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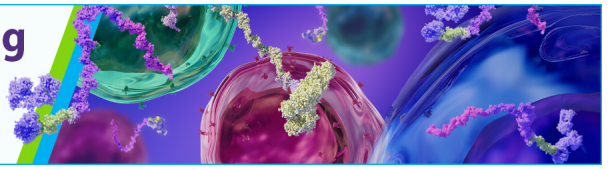

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Postthymic Development of CD28$^-$CD8$^+$ T Cell Subset: Age-Associated Expansion and Shift from Memory to Naive Phenotype[1]

Marcelo M. Nociari,* William Telford,† and Carlo Russo[2]*

During human aging, one of the major changes in the T cell repertoire is a dramatic expansion of T cells with the atypical CD28$^-$CD8$^+$ phenotype. In this study, we show that this increase is a consequence not only of an expansion in the CD28$^-$CD8$^+$ population but also of a decrease in the number of CD28$^+$CD8$^+$ T cells. The decrease in circulating CD28$^+$CD8$^+$ T cells is dramatically accelerated after the age of 50 and is not accompanied by an equivalent reduction in the CD28$^+$CD4$^+$ subset. Our findings confirm that aging leads to an accumulation of CD45RO$^+$ T cells within the CD28$^+$CD8$^+$ subset as previously observed. Surprisingly, we found an increase in CD45RA$^+$ expression with age in the CD28$^-$CD8$^+$ subset. Immune-phenotyping for activation markers, measurement of telomere DNA content, and cytokine production analysis indicate that the large majority of CD28$^-$CD8$^+$ T cells are Ag-experienced, despite their CD45RA$^+$ phenotype. Our study further demonstrates that the poor proliferative response displayed by CD28$^-$CD8$^+$ T cells is not a consequence of telomere shortening. Also, analysis of cytokine production at the single cell level revealed that the proportions of IFN-γ^+, IL-4$^+$, and IL-10$^+$ T cells are considerably higher among the CD28$^-$CD8$^+$ than the CD28$^+$CD8$^+$ subset. In summary, these data explain the presence of CD45RA$^+$ T cells in the elderly, shed light on the phylogenetic origin of CD28$^-$CD8$^+$ T cells, and suggest a role for these cells in the immune senescence process. ***The Journal of Immunology,*** **1999, 162: 3327–3335.**

The increased susceptibility to infectious, neoplastic, and degenerative diseases observed in the elderly is, in part, the consequence of an impaired ability to mount effective humoral and cellular immune responses against new Ags (1, 2). A decrease in both T and B cell-mediated protection seems to be driven by age-related changes in the T lymphocyte population (3). Indeed, T cells in the aged show decreased ability to promote B cell activation and differentiation (4), decreased proliferative response to mitogens and Ags (5), and decreased ability to generate allospecific CTLs (6).

T cell immune senescence has traditionally been associated with thymic involution, since a striking decline in the output of newly thymus-derived T cells occurs with age (7). Nonetheless, the number of T lymphocytes in circulation remains relatively constant throughout life (8), and a significant number of T cells with unprimed-naive (CD45RA$^+$) phenotype are readily detectable in the aged (9). Indeed, it was recently shown that the percentage of CD45RA$^+$ T cells reaches about 50% of the total T lymphocyte population in centenarians, a value only slightly lower than in young donors (10). Furthermore, it was noticed that CD45RA$^+$ T cells in these individuals were unequally distributed, being more common in the CD8$^+$ than the CD4$^+$ subset (11). Since a lifespan of several decades is highly improbable for most T cells, the origin and continuous renewal of T cells in the elderly remains unexplained.

One intriguing change observed in the T cell pool with aging is the marked increase in the proportion of CD8$^+$ lymphocytes lacking expression of CD28 Ag (12). CD28 is a major costimulatory molecule required for functional T cell activation (13). In a prior study (14), we have found that clonal expansions of CD28$^-$CD8$^+$ T cell occurs in virtually all healthy elderly subjects. These clonally expanded T cells can persist in humans for years (15). Elevated numbers of CD28$^-$CD8$^+$ T cells in blood have been also associated with numerous immunocompromised conditions, such as systemic lupus erythematosus (16), rheumatoid arthritis (17), Chagas disease (18), allograft transplants (19), and HIV infection (20). However, the role played by CD28$^-$CD8$^+$ T cells in disease progression remains to be determined. Previous studies have suggested a suppressor role for CD28$^-$CD8$^+$ T cells on B and T cell function (21–23). Moreover, these atypical T cells exhibit unique cytotoxic properties. CD28$^-$CD8$^+$ T cells can exert direct lysis of anti-CD3-coated P815 target cells (12, 24) and may mediate HLA-unrestricted cytolysis (25). The nature of the target they recognize in the elderly is unknown. In contrast to their in vivo predominance, CD28$^-$CD8$^+$ T cells do not proliferate readily in vitro, regardless of the stimulus used (22, 24). CD28$^-$CD8$^+$ T cells frequently are CD11b$^+$, and to a lesser extent CD57$^+$, two molecules associated with differentiated cytotoxic and suppressor T cells (24). Coexpression of some activation Ags like CD38 and HLA-DR has been described in CD28$^-$CD8$^+$ recovered from HIV$^+$ patients (26). CD28$^-$CD8$^+$ T lymphocytes are very infrequent in cord blood and are uncommon in thymus and lymph nodes but are widely distributed in the gut and lung mucosal tissues (27–30). It is still unclear whether they arise from the CD28$^+$ subset or whether they belong to a separate T cell lineage of extrathymic origin. CD28$^-$CD8$^+$ cells have been shown to have shorter telomeres than CD28$^+$CD8$^+$ cells (31, 32). It has therefore

*Laboratory of Immunology, Divisions of Geriatrics and Gerontology and International Medicine and Infectious Diseases, Cornell University Medical College, New York, NY 10021; and †Section of Immunology and Inflammation, Hospital for Special Surgery, New York, NY 10021

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[2] Address correspondence and reprint requests to Dr. Carlo Russo, Merck Research Laboratories, P.O. Box 4, BLA 34, West Point, PA 19486. E-mail address: carlo_russo@merck.com

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been proposed that CD28$^-$CD8$^+$ T cells represent the replicative senescent progeny of CD28$^+$CD8$^+$ T cells. If that is the case, progression of age-associated loss of immune function may be related to accumulation of the CD28$^-$CD8$^+$ subset and exhaustion of the regenerative capacity of the CD8$^+$ population (33–35).

