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Early Antigen-Specific Response by Naive CD8 T Cells Is Not Altered with Aging

Shaokang P. Li,* Zeling Cai,† Weixing Shi,† Anders Brunmark,† Michael Jackson,† and Phyllis-Jean Linton‡*#

Both a dramatic decline in CD8 responses and a switch to memory T cell predominance occur with aging. The extent to which the loss of responsiveness is the consequence of the accumulation of more differentiated vs intrinsically defective T cells (or both) has been unclear. Using similar conditions of Ag stimulation, we have examined the responses generated by CD8⁺ T cells isolated from aged TCR transgenic mice. We found that the naive transgene⁺ CD8⁺ cells from aged 2C mice expressed activation markers, produced IL-2, proliferated, and differentiated into cytotoxic T cells as efficiently as their young counterparts. The extent of responsiveness and the level of the responses were comparable in both age groups regardless of the stimulatory conditions used, i.e., partial costimulation/adhesion molecule expression on APCs, or presentation of lower affinity peptide or diminished peptide concentrations. By day 4 after Ag stimulation, no significant age-related differences were observed in the number of effector cells generated nor in the levels of secreted IL-2 or IFN-γ. Upon restimulation of effector cells, IL-2 secretion and to a lesser extent TNF-α expression, but not IFN-γ secretion, were diminished with age. These findings suggest that age-associated alterations in naive CD8 T cell function are not found after primary stimulation, but may become apparent upon restimulation. The Journal of Immunology, 2002, 168: 6120–6127.

The decline in cell-mediated immunity during aging has been implicated as a factor in the increased susceptibility of the elderly to infections. Substantial changes in both the function and phenotype of T cells in aged humans and rodents have been found (1, 2). Among the functional changes are alterations in the profile of cytokines produced (3–5), changes in the early events of signal transduction (6–9), decreased proliferation in response to TCR and costimulus-mediated stimulation (10–12), and decreased IL-2 production (13). Among the phenotypic changes is a dramatic shift toward an increased representation of T cells expressing determinants typical of memory cells with a concomitant decline in the proportion of T cells expressing determinants typical of naive cells (3, 11, 14). Since many of the functional alterations found in the aged may be the consequence of the accumulation of memory T cells rather than deficiencies intrinsic to the T cells of the aged, many studies of T cell function have been reevaluated using purified subset populations.

Understanding the changes in CD8 T cell function with aging has been difficult due to the dependence of many CD8 responses on CD4 cells for sufficient stimulation and/or population shifts that are evident in the CD4 T cell subset. Moreover, other alterations in the aged environment, such as those in the cytokine milieu (15, 16) and APC function (17–19), may also impact CD8 T cell responses. Studies of CD8 cell function using unseparated T cell preparations (i.e., CD4 + CD8) have shown a decline in the proportion of T cells that can respond to polyclonal stimulation in the presence of excess IL-2 by proliferation or by the generation of cytotoxic effectors (11, 12). A limited number of studies have been done to define those changes intrinsic to the CD8 T cells of the aged using purified naive vs memory phenotype CD8⁺ cells. In one such study, an increase in the production of IFN-γ by memory CD8 T cells of the aged was observed (3). However, an extensive functional analysis of the age-associated changes intrinsic to purified naive vs memory CD8 cell populations is still lacking. Therefore, we have undertaken a comprehensive study of Ag-specific CD8 T cell function using young and old TCR transgenic (Tg) mice.

In the past, assays to elucidate which aspects of CD8 T cell responsiveness decrease with aging were difficult due to the low cell numbers in the purified naive vs memory CD8 population and the dependency of some CD8 responses on CD4 cells for sufficient IL-2 production. MHC class I-restricted TCR Tg mice, such as the 2C Tg mice, provide a well-defined Ag-specific system to study deficiencies that may be intrinsic to the CD8 cells of the aged. The Tg⁺ CD8 T cells from 2C TCR transgenic mice (20) express the 1B2 clonotype (21) that recognizes allo-Ld molecules linked to either of two self peptides, p2Ca and QL9, derived from a Krebs cycle enzyme (22, 23). In the 2C system, the binding affinities involved in the TCR-MHC/peptide interaction are well defined. The p2Ca and QL9 peptides have been widely used and characterized; QL9 (a 9-mer peptide) has 100-fold higher binding affinity for Ld than p2Ca (an 8-mer) and ~10-fold higher affinity for 2C TCR (22, 23). Importantly, in vitro responses by CD8 cells from 2C mice are CD4 independent (24). Moreover, in the 2C system, a variety of Drosophila cell transfectants expressing varying costimulation/adhesion molecules is available as APCs (25). Thus,
using the p2Ca and QL9 peptides, and a panel of Drosophila transfectants expressing L^{d} plus B7-1 (L^{d}B7), L^{d} plus ICAM-1 (L^{d}I-CAM), or L^{d} plus B7-1 plus ICAM-1 (L^{d}B7/ICAM), alterations in Ag-specific activation, proliferation, cytokine production, and CTL function that are intrinsic to the Tg^{+} CD8 cells of aged 2C mice have been determined.

