells from the thymus has not been identified. Although the thymus does decrease in size with age in these mice, subtle changes in the cell populations present remain unknown. The presence of significant numbers of naive transgene-expressing cells in the secondary lymphoid organs of the aged mice raises the question of whether the thymus is productive in the aged and/or whether the naive cells in the aged are long-lived. From the studies presented here, it is clear that in the aged the shift to cells expressing predominantly the memory phenotype is likely the result of antigenic stimulation, the increase in the number of memory phenotype cells is most likely due to the expansion and/or persistence of such cells, and the decrease in antigen-induced responsiveness in naive cells of the aged is in part the consequence of intrinsic changes. The findings suggest that strategies to vaccinate elderly individuals must take into account both the decrease in naive T cell numbers and their decreased responsiveness.

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