Therefore, postthymic or extrathymic developmental models are needed to explain the maintenance of the peripheral pool size in the face of reduced thymic output and to understand the senescent changes occurring in T cell population in adults. In the present study, we investigate the phylogenetic origin of the CD28$^-$CD8$^+$ and CD45RA$^+$ T cells found in the elderly. Overall, the results support the hypothesis that CD28$^-$CD8$^+$ lymphocytes derive from CD28$^+$CD8$^+$CD45RA$^-$ precursors, which become CD28$^-$CD8$^+$ CD45RA$^-$ first and CD28$^-$CD8$^+$CD45RA$^+$ later. In addition, they suggest that the presence and persistence of CD45RA$^+$ T cells in the elderly are due to an extensive CD45RO to CD45RA reversion process. Our data also reveals that CD28$^-$CD8$^+$ T cells are highly differentiated T cells rather than replicative exhausted lymphocytes. Finally, we present evidence that, based on their cytokine profile, CD28$^-$CD8$^+$ may play an important role in the immune senescence process.

Materials and Methods

Abs and reagents

Phycoerythrin (PE)[3]-goat anti-mouse Ig and RED-613-streptavidin were obtained from Life Technologies (Grand Island, NY). PE-anti-CD28, anti-CD28, anti-CD4, anti-HLA-DR, FITC-anti-TCR$\alpha\beta$, and PE-anti-IL-2 were purchased from Becton Dickinson (San Jose, CA). Anti-CD8, PE-anti-CD4, and anti-CD11b were obtained from Coulter (Miami, FL). FITC-anti-CD45RO was obtained from AMAC (Westbrook, ME). Biotin-anti-CD3, anti-CD8β, and anti-CD25 were obtained from Immunotech (Westbrook, ME). Conjugation of purified anti-CD3 with the fluorochrome Cy5 was performed using a kit from Amersham (Arlington Heights, IL). All other surface marker and cytokine Abs were obtained from PharMingen (San Diego, CA). Complete medium consisting of RPMI 1640 supplemented with 10% FBS (Gemini Biological Products, Calabasas, CA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies) was used as culture medium for T cells. PMA and ionomycin were obtained from Calbiochem (San Diego, CA). Monensin was obtained from Sigma (St. Louis, MO). Human rIL-2, rIL-4, and rIFN-γ were obtained from Life Technologies. FACS permeabilizing solution (10×) was obtained from Becton Dickinson.

Isolation of PBMC

Heparinized peripheral blood was obtained from healthy donors, 18-87 yr old. Mononuclear cells were isolated by centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ) and further depleted of adherent cells by incubation in plastic plates for 90 min at 37°C.

Magnetic purification of CD8$^+$ T lymphocytes

Nonadherent cells were incubated with anti-CD8 mAb for 30 min at 4°C, washed twice, and incubated with goat anti-mouse-conjugated immunomagnetic beads (Dynal, Lake Success, NY) at a ratio of 20 beads/cell for 30 min. CD8$^+$ cells were then separated by positive selection with a magnet and incubated overnight at 37°C to release the beads. For some experiments, cells were additionally purified by subsequent removal of CD4$^+$ cells via magnetic negative selection. The purity of CD8$^+$ T cells was routinely 95–97%. TCR$\alpha\beta$ expression was >98% within the CD3$^+$ subset as measured by flow cytometry.

FACS

Before sorting, nonadherent PBMCs were incubated for 1 h on ice with 2-aminoethylisothiouronium bromide (AET)-treated SRBC (Hazleton-Dutchland, Denver, PA) prepared as previously described (36). T cells (rosetted cells) were separated from non-T cells by Ficoll-Hypaque density gradient centrifugation, and washed with SRBC lysis buffer. The resulting T cells were then labeled for four-color flow analysis and sorting with FITC-anti-CD45RA, PE-anti-CD28, APC-anti-CD8α, biotin-anti-CD3, and Red-613-streptavidin. Cell sorting was performed using a FACSVantage cell sorter (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with argon ion and helium neon lasers emitting spatially separated beams at 488 nm (for FITC, PE, and Red-613 excitation) and 632 nm (for APC excitation). FITC, PE, and Red-613 signals were separated using 610-nm and 560-nm short pass reflecting dichroics, and collected through 535/30-, 575/20-, and 610/20-nm narrow bandpass filters, respectively. APC signals were collected through a 660/20-nm narrow bandpass filter. CD28$^+$CD45RA^{high}, CD28$^+$CD45RA$^-$, CD28$^-$CD45RA^{high}, and CD28$^-$CD45RA$^-$ CD8$^+$CD3$^+$ T cell subsets were gated and sorted using a FACSVantage MacroSort sort module (Becton Dickinson). All sorted cell populations exhibited >95% purity as evidenced by back-analysis of sorted fractions.

Three- and four-color flow cytometric analysis

Three-color flow cytometry was performed by incubating PBMCs or purified CD8$^+$ cells with three directly conjugated mAbs (FITC-, PE-, and Cy5 conjugated) for 30 min at 4°C. Cells were fixed in 2% paraformaldehyde and analyzed using a Coulter EPICS XL flow cytometry equipped with a single argon ion laser emitting at 488 nm. Absolute subset cell numbers were determined by multiplying the total cell count by the percentage of cells exhibiting the indicated phenotype. For four-color flow cytometry, purified CD8$^+$ cells were labeled with an unconjugated mAb (as indicated) followed by PE-goat anti-mouse, FITC-anti-CD45RA, Cy5-anti-CD3, biotin-anti-CD28, and Red-613-streptavidin. Cells were then washed and fixed as described above. Acquisition was performed using a FACSCalibur flow cytometer (Becton Dickinson) equipped with an argon ion laser emitting at 488 nm (for FITC, PE, and Red-613 excitation) and a spatially separated diode laser emitting at 631 nm (for Cy5 excitation). For each sample, 20,000 events were acquired and analyzed using CellQuest software (Becton Dickinson).