We found that the Tg^{+} CD8 cells from aged 2C mice retain a naïve phenotype. Unlike naïve CD4 T cells (26), no alterations intrinsic to naïve CD8 T cells of the aged were detected. Under similar stimulatory conditions, the naïve Tg^{+} CD8 T cells from aged 2C mice expressed activation markers, produced IL-2, proliferated, and differentiated into cytotoxic T cells as efficiently as Tg^{+} CD8 cells from young mice. These age-related similarities in responsiveness were still evident if less overt stimulatory conditions were used, i.e., responses generated with reduced peptide concentration or with peptides of lowered affinity or with APCs that express either B7 or ICAM as costimulation or adhesion molecules, respectively. Cell numbers and cytokine levels late in culture were also found to be comparable between the age groups. However, upon Ag restimulation of effector cells, IL-2 secretion and intracellular TNF-α expression, but not IFN-γ production, were diminished in the aged. These findings suggest that naïve CD8 cell function is not altered with age after primary Ag exposure; however, altered function may become apparent later in the ensuing response upon Ag restimulation. Thus, it is likely that a defect intrinsic to naïve CD8 T cells of the aged may not have as major an impact on the decline in CD8 responses of the elderly. Instead, other age-associated alterations, such as a decline in the frequency of Ag-specific CD8 cells (27), reduced CD4 or APC function (2, 17), and/or an altered cytokine milieu (15), may have a greater impact on the reported decline in CD8 responsiveness with aging.

Materials and Methods

Mice

The 2C TCR-transgenic mice were originally generated by Sha et al. (20). The CD8 T cells express the αβ Ag receptor from the cytotoxic T lymphocyte clone 2C (28). The mice were bred and maintained under specific pathogen-free conditions at The Scripps Research Institute (La Jolla, CA) and the Sidney Kimmel Cancer Center until their use at 2 mo (young), 9–10 mo, and 16–18 mo (aged). Quarterly serology was performed on sentinel mice sent to the University of Missouri (St. Louis, MO) to verify that specific pathogen-free conditions were maintained. Based on ~120 mice used for this study, the average life span for the 2C mice is 9–10 mo, and ~28% of the mice survive to 14 mo of age. Several of the old mice that died at ~10 mo of age were sent for pathology tests, and all tested mice were found to have developed thymomas. Necropsy was performed at the time of mouse sacrifice to exclude individual animals with gross pathology. Findings with 9- to 10-mo-old 2C mice were similar to those obtained with 16- to 18-mo-old mice and, therefore, were not shown.

CD8 cell isolation

The CD8 T cells were isolated from the spleens and lymph nodes of 2C mice by negative selection using magnetic beads and columns for murine CD8 cell enrichment, according to the manufacturer (Stem Cell Technologies, Vancouver, Canada). This was followed by positive selection of CD8^{+} cells by sorting on the FACSVantage (BD Biosciences, San Jose, CA). Briefly, after a preincubation with 5% normal rat serum, the cell suspension at 5 × 10^{6}/ml was incubated 15 min at 4–8°C with 10 μl murine CD8 enrichment Ab mixture/million cells. The cells were washed and resuspended at 5 × 10^{6}/ml, 100 μl anti-biotin tetrameric Ab complexes/ml cells were added, and the cells were incubated as above. Without washing, the cells were further incubated with magnetic colloid (60 μl/ml) before loading on a column in a magnetic field. The collected nonmagnetic fraction was enriched for CD8 cells, but contained a significant portion of Tg^{+} double-negative (i.e., CD4^{−} CD8^{+}) cells. Therefore, the CD8^{+} population was isolated by staining with allophycocyanin-conjugated anti-CD8a Ab (BD Pharmingen, San Diego, CA), followed by sorting on a fluorescent activated cell sorter (FACSVantage; BD Bio-

The enriched population contained >99% CD8^{+} cells with the average percentage of 1B2^{+} (Tg^{+}) cells from young and aged donors, 98.5 and 94.6%, respectively.

Ag-presenting cells

The generation of the transfected Drosophila cell lines expressing murine class I molecule L^{d} and costimulation molecules B7-1 (L^{d}B7), ICAM-1 (L^{d}I-CAM), or both (L^{d}B7/ICAM) was described elsewhere (25). The Drosophila cells were grown at room temperature in Schneider’s Drosophila medium. Expression of the transfected genes was induced by the addition of CuSO_{4} to a final concentration of 1 mM 1 day before use of these cell lines as the artificial APCs.

Peptides

p2Ca, an 8-mer peptide (LSPFFPDLE) with intermediate binding affinity for L^{d} molecules (4 × 10^{-6} M^{-1}) and high affinity for 2C TCR molecules (2 × 10^{6} to 1 × 10^{7} M^{-1}), and QL9, a closely related 9-mer peptide (QLSPPFDFL) with higher affinity for those molecules (2 × 10^{8} M^{-1} for L^{d} and 2 × 10^{7} M^{-1} for 2C TCR), were used as the Ag for 2C Tg^{+} cells (25, 29).

Media

RPMI 1640 medium supplemented with 10% FBS (Irvine Scientific, Santa Ana, CA), 5% NCTC 109 (Life Technologies, Germantown, MD), 2 mM glutamine, 5 × 10^{-3} M 2-ME, and antibiotics was used in the culture of 2C mice. Schneider’s Drosophila medium containing 10% FBS (Life Technologies), 500 μg/ml G418 sulfate (Calbiochem, La Jolla, CA), 2 mM glutamine, and antibiotics was used to grow the transfected Drosophila cells.

Immunofluorescent staining and flow cytometry

The following Abs and fluorescent reagents were purchased from BD Pharmingen: FITC-conjugated anti-CD11a (M174), anti-CD25 (7D4), anti-CD4 (12/2F1), anti-CD8α (53-6.7), and hamster IgG; allophycocyanin-conjugated anti-CD8α (53-6.7-2); and biotinyl anti-VJ81.8, 8.2 TCR (MR5-2). PerCP-conjugated streptavidin was from BD Biosciences. Biotinyl anti-clonotopic Ab (1B2) was prepared (30) from the ascites of the hybridoma-producing 1B2 mAb, which was kindly provided by H. Eisen (Massachusetts Institute of Technology, Cambridge, MA).

The phenotype of the Tg^{+} CD8^{+} cells from young and aged 2C mice was analyzed on a FACScalibur flow cytometer using CellQuest software (BD Biosciences). A minimum of 1000 gated events (i.e., 1B2^{+} CD8^{+} cells) was collected for each analysis. Since the TCR is down-regulated upon Ag stimulation in vitro, cultured Tg^{+} CD8 cells were analyzed gating on CD8^{+} cells. The procedure for intracellular TNF-α staining has been described previously (31).

Proliferation assay

Purified 1B2^{+} CD8^{+} cells were cultured in duplicate at 5 × 10^{6}/well with 15 × 10^{6} APCs in 200-μl wells (microculture) in the presence or absence of peptides. Cells were pulsed 3 days later with 1 μCi [H]thymidine and harvested 16 h later.

Bulk culture and generation of CTLs

CTLs were generated in bulk culture. A total of 5 × 10^{6} Tg^{+} CD8^{+} cells was cultured with 1.5 × 10^{6} APCs and peptide in a 2 ml vol. This time point was optimal for CTL generation from young and aged Tg^{+} CD8 cells. CTL activity was assessed 4 days later. The target cells, RMA-S.L1.4, were labeled 90 min at 37°C with 51Cr (100 μCi/1–2 × 10^{5} cells) in the presence or absence of peptides. The target cells and CD8 effectors were thoroughly washed before 4-h culture at varying E:T ratios in the presence or absence of peptides. Specific 51Cr release was calculated as previously described (25, 29).

Detection of IL-2 and IFN-γ secretion

Culture supernatants were collected 1, 4, or 5 days after initiation of culture or 24 h after restimulation with Ag on day 4. The supernatants were kept at −80°C until assayed for IL-2 (26), IFN-γ (32), and TNF-α. Briefly, IL-2 activity was detected either by ELISA or by measuring the proliferation of NK3 cell line that is both IL-2 and IL-4 dependent (L. Bradley, The Scripps Research Institute). To block any IL-4-induced proliferation by NK3 cells, anti-IL-4 Ab (1B11; BD Pharmingen) was added at a final concentration of 2 μg/ml. Under these conditions, IL-4-dependent proliferation by NK3 cells is blocked in the presence of 1250 U/ml or 1.25 ng/ml IL-4. The data

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were quantitated from standard curves using rIL-2 and are expressed as U/ml, in which 50% of the maximum response represents 1 U of activity (26). The detection limit of the assay was ~10 U/ml.

An amplified ELISA was used for the detection of IFN-γ and IL-2 activity. Briefly, the samples and recombinant IFN-γ or IL-2 standard were added to wells previously coated with purified anti-IFN-γ Ab (2 μg/ml R46A.2; BD PharMingen) or anti-IL-2 Ab (1 μg/ml Jes6 capture; BD PharMingen) and blocked. The plates were incubated overnight at 4°C and washed. The following reagents were added to each well successively, with incubation and washes between each step: biotinyl anti-IFN-γ or anti-IL-2 Ab (1 μg/ml XMGI 1.2; BD PharMingen) or biotinyl anti-IL-2 Ab (1 μg/ml Jes6 detection; BD PharMingen), Extravidin (1 μg/ml; Sigma-Aldrich, St. Louis, MO), anti-avidin-biotin (0.5 μg/ml; Sigma-Aldrich), streptavidin-peroxidase (1:2000; Jackson ImmunoResearch Laboratories, West Grove, PA), and o-phenylenediamine (1:10 of 2 mg/ml; Sigma-Aldrich) plus urea hydrogen peroxide (1:200 of 40 mg/ml; Sigma-Aldrich). The detection limits of the assay were ~10 ng/ml IFN-γ and 5 pg/ml IL-2 (32). TNF-α was determined using an ELISA kit according to the instructions of the manufacturer (Endogen, Woburn, MA). The detection limit of the assay was 40 pg/ml TNF-α.