Detection of cytokine production at single cell level

Flow cytometric measurement of cytokine production was performed as previously described (37, 38) with some modifications. Briefly, 10^6 CD8$^+$ cells were stimulated for 5 h with 10 ng/ml PMA and 1 μM ionomycin in the presence of 1 μM monensin. This short-term incubation did not affect membrane expression of CD28 and CD45RA molecules as confirmed by flow cytometry (data not shown). Cells were then labeled with FITC-anti-CD45RA, Cy5-anti-CD3, biotin-anti-CD28, and Red-613-streptavidin. Cells were then permeabilized and fixed with FACS permeabilizing solution for 10 min at room temperature. Permeabilized cells were subsequently incubated with a blocking solution followed by labeling with PE-anti-cytokine mAb (0.2 μg/10^6 cells) for an additional 30 min. Recombinant cytokine-blocking controls using a 100-fold molar excess of the relevant cytokine added 2 h before labeling were performed in parallel to differentiate specific labeling from background. Stained cells were then fixed with paraformaldehyde and analyzed on a FACSCalibur flow cytometer as described above.

Telomere DNA content

Telomere DNA content was determined as previously described (39) with minor modifications. Genomic DNA was isolated from 3–5 × 10^5 cell sorter-purified T cells using the Wizard Genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions. DNA was diluted and denatured in 0.5 M NaOH, 1.5 M NaCl buffer at 55°C for 30 min. DNA samples were then vacuum blotted onto ZetaProbe nylon membranes (BioRad Laboratories, Hercules, CA) and UV-cross-linked. The telomere-specific oligonucleotide $[TTAGGG]_4$ and centromere specific oligonucleotides [GTTTTGAAACACTCTTTTTGTAGAATCTGC] were end-labeled with 100 μCi [γ-^{32}P]ATP (>5000 Ci/mmol; Amersham) with polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN). Duplicated membranes were then hybridized with either the telomere or centromere for 24 h at 56°C. Membranes were then washed, air-dried, and exposed to a storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA) for 16 h. The density of the dots was determined with a PhosphorImager (Molecular Dynamics) and quantified using the volume-integration function of the ImageQuant software (Molecular Dynamics). Telomere DNA content of each population was expressed as the ratio between telomere and centromere signals, as a percentage of the control placental DNA measurement.

Proliferation assays

T cell proliferation was induced with anti-CD3 mAb immobilized on 96-well flat-bottom plates. Freshly and highly purified CD8$^+$ T cell subsets were added in triplicate wells at 5 × 10^4 cells/well and cultured for 4 days

[3] Abbreviations used in this paper: PE, phycoerythrin; L, ligand; T:C, telomere:centromere ratio; AET, 2-aminoethylisothiouronium bromide.

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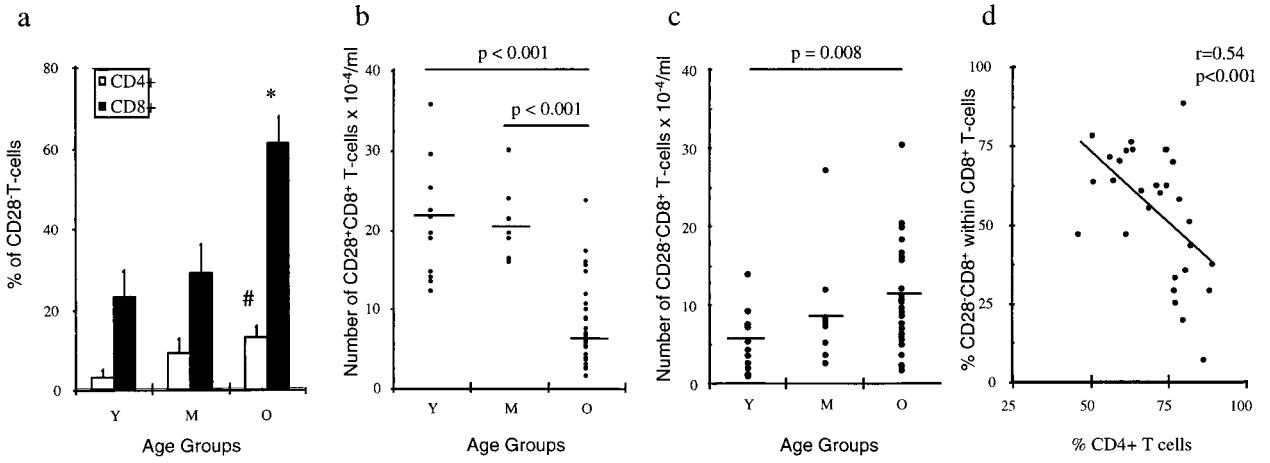

FIGURE 1. Changes in the proportion of $CD28^-$ T cells within the $CD8^+CD3^+$ and $CD4^+CD3^+$ subsets with aging. PBMC from 51 healthy donors (11 younger than 25, 10 between 37 and 50, and 30 older than 66 years of age) were three-color stained using FITC anti-CD28, PE anti-CD8, or PE anti-CD4, and Cy5 anti-CD3. Appropriated fluorochrome-conjugated control-Ig were used to set the position of the quadrant markers. *a*, Median percentages (±95% confidence interval) of $CD28^-$ cells within the $CD4^+$ (□) and $CD8^+$ T cell subsets (■) are represented for each age group. All values were compared by Mann-Whitney *U* test. * indicates significant differences between the old and the two youngest groups ($p < 0.001$). # indicates significant difference between the old and the youngest group ($p = 0.008$). *b* and *c*, Absolute cell numbers of $CD28^+CD8^+$ and $CD28^-CD8^+$ T lymphocytes among the different age groups, respectively. Absolute cell numbers were calculated by multiplying the total lymphocyte count by the percent of lymphocytes exhibiting the indicated phenotypes. Median and individual values are displayed correspondingly. *d*, Correlation between the percentage of $CD28^-CD8^+$ T cells in the $CD8^+CD3^+$ population and the proportion of $CD4^+$ T cells in the blood of elderly volunteers. The regression line with *r* value is indicated. The *p* value was calculated by linear regression analysis.

in complete culture medium RPMI 1640 in the presence and absence of 40 U/ml of rIL-2. Cultures were pulsed for the final 24 h of culture with 1 μCi/well [^{3}H]TdR (Amersham) and subsequently analyzed for incorporation with a β scintillation counter.

Statistical analysis

Nonparametric analyses were performed on most data, since most data did not fit a Gaussian distribution. Data are therefore represented by the median value and confidence interval unless otherwise indicated. The statistical significance of differences was assessed using Mann-Whitney *U* tests (40).

Results

Changes in the $CD8^+$ T cell peripheral pool during aging

The frequency of $CD28^-CD8^+$ T cells in 51 healthy individuals was measured by three-color immunofluorescence analysis of PBMC. The subjects were arbitrarily divided into young (mean 22 years of age, range 18–25), middle-aged (mean 43.9, range 37–50), and old (mean 75.4, range 66–89). The proportion of $CD28^-CD8^+$ T cells within the $CD8^+$ T cell population increased with age (Fig. 1*a*). $CD28^-CD8^+$ T cells represented 23% (±6.5), 29% (±7.0) and 61% (±6.1) of total $CD8^+$ T cells within the young, middle-aged, and old groups, respectively. The kinetics of $CD28^-CD8^+$ T cell increase was not progressive; instead, the increase occurred suddenly and relatively late in life.

Since the percentage of $CD28^-CD8^+$ T cells does not reflect the actual number of cells and is dependent upon the proportion of other T cell subsets, we investigated whether the age-associated increase in the percentage was due to an increase in the absolute number of $CD28^-CD8^+$ T cells or to a decrease in the number of $CD28^+CD8^+$ T cells. As shown in Fig. 1, *b* and *c*, $CD28^+CD8^+$ cell number/ml did not differ significantly between the young and middle-aged groups, but were drastically decreased in the old group ($p < 0.001$). Conversely, the number of $CD28^-CD8^+$ T cells/ml significantly increased with age ($p = 0.008$). Thus, the increase in the percentage of $CD28^-CD8^+$ T cells in the elderly was due to the disappearance of the $CD28^+CD8^+$ population and the concomitant emergence of a $CD28^-CD8^+$ subpopulation.

Changes in the $CD4^+$ T cell peripheral pool during aging

The frequency of $CD28^-$ T cells within the $CD4^+$ T cell pool also increased with age (Fig. 1*a*). However, this augmentation was less pronounced than that observed in the $CD8^+$ population. $CD28^-CD4^+$ T cells represented 3% (±2.0), 7% (±3.7), and 10% (±2.7) of total $CD4^+$ T cells within the young, middle-aged, and old groups, respectively. Absolute numbers of circulating $CD28^+CD4^+$ did not undergo an equivalent age-related reduction as was observed in the $CD28^+CD8^+$ subset. In fact, the $CD28^+CD4^+$:$CD28^+CD8^+$ T cell ratio increased significantly with age, although the absolute number of $CD28^+CD4^+$ T cells did not rise (Table I). On the other hand, increases in the absolute number of peripheral $CD28^-CD4^+$ T cells paralleled the increases in the number of $CD28^-CD8^+$ T cells as suggested by the constancy in the $CD28^-CD4^+$:$CD28^-CD8^+$ T cell ratio among the three age groups (Table I). These findings suggest that the age-associated increase in the percentage of $CD28^-$ within the $CD4^+$ population was mainly due to the increase in the number of peripheral $CD28^-CD4^+$ T cells.

Table I. *T cell ratios in different age groups*[a]

T Cell Ratios	Age Groups		
	<25 (n = 11)	37–47 (n = 10)	>66 (n = 30)
$CD28^+CD4^+$:$CD28^+CD8^+$	1.8 ± 0.3	2.4[b] ± 0.6	6.5[c] ± 1.7
$CD28^-CD4^+$:$CD28^-CD8^+$	0.46 ± 0.4	0.66 ± 0.2	0.43 ± 0.5

[a] Mean ± SD.
[b] Significant differences ($p < 0.03$) compared to the youngest group.
[c] Significant differences ($p < 0.003$) compared to the younger groups (Wilcoxon rank test).

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Relationship between the presence of CD28$^-$CD8$^+$ T cells and the proportion of CD4$^+$ T cells in circulation

It has been recently shown that in asymptomatic HIV$^+$ patients, there is a homeostatic mechanism by which variations in the percentage of CD4$^+$ T cells inversely correlate with variations of CD28$^-$CD8$^+$ T cells in blood (41). We therefore analyzed the proportions of CD4$^+$ and CD28$^-$CD8$^+$ T cells in PBMCs from 32 healthy volunteers. An apparently inverse correlation between the percentage of CD28$^-$CD8$^+$ T cells within the circulating CD8$^+$CD3$^+$ population and the percentage of CD4$^+$ T cells existed in some, but not other individuals (Fig. 1*d*). Although regression analysis did not reveal a linear relationship between the two subsets, this result may suggest that changes in the number of CD4$^+$ and CD28$^-$CD8$^+$ T cells in the elderly are not random events but instead are mutually regulated.

Expression of CD45RA and CD45RO molecules on CD28$^+$CD8$^+$ and CD28$^-$CD8$^+$ T cell subsets

To clarify the process that leads to the partial replacement of the CD28$^+$CD8$^+$ by CD28$^-$CD8$^+$ T cells, we studied the maturational stage of these subsets via expression of CD45RA and CD45RO molecules. More than 95% purified CD8$^+$ lymphocyte populations from 10 young donors (mean 21.9 years of age, range 18–25) and 20 old donors (mean 75.5 years of age, range 68–89) were analyzed for CD3, CD28, and CD45RA or CD45RO expression by three-color flow cytometry. In young subjects, the percentages of CD45RA$^+$ and CD45RO$^+$ T cell subsets did not differ significantly within the CD28$^+$CD8$^+$ and CD28$^-$CD8$^+$ T cell subpopulations (Fig. 2*a*), in accordance with previous reports (24). In contrast, the CD28$^-$CD8$^+$ T cell subset from elderly individuals contained a high percentage of cells with CD45RA$^+$ phenotype, while the CD28$^+$CD8$^+$ subset was rich in T cells with CD45RO$^+$ phenotype (Fig. 2*b*). This dichotomy in CD45 expression was reflected in the CD45RA:CD45RO T cell ratios (Fig. 2*c*).