Results

The phenotype of Tg⁺ CD8⁺ T cells in aged 2C mice

The Tg⁺ cells in young mice express the naive phenotype (25, 29, 33). To investigate whether aged 2C mice retained naive Tg⁺ cells or whether the CD8 population shifted to a Tg⁻ or memory phenotype, we examined the expression of the TCR transgene (i.e., the 1B2 clone) on CD8⁺ cells of young and old mice. In general, no striking differences were observed in the total number of CD8⁺ cells isolated from the spleens of young (2–3 mo) vs aged (16–18 mo) 2C mice. In addition to CD8⁺ 1B2⁺ cells, ~50% of the Tg⁺ spleen cells in young 2C mice were both CD8⁻ and CD4⁻. These double-negative Tg⁺ cells were slightly increased with age (data from representative individual young and aged 2C mouse are shown in Fig. 1A). Similar to, but not as dramatic as the findings with CD4 cells isolated from aged TCR transgenic mice (26), the percentage of Tg⁺ cells in the CD8⁺ population decreased slightly with age (Fig. 1). Whereas ~98.5 ± 1.1% (mean ± SD) of the CD8⁺ cells of young mice were 1B2⁺, 94.6 ± 3.2% of the CD8⁺ cells of aged mice were found to express the transgene (Fig. 1B).

In conventional animals, a phenotypic shift occurs in the CD8⁺ T cell population such that cells expressing determinants typical of memory cells, i.e., increased levels of CD44, increase in representation with aging (3). This phenotypic shift is presumably the accumulation of memory cells that have been generated through exposure to environmental Ags. We examined the phenotype of the Tg⁺ CD8⁺ cells to determine whether these cells isolated from aged 2C mice remained naive. Unless Ags found in the environment cross-react with the determinants recognized by the 1B2 TCR, the Tg⁺ CD8⁺ T cells of the aged 2C mice would be expected to retain the naive phenotype. Gating on the Tg⁺ CD8⁺ cells from aged and young 2C transgenic mice, we determined the level of expression of several adhesion and activation molecules that are known to be differentially expressed on naive vs memory/activated T cells (34). Indeed, as shown in Fig. 2, the Tg⁺ CD8⁺ cells from aged mice retained a naive phenotype, i.e., CD44low−mesCD62LhighCD11a−mesCD25lowCD69high. Importantly, these cells were able to switch to an activated phenotype, as determined 60 h after exposure to Ag in vitro (Fig. 2B).

Responses of aged Tg⁺ CD8⁺ T cells after maximal stimulation

Initially, we determined whether any age-associated differences were evident in the activation, proliferation, cytokine production, and cytotoxic activity of CTLs generated from purified Tg⁺ CD8⁺ cells under conditions of maximal stimulation. Earlier studies have shown that maximal stimulation of the naive Tg⁺ CD8⁺ T cells of young 2C mice was obtained with the QL9 peptide presented on Drosophila transfectedants expressing H-2Ld, B7-1, and ICAM-1 (LdB7.ICAM) (25). The alloreactivity of 2C to Ld is specific for an endogenous 8-mer peptide, p2Ca, derived from a Krebs cycle enzyme, 2-oxoglutarate dehydrogenase (22, 23). QL9 is a closely related 9-mer peptide that has a higher affinity for Ld (2 × 10^8 M⁻¹) and for the 2C TCR molecules (2 × 10^7 M⁻¹) as compared with p2Ca (35). Since Drosophila cells lack peptide transporters (TAP-1 and TAP-2 (36)), after transfection with MHC class I heavy chain and β₂-microglobulin, large amounts of class I molecules reach the cell surface, but are not stably associated with peptide (37). The class I molecules can then be loaded at high concentration with exogenous QL9 peptide before experimentation, thus allowing for maximal Ag presentation to the Tg⁺ cells of young vs aged 2C mice.

Since a significant portion of the Tg⁺ (i.e., 1B2⁺) cells in the spleens of young and old 2C mice is also CD8⁻, we enriched the CD8⁻ cells by negative selection with magnetic beads followed by FACS sorting CD8⁻ cells. Essentially 92–99% of the recovered CD8⁻ cells expressed the 1B2 cloneotype. Moreover, these cells displayed comparable levels of CD28 expression (data not shown). An equivalent number of 1B2⁺ CD8⁺ cells from young and aged 2C mice was cultured per well along with 10^−7 M QL9 peptide and Ld.B7.ICAM APCs. Activation, proliferation, cytokine production, and CTL activity were assessed.

Activation

As a measure of cell activation, we examined the expression of CD44, CD25 (IL-2Rα), and CD69 on Tg⁺ CD8⁺ cells 16 h after Ag stimulation. Up-regulation of both CD25 and CD69 is among

FIGURE 1. Transgene and CD8 expression on spleen cells of aged 2C mice. The expression of the 1B2 cloneotype, i.e., Tg⁺ cells, and CD8 on spleen cells isolated from a young (2-mo) and an old (17-mo) 2C transgenic mouse is shown in A. The percentage of CD8⁺ cells expressing 1B2 for each mouse is shown in B. The mean for each age group, designated by a dash, is 98.5% (young) and 94.6% (old).
The early events in T cell activation. As shown in Fig. 3, the expression of CD25 and CD69 increased on cells from young and aged 2C mice. Importantly, the levels of expression were similar on the cells from aged vs young mice. Although not markedly increased by 16 h, CD44 expression was up-regulated completely by 60 h, and similar levels were obtained in both age groups (see Fig. 2B). Thus, Tg<sup>+</sup> CD8<sup>+</sup> cells from aged 2C mice were activated as well as their young counterparts.