The exclusive expression of CD45 isoforms on CD28$^+$CD8$^+$ and CD28$^-$CD8$^+$ T cells during aging was confirmed by four-color flow cytometry. Purified CD8$^+$ cells were simultaneously stained with fluorochrome-labeled Abs against CD45RA, CD45RO, CD28, and anti-CD3. Representative results from a young and old individual are shown in Fig. 3, *a-d*. These results confirmed the results obtained by three-color analysis, showing that CD28$^+$CD8$^+$ and CD28$^-$CD8$^+$ T cells contained significant CD45RA$^+$ and CD45RO$^+$ subpopulations, respectively, in young individuals (Fig. 3, *a* and *b*). Conversely, old individuals showed few CD28$^+$CD8$^+$ T cells with a CD45RA phenotype and a dramatic increase in CD28$^-$CD8$^+$CD45RA$^+$ cells (Fig. 4, *c* and *d*). Four-color analysis also demonstrated that there was minimal CD45RA and CD45RO coexpression. Thus, CD28$^-$CD8$^+$CD45RA$^+$ cells showed no CD45RO expression (Fig. 3*d*).

Telomere DNA content among the four CD8$^+$ T cell subsets defined by CD45RA and CD28 expression

We then attempted to determine whether this RO to RA phenotypic change within the CD28$^-$CD8$^+$ subset was due to an age-related differentiation process or to the de novo emergence of a nonphylogenetically related CD28$^-$CD45RA$^+$ population. Telomere DNA content is a powerful tool used to assess the amount of cellular divisions undergone by a population (42, 43). We used this procedure to estimate the phylogenetic relationship among the CD8$^+$ T cell subsets. For this purpose, CD28$^+$CD45RA$^+$, CD28$^+$CD45RA$^-$, CD28$^-$CD45RA$^+$, and CD28$^-$CD45RA$^-$ CD8$^+$ T cells were isolated from the peripheral blood of seven elderly volunteers 66 years of age or older using four-color cell

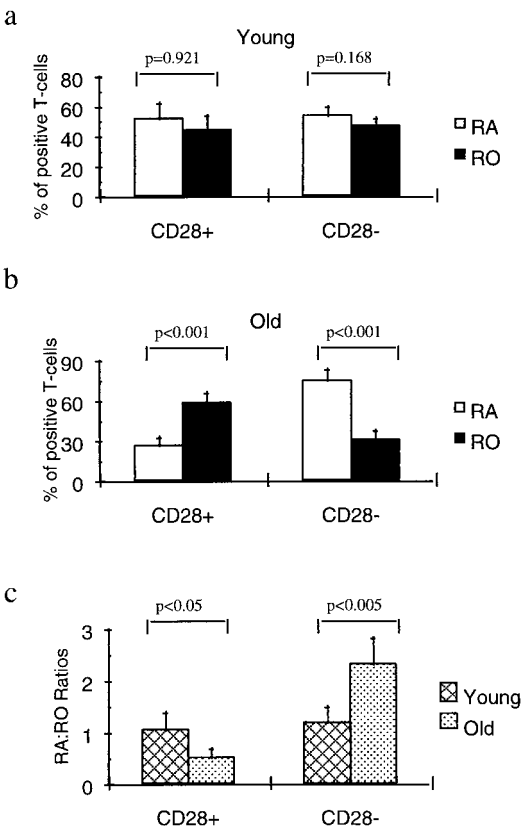

FIGURE 2. Expression of CD45RA and CD45RO Ags within the CD28$^+$CD8$^+$ and CD28$^-$CD8$^+$ T cell subsets. CD8$^+$ purified cells from 10 individuals younger than 25 yr, and 20 older than 67 were three-color analyzed using FITC anti-CD45RA or FITC anti-CD45RO, PE anti-CD28, and CyChrome anti-CD3 Abs. *a*, Proportions of CD45RA and CD45RO cells within the CD28$^+$CD8$^+$ and CD28$^-$CD8$^+$ T cell subsets in young individuals. *b*, Proportions of CD45RA and CD45RO cells within the CD28$^+$CD8$^+$ and CD28$^-$CD8$^+$ T cell subsets in old individuals. *c*, Changes in the CD45RA:CD45RO ratio among the CD28$^+$CD8$^+$ and CD28$^-$CD8$^+$ T cell subset with age. For each subset, the medians (±95% confidence intervals) of percent of positive cells were represented. Statistical differences were established using the Mann-Whitney *U* test.

sorting as described in *Materials and Methods*. Telomere DNA content was analyzed by determining the telomere:centromere ratio (T:C) as previously described (39). Results are shown in Fig. 4*a*. The means of the T:C ratios for the CD28$^+$CD45RA$^+$, CD28$^+$CD45RA$^-$, CD28$^-$CD45RA$^-$, and CD28$^-$CD45RA$^+$ T cell subsets were 1.60 (±0.24), 1.06 (±0.31), 1.32 (±0.36), and 1.16 (±0.18), respectively. We found that for all seven donors, the CD28$^+$CD45RA$^+$ subset had more telomere DNA than CD28$^+$CD45RA$^-$ T cells, consistent with the idea that, upon proper Ag presentation, CD28$^+$CD45RA$^+$ (unprimed-naive) T cells become activated, divide, and transform into CD28$^+$CD45RA$^-$ (primed-memory) T cells. CD28$^-$CD45RA$^+$ T cells had significantly less telomere DNA than naive (CD28$^+$CD45RA$^+$) T cells but similar amounts compared with primed-memory (CD28$^+$CD45RA$^-$) T cells, indicating that CD28$^-$CD45RA$^+$ T cells were not de novo generated lymphocytes. In most individuals analyzed, CD28$^-$CD45RA$^-$ T cells exhibited higher telomere DNA content than CD28$^-$CD45RA$^+$ T cells. However, in contrast to what we observed within the CD28$^+$CD8$^+$ subset, the difference in the telomere content between the CD28$^-$CD8$^+$ subsets was not statistically significant. This observation suggests that in vivo CD45RA isoform shifting

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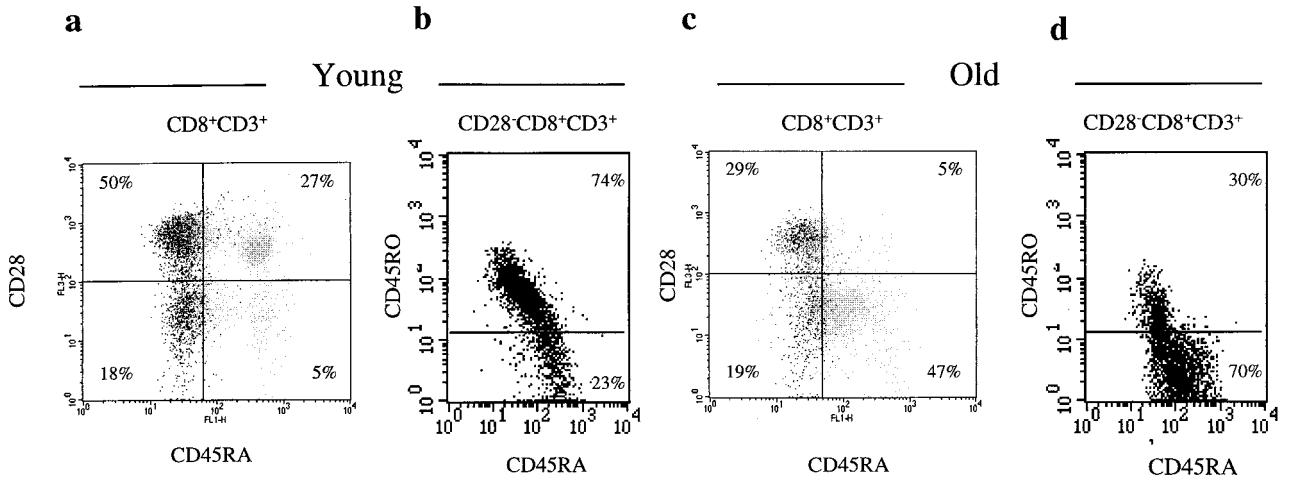

FIGURE 3. Coexpression of CD45RA and CD45RO Ags on $CD28^+CD8^+$ and $CD28^-CD8^+$ T cells. $CD8^+$ cells from four young donors and four old donors were four-color stained using FITC anti-CD45RA, PE anti-CD45RO, Red 613 anti-CD28, and Cy5 anti-CD3. *a*, Expression of CD45RA, CD45RO, and CD28 Ags on $CD3^+$ gated lymphocytes from a 24-yr-old donor. Dot plots show distribution of CD45RO positive cells (black) among the total $CD3^+$-gated population (gray). *b*, Simultaneous expression of CD45RA and CD45RO Ags on $CD28^-CD8^+CD3^+$-gated lymphocytes in the same 24-yr-old subject analyzed before. *c* and *d*, identical to *a* and *b* but in an 84-yr-old subject.

within the $CD28^-CD8^+$ subset requires less extensive cell division than within the $CD28^+CD8^+$ T cell subset.

Telomere DNA content may play a critical control in regulating cell division (44, 45). Therefore, to test if the poor response to mitogens described for $CD28^-CD8^+$ T cells was a consequence of their shortened telomeres, we compared the proliferative response of $CD28^-CD8^+$ T cells to $CD28^+CD8^+$ T cells with equivalent telomere DNA content. The $CD28^+CD8^+CD45RA^-$ and $CD28^-CD8^+CD45RA^+$ T cells satisfied this condition. Furthermore, the proliferative response of the $CD28^+CD8^+CD45RA^+$ subset, a population with significant more telomere DNA than the other two, was also evaluated. Freshly four-color sorted T cells were stimulated with plate-coated anti-CD3 for 4 days in the presence or absence of rIL-2. Although CD25 (IL-2R α-subunit) upregulation was confirmed in the $CD28^-$ subpopulation (data not shown), [^{3}H]thymidine incorporation by $CD28^-CD8^+CD45RA^+$ T cells was considerably lower than in the $CD28^+CD8^+$ $CD45RA^-$ and $CD28^+CD8^+CD45RA^+$ T cell subsets (Fig. 4*b*). Thus, in spite of having similar or even higher telomere content, the proliferative response of these $CD28^-CD8^+$ T cells was considerably reduced with respect to the proliferative response of the $CD28^+CD8^+$ T cells. These results show that the low proliferative capacity of the $CD28^-CD8^+$ T cells is not directly related to their telomere length.

Phenotypic and functional characterization of $CD8^+$ T cell subsets defined by CD45RA and CD28 expression

To define whether the $CD28^-CD45RA^+$ $CD8^+$ T cells found in elderly donors were Ag-experienced cells, we analyzed the expression of other markers indicative of unprimed- and primed-memory status, including CD29, CD11a, and CD62 ligand (CD62L). CD29 and CD11a, which are expressed at low levels on naive cells but increase in primed-memory cells, were both constantly expressed on all the $CD28^-CD8^+$ T cells at levels consistent with primed-memory cells (Fig. 5*a*). Conversely, the homing Ag receptor CD62L, which is associated with naive T cells, was poorly expressed on $CD28^-CD8^+$ T cells (Fig. 5*b*). Expression of the activation markers HLA-DR and CD25 was