### Proliferation

Similar to the findings of cell activation, no age-related differences were evident in cell proliferation measured on day 3 after Ag stimulation (Fig. 4, A and B). Moreover, the kinetics of expansion was similar for both age groups, that optimal proliferation of CD8 cells from young and aged 2C mice occurred by day 3 (data not shown).

### Cytokine production

Culture supernatants, collected 24 h after Ag stimulation, were assayed for the presence of IL-2 and IFN-γ. Although IL-2 was detected, no age-related difference in the amount of IL-2 produced in culture was evident (Fig. 4, C and D). Very little IFN-γ (≤ 20 ng/ml) was detected in the culture supernatants of young and aged cells (detection limits of the assay ∼10 ng/ml; data not shown). The finding of IL-2 and little/no IFN-γ production is consistent with the naive status of the Tg<sup>+</sup> CD8<sup>+</sup> cells in young and aged 2C mice, in that memory CD8 cells secrete high levels of IFN-γ and naive CD8 cells secrete low detectable levels of IFN-γ (3, 34, 38).

### CTL function

CTLs obtained 4 days after stimulation with QL9 peptide and L<sup>d</sup>.B7.ICAM APCs were examined for cytolytic activity to peptide Ags. RMA-S cells, a TAP-2-deficient cell line, transfected with L<sup>d</sup> and loaded with p2Ca or p1A (control) peptide, were used as targets (22). As shown in Fig. 4, E and F, CTL activity in both young and old age groups was comparable. Although data obtained from young (2-mo) and old mice are shown throughout the study, no differences in CD8 cell responses were observed in 9- to 10-mo mice (data not shown). Thus, the absence of age-associated changes in CD8 responses in the 16- to 18-mo group is not likely due to selection of the most fit aged mice.

### Minimal requisites for activation, proliferation, and CTL generation

An extensive study of the minimal requisites for activation, proliferation, and CTL function revealed subtle differences in the responses by Tg<sup>+</sup> CD8<sup>+</sup> cells from young 2C mice that were dependent upon Ag dose and affinity (24, 25, 29). Although class I-transfected Drosophila cells were not immunogenic, Drosophila cells that were cotransfected with either B7-1 or ICAM-1 or both could elicit CD8 responses under a restricted set of culture conditions. Responses could be generated with high concentrations of low affinity peptide or very low concentrations of high affinity peptide if the APCs expressed high levels of both B7-1 and ICAM-1. Alternatively, if the APCs expressed either B7-1 or ICAM-1 alone, significantly higher concentrations of peptide possessing higher affinity were necessary to elicit responses; however, the full array of CD8 responses (i.e., activation, proliferation, cytokine production, and CTL function) were not necessarily elicited (25). Since no age-associated differences in T cell responses were observed with optimal stimulatory conditions, we determined whether age-associated differences in activation, proliferation, IL-2 production, and CTL activity become evident using less overt stimulatory conditions (i.e., p2Ca vs QL9 and L<sup>d</sup>.B7 vs L<sup>d</sup>.ICAM vs L<sup>d</sup>.B7.ICAM APCs). This may be of importance since suboptimal APC function might occur in the aged environment (17).

### Activation

As mentioned above, up-regulation of CD25 and CD69 occurs early after T cell activation. Therefore, we examined the expression of these determinants 16 h after stimulation with a lower affinity peptide or with APCs that varied in their costimulation molecule expression. In the presence of L<sup>d</sup>.B7.ICAM APCs, we found that similar to the findings with the high affinity peptide,
from individual experiments in which thymidine incorporation by Tg CD8+ cells was measured. The generation of primary CTL in vitro was determined. Purified CD8+ cells from 2-mo and 17-mo 2C mice were stimulated with QL9 (0.1 μM) and Ld.B7.ICAM APC (1.5 × 105). Shown in A are representative data from one experiment in which proliferation by varying numbers of Tg CD8+ cells was measured. B, Data from individual experiments in which thymidine incorporation by 5 × 10^6 sorted Tg CD8+ cells is shown. IL-2 production by purified CD8+ cells 24 h after stimulation with QL9 (0.1 μM) and Ld.B7.ICAM APC (1.5 × 10^5) was determined. Supernatants collected 24 h after the start of culture were tested for IL-2 using a bioassay described in Materials and Methods. Shown in C are representative data from one experiment in which IL-2 secretion by varying numbers of sorted Tg CD8+ cells was measured. D, Data from individual experiments in which IL-2 secretion by 5 × 10^6 sorted Tg CD8+ cells is shown. The generation of primary CTL in vitro was determined. Purified CD8+ (5 × 10^5) were incubated with QL9 (0.1 μM) and Ld.B7.ICAM APC (1.5 × 10^5). After 4 days, the cells were harvested, and CTL activity was tested against 51Cr-labeled RMA-S. Ld target cells loaded with QL9 (diamond) or control P1A (circle) peptide. Shown in E and F are data from two experiments.