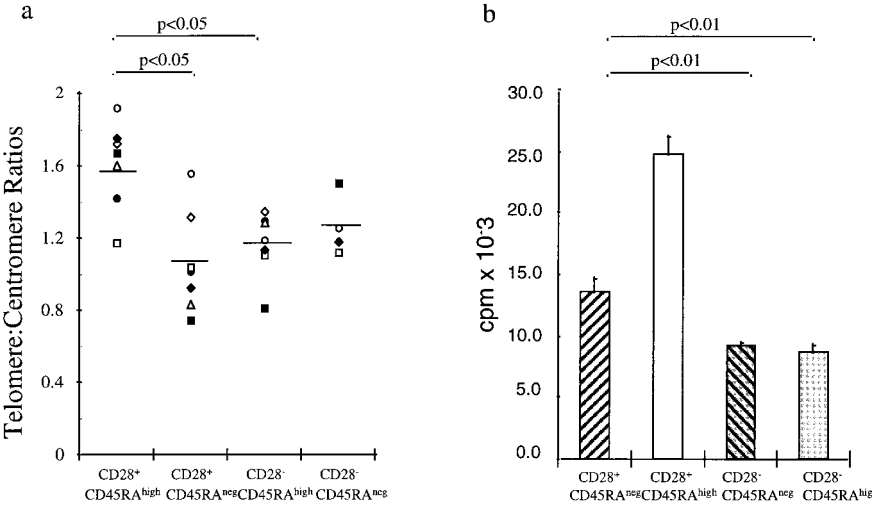

FIGURE 4. Replicative history and proliferative capacity of $CD8^+$ T cell subsets. $CD28^+CD45RA^-$, $CD28^+CD45RA^{high}$, $CD28^-CD45RA^-$, and $CD28^-CD45RA^{high}$ $CD8^+$ T cells were isolated by four-color cell sorting from seven individuals older than 66 yr. For this purpose PBMC cells were stained with FITC anti-CD45RA, PE anti-CD28, Red-613 anti-CD3, and APC anti-CD8. *a*, Replicative history of each $CD8^+$ T cell subset as depicted by their T:C DNA ratio. For each individual, a different symbol was utilized to depict his T:C ratios, and the means of the T:C ratios for each subset are indicated. *b*, Comparison of the proliferative capacity of $CD28^-$ T cells with the proliferative capacity of $CD28^+$ T cells that bear similar or lower telomere DNA content. T lymphocytes from donors ◇, ■, and ◆ (see *a*) were investigated. Representative results obtained with T lymphocytes from donor ◆ are shown. Statistical differences were established using the Mann-Whitney *U* test.

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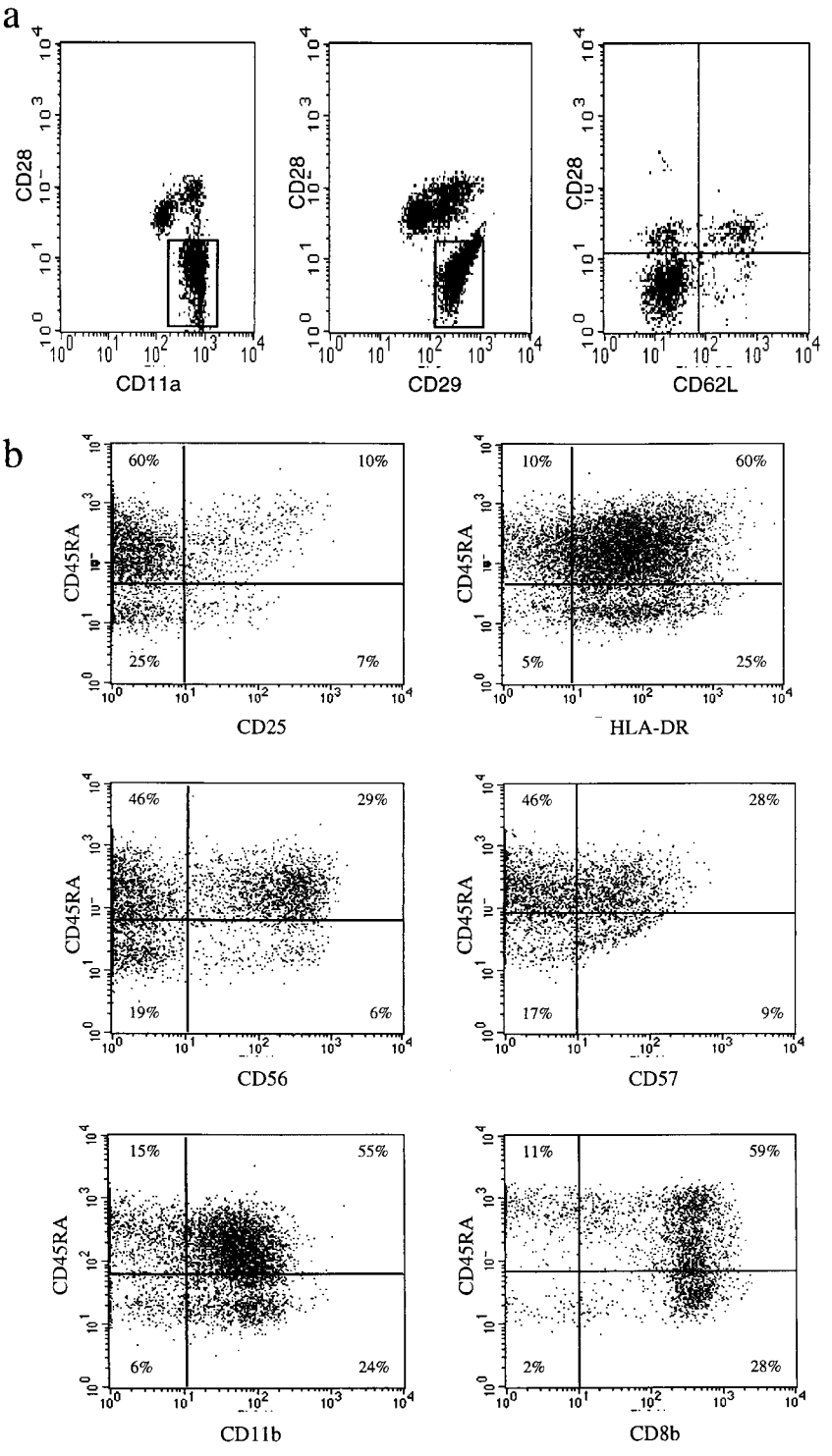

FIGURE 5. Phenotypic characteristics of CD8$^+$ T cell subsets. Purified CD8$^+$ cells from five donors older than 67 yr were four-color stained using FITC-anti-CD45RA; PE-anti-CD29, anti-CD11a, anti-CD62L, anti-HLA-DR, anti-CD25, anti-CD57, anti-CD11b, or anti-CD8b; Red-613 anti-CD28; and Cy5-anti- CD3. *a*, Expression of CD29, CD11a, and CD62L by CD3$^+$CD8$^+$ lymphocytes with differential expression of CD28 molecule. *b*, Dot plots show expression of HLA-DR, CD25, CD56, CD57, CD11b, and CD8β within the CD28$^-$CD8$^+$CD3$^+$ vs CD45RA expression.

also evaluated. The largest incidence of HLA-DR$^+$ cells was found within the CD28$^-$CD8$^+$ subset independent of CD45RA expression (Fig. 5*b*). Expression of CD25 was also significant within the CD28$^-$CD8$^+$CD45RA^{high} subset in some individuals (Fig. 5*b*). Taken together, these data support the concept that the CD28$^-$CD45RA^{high} T cells represent a population of Ag-experienced lymphocytes.