Young CD8+ cells stimulated with Ld.B7.ICAM APC plus QL9 (0.1 μM) or p2Ca (10 μM), and B, Ld.B7.ICAM, Ld.B7, and LAICAM APC plus QL9 (0.1 μM). Ab isotype controls are shown with cells from the aged (dotted lines).

FIGURE 4. Responses of CD8+ cells from young and old 2C mice to peptide presented by Ld.B7.ICAM APC. Tg CD8+ cells from 2-mo (filled symbols and bars) vs 16- to 18-mo (open symbols and bars) 2C mice were assessed for proliferation (A and B), IL-2 production (C and D), and CTL generation (E and F). Proliferation of purified CD8+ cells from 2-mo and 17-mo 2C mice was determined in 3-day cultures with QL9 (0.1 μM) and Ld.B7.ICAM APC (1.5 × 10^5). Shown in A are representative data from one experiment in which proliferation by varying numbers of Tg CD8+ cells was measured. B, Data from individual experiments in which thymidine incorporation by 5 × 10^6 sorted Tg CD8+ cells is shown. IL-2 production by purified CD8+ cells 24 h after stimulation with QL9 (0.1 μM) and Ld.B7.ICAM APC (1.5 × 10^5) was determined. Supernatants collected 24 h after the start of culture were tested for IL-2 using a bioassay described in Materials and Methods. Shown in C are representative data from one experiment in which IL-2 secretion by varying numbers of sorted Tg CD8+ cells was measured. D, Data from individual experiments in which IL-2 secretion by 5 × 10^6 sorted Tg CD8+ cells is shown. The generation of primary CTL in vitro was determined. Purified CD8+ cells (5 × 10^5) were incubated with QL9 (0.1 μM) and Ld.B7.ICAM APC (1.5 × 10^5). After 4 days, the cells were harvested, and CTL activity was tested against 51Cr-labeled RMA-S. Ld target cells loaded with QL9 (diamond) or control P1A (circle) peptide. Shown in E and F are data from two experiments.

QL9 (10^-7 M), high concentrations of the lower affinity peptide, p2Ca (10^-5 M), induced comparable up-regulation of CD25 and CD69 on Tg CD8+ cells from aged and young 2C mice (Fig. 5A). Similarly, no age-related differences in CD25 up-regulation were observed upon stimulation with QL9 and APCs expressing either or both B7.1 and ICAM-1 (Fig. 5B), thus suggesting no intrinsic differences in the Ag-induced activation of naive Tg CD8+ cells of the aged. Evident in the cultures of young and aged cells (Fig. 5B) and as reported earlier in young Tg CD8+ cells of young 2C mice, Ld.B7.ICAM APCs induced a slightly higher-to-equivalent level of CD25 compared with Ld.B7, and Ld.ICAM APCs induced the lowest level of CD25.

Proliferation
Consistent with their ability to up-regulate CD25 expression, Ld.B7 vs Ld.ICAM vs Ld.B7.ICAM APCs induced proliferation in Tg CD8+ cells from young and aged mice in the same hierarchical fashion (i.e., Ld.B7.ICAM > Ld.B7 > Ld.ICAM) (Fig. 6). Ld.B7 as well as Ld.ICAM APCs were able to induce a proliferative response if higher concentrations of Ag were available. Moreover, high concentrations of the low affinity peptide could induce proliferation if presented on Ld.B7.ICAM APCs (Fig. 6B). No responses to p2Ca were observed with Ld.B7 or Ld.ICAM APCs (data not shown). Importantly, as shown in Fig. 6, no differences in proliferation were noted between the young and old under any of the culture conditions tested.

Cytokine production
Shown in Fig. 7 is a comparison of IL-2 production by naive Tg CD8+ cells stimulated with Ld.B7.ICAM APCs and optimal concentrations of peptides with varying affinities for TCR and MHC class I (10^-7 M QL9 vs 10^-5 M p2Ca). Significant levels of IL-2 were not detected with 10^-7 M QL9 peptide and Ld.B7 or Ld.ICAM APCs, nor with lower concentrations of p2Ca (data not shown). Although a hierarchy in responses similar to that found in activation and proliferation was observed (i.e., Ld.B7.ICAM > Ld.B7 or Ld.ICAM and QL9 > p2Ca), no age-associated differences in IL-2 production were revealed. This was not surprising
given that no age-related differences were observed in CD8 expansion (Fig. 6). In addition, IFN-γ was observed only in cultures with high concentrations of QL9 and p2Ca (data not shown). No age-related differences could be detected.

**CTL function**

Finally, we examined the cytolytic activity of CTLs that were generated from naive CD8 cells stimulated with 10^{-2} M QL9 in the presence of L^{d}.B7.ICAM or L^{d}.B7 APCs. L^{d}.ICAM APCs were not included in the study since they were shown not to generate CTL from young 2C mice without the addition of IL-2 (25). CTL activity was tested on 3H Tdr the last 16 h of culture, harvested, and counted. Shown are data from two representative experiments.

![Figure 6.](image)

**FIGURE 6.** Proliferative response of CD8^{+} cells from young and old 2C mice to peptide presented by transfected Drosophila cells. A, Influence of QL9 concentration on the day 3 response of CD8^{+} cells with different APCs. B, Influence of p2Ca concentration on the day 3 response of CD8^{+} cells with different APCs. Purified CD8^{+} cells (5 \times 10^5) from 2-mo (filled symbols) and 17-mo (open symbols) 2C mice were cultured for 3 days with peptide and 1.5 \times 10^6 L^{d}.B7.ICAM APCs (diamond) or L^{d}.B7 APCs (triangle) or L^{d}.ICAM APCs (circle). The microcultures were pulsed with [3H]Tdr the last 16 h of culture, harvested, and counted. Shown are data from two representative experiments.

shown in Fig. 8. As expected, both L^{d}.B7.ICAM and L^{d}.B7 APCs were highly efficient in generating CTL, and no differences in the cytolytic responses by the CTL generated from naive Tg^{+} CD8 cells of young vs aged donors were observed.

**Late response cytokine production**

Several studies have shown alterations in cytokine production by T cells of the aged that are manifested later in the response (31, 39, 40). Among those cytokines shown to be elevated in stimulated CD8 cells from the aged are IFN-γ and intracytoplasmic TNF-α (40). Therefore, we determined whether these changes were also present in effectors generated 4 and 5 days in culture after stimulation of naive Tg^{+} CD8 cells. On days 4 and 5, similar numbers of CD8 effectors were recovered from cultures of cells from young or aged 2C mice. No age-related differences were observed in the levels of IFN-γ and IL-2 from supernatants collected on day 4 or 5 (p > 0.05; only day 4 data are shown in Fig. 9A). Upon restimulation of 4-day effectors with QL-9 peptide and L^{d}.B7.ICAM APCs, both IL-2 and IFN-γ production were elevated, but only IL-2 production was significantly diminished in the cultures of aged cells (p < 0.02) (Fig. 9A).

Previous studies demonstrated an age-associated increase in intracytoplasmic TNF-α expression by anti-CD3 plus anti-CD28-stimulated T cells that were polyclonally restimulated in the presence of monensin or brefeldin (40). We repeated these studies using FACS-purified CD8 cells that were stimulated with APC and Ag on day 0 and restimulated on day 4 with a second challenge of Ag in the presence of brefeldin. As shown in Fig. 9B, TNF-α expression was slightly lower in the aged (44% young; 34% aged). It should be noted that TNF-α was not detected by ELISA in the supernatants of 4- or 5-day cultures or in the supernatant of re-stimulated day 4 effector cell cultures (the detection limit of the assay was 40 pg/ml; data not shown.). Although our finding of slightly diminished TNF-α expression is contrary to the findings of others, diminished TNF-α expression in the aged has been observed in caloric restricted animals that possess improved T cell function (41). In any case, the finding of diminished IL-2 and possibly TNF-α upon Ag restimulation suggests that age-related differences intrinsic to CD8 cells may become apparent later in the response upon Ag reexposure. This may be indicative of an increased rate of maturation to the effector cell stage; however, more thorough studies are necessary to determine whether effector cell function is dramatically altered.
Discussion

Using aged 2C transgenic mice, we have presented data supporting the hypothesis that the age-associated shift of the T cell population to a more memory phenotype is Ag driven and does not happen if Ag is unavailable. More importantly, we have shown that although CD8 cells display a reduced response in vivo with aging, the CD8 cells that retain the naive phenotype with aging also retain their functional capacity, as measured by activation, proliferation, cytokine production, and CTL generation. These findings suggest that the decline in CD8 responses of the aged is not likely the consequence of alterations intrinsic to naive CD8 T cells.

Unlike conventional aged animals (3, 11), the Tg⁺ CD8 cells from aged 2C mice retain a naive phenotype. This finding is similar to that with aged AND TCR transgenic mice in which the Tg⁺ CD4 cells also remained naive. Also similar to the AND model, the Tg⁺ CD8 cells of 2C mice recognize a determinant that is not likely to be encountered in the environment without intentional immunization. The 1B2⁺, Tg⁺, CD8 cells of 2C mice recognize allo-Ld molecules linked to a self peptide derived from the Krebs cycle. The finding that the Tg⁺ CD8 T cells of aged 2C mice retain their naive status is consistent with the hypothesis that the acquisition of the memory phenotype with aging is Ag driven (26, 42). The retention of naive status by the Tg⁺ CD8 T cells in aged 2C mice was shown by phenotypic and functional parameters. The Tg⁺ CD8 cells not only expressed determinants typical of naive cells, i.e., CD44lowCD11a lowCD25 negCD69 neg, but upon Ag stimulation secreted a cytokine profile typical of naive cells, i.e., IL-2 secretion and little/no detectable IFN-γ (3, 33, 34, 38, 43, 44).

The mechanism underlying the age-associated decline in CD8 T cell responsiveness remains unclear. Several studies of aged rodents and humans have shown that cytotoxic T cells exhibit decreased responses to mitogens and Ags, decreased cell division, and increased IFN-γ production (3, 10–12, 39, 40). These functional changes may be the consequence of alterations intrinsic to the T cells of the aged, the switch in T cell subset representation, and/or a manifestation of extrinsic factors that are altered with aging and affect CD8 cell function. Although some or all of these factors may play some role in reduced CD8 responses, it is certain that the decreased frequency of relevant Ag-specific naive cells that emigrate from the thymus of the aged contributes to the overall decline in T cell responses (27, 45).

In this study, we have addressed whether naive CD8 T cells of the aged function differently from their young cohorts when stimulated with the same conditions of optimal Ag presentation. We detected no differences in the level of responses, as measured by activation, proliferation, IL-2 production, and CTL activity. QL9 peptide presented on APCs expressing both B7 and ICAM induced comparable responses in naive CD8 cells isolated from either aged or young 2C mice. These findings were obtained regardless of the kinetics or peptide concentration, suggesting no age-associated differences in the ability or capacity of the cells from the aged to respond. If submaximal stimulation was used, either in the form of partial costimulation/adhesion molecule expression, or in the presentation of lower affinity or diminished concentrations of peptide, the extent of CD8 responsiveness (i.e., activation through CTL differentiation and function) and the level of CD8 response remained comparable for both age groups. These findings suggest that suboptimal Ag presentation would lead to similar, albeit decreased, responses by naive CD8 cells from either age group. Thus, based on our findings, we would predict that the observed decline in CD8 function in aged conventional animals and humans is not likely the consequence of alterations intrinsic to the naive CD8 cells.

Dysfunction in cytokine production by T cells of the aged has been shown to manifest itself late in the ensuing T cell response. In this study, we found no significant age-related differences in the number of effector cells or in the amount of IL-2 or IFN-γ present in cultures generated 4 or 5 days after Ag stimulation. However, an age-associated decline was evident in IL-2 secretion and to a lesser extent in TNF-α expression upon Ag restimulation of effector cells. The decline in IL-2 production in the aged may lead to diminished effector cell expansion and possibly a reduction in the frequency of Ag-specific memory cells that are generated.

The nature of the impact of an aged environment on naive CD8 responses remains to be elucidated. There is some evidence for age-associated changes at the level of the accessory cell. In mice, the precursor frequency of memory cytotoxic T cells that respond to influenza is entirely dependent upon the age of the APC donor (17). Responses to trypanosome (18) and pneumococcal Ags (19) are also compromised in aged mice due to suboptimal accessory cell function. Alternatively, or in conjunction with alterations in APC function, is the possibility that the switch in cytokine milieu with aging may affect the generation of cell-mediated immune responses (16). Cytokines present during Ag stimulation are known to influence the type of effector cells generated. Consequently, the type 2 cytokine profiles that dominate in the elderly (15) may impact on the ensuing T cell responses by inhibiting the production of type 1 cytokines, such as IFN-γ, thereby resulting in the inhibition of cell-mediated immune responses and CD8 responsiveness (16, 46). Finally, the age-associated decline in CD4 T cell function may further affect the reported decline in responses by CD8 cells of the aged.

Contrary to the current findings on naive CD8 cells, Linton et al. (26) have earlier reported an age-associated intrinsic defect in naive CD4 cell function, in which IL-2 production by Ag-stimulated naive CD4 T cells was dramatically reduced in the aged. Why are the two naive populations so different in their functional capacity? It is possible that although thymic output of CD4 and CD8 T cells declines with aging, newly emigrated naive CD8 cells are fully
functional, whereas CD4 cells are lacking in their ability to secrete significant levels of IL-2. Alternatively, differences in naive CD4 vs CD8 cell turnover/longevity in the periphery of the aged may lead to differences in their functional capacities (47, 48). Naive CD8 T cells in young and aged mice turn over similarly, whereas naive CD4 T cells of aged mice turn over at a greater rate compared with their young counterparts.

Although some studies of memory phenotype CD8 cells have reported decreased proliferative capacity (10, 11) and others have found normal levels of cytokine production (3, 39), no studies have demonstrated a decline in responsiveness using purified naive CD8 cells. Since memory phenotype CD8 cells accumulate with age (11), it is conceivable that some of the functional changes that are observed in CD8 responses can be attributed to the increased representation of this subset. Indeed, only memory phenotype CD8 cells have been shown to secrete high levels of IFN-γ, and the increase in frequency of these cells accounts for the reported increase in IFN-γ levels upon TCR stimulation in the aged (3). Some controversy still exists regarding the functional ability of the memory phenotype CD8 cells since the observed reduced proliferation may be the consequence of increased IFN-γ inhibiting IL-2 production and CD8 proliferation and/or a deficiency intrinsic to aged memory CD8 cells. Regardless, it is clear from this study that the naive CD8 cells from aged mice display comparable function to their young naive counterparts.

References