We then measured CD56, CD57, CD11b, and CD8β expression to see whether CD28$^-$CD8$^+$ T cell subsets shared the same atypical phenotype. CD56, CD57, and CD11b were expressed exclusively in the CD28$^-$CD8$^+$ subset by both CD28$^-$CD45RA^{high} and CD28$^-$CD45RA$^-$ T cells (Fig. 5*b*). We also observed that both CD28$^-$CD8$^+$ T cell subsets expressed NK inhibitory receptors (data not shown). Furthermore, CD8β was present in >90% of the circulating CD8$^+$ T lymphocytes. The majority of the CD28$^-$CD8$^+$ T cells expressed CD8β, and no preferential accumulation of CD8α T cells was observed within the CD28$^-$CD8$^+$ subsets (Fig. 5*b*).

To assess the functional capabilities of the different CD8$^+$ T cell subsets found in elderly subjects, synthesis of IL-2, IFN-γ, IL-4, and IL-10 was measured in cells stimulated with PMA and ionomycin. As illustrated in Fig. 6, IL-2 was almost exclusively produced by the CD28$^+$CD8$^+$ T cells. The low percentage of IL-2$^+$

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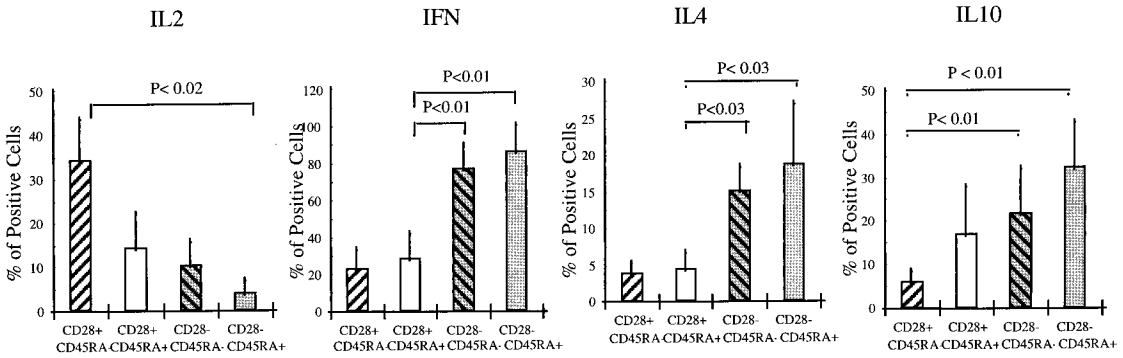

FIGURE 6. Intracellular measurement of cytokine production by $CD8^+$ T cell subsets from aged individuals. Purified $CD8^+$ T cells were stimulated for 5 h with PMA and ionomycin in presence of monensin. After surface staining with CD45RA, CD28, and CD3, cells were fixated and permeabilized, and intracellular cytokines were detected with specific PE-conjugated mAbs. The positions of the quadrant markers were set using as negative controls the PE-conjugated specific anti-cytokine mAbs pre-blocked with the corresponding cytokine. Percentage of positive cells among each $CD8^+$ subset (median ± confidence interval, $n = 5$).

cells within $CD28^-CD8^+$ population was inversely associated with CD45RA expression. In contrast, IFN-γ was mainly produced by the $CD28^-CD8^+$ subset. Remarkably, the largest percentage of IL-4-producing cells was found among both $CD28^-CD8^+$ subsets. Finally, IL-10 was preferentially produced by $CD28^-CD8^+$ $CD45RA^+$ subset. The presence of cells with the same unusual phenotype and with the same unique pattern of lymphokine expression support the notion that $CD28^-CD45RA^-$ and $CD28^-$ $CD45RA^+$ subsets contain highly specialized and phylogenetically related T lymphocytes. Expression of NK markers such as CD56 and cytokine profile of human $CD28^-CD8^+$ T cells may suggest that these lymphocytes represent the equivalent to the $NK1.1^+$ T cells in mice (46).

Discussion

Human aging has been associated with an increased percentage of $CD28^-CD8^+$ T cells in the peripheral blood (12). In this study, we demonstrated that this is due not only to the expansion of a $CD28^-CD8^+$ T cell population but also to the disappearance of the $CD28^+CD8^+$ T cell subset. This loss of circulating $CD28^+CD8^+$ T cells dramatically increased after the age of 50 and was not accompanied by an equivalent reduction of $CD28^+CD4^+$ T cells. Previous studies have shown that $CD28^+CD8^+$ T cells preferentially migrate to peripheral sites such as the gut (47–49). These sites are particularly rich in $CD28^-CD8^+$ T cells (27–29) and may be a source of circulating $CD28^-CD8^+$ T cells in HIV infection (30). We hypothesize that our observed decrease of $CD28^+CD8^+$ T cells may be a result of changes in peripheral tissue milieus to which migratory $CD28^+CD8^+$ T cells are exposed. Consequently, migrated T cells may become trapped at these peripheral sites (by binding to tissue Ags, cytokines, or chemokine signals) or induced to differentiate to $CD28^-CD8^+$ phenotype.

The phylogenetic origin of circulating $CD28^-CD8^+$ T cells in the elderly is unclear. It has been suggested that they may derive from Ag-experienced $CD28^+CD8^+$ T cells (32), but they may also represent de novo-generated T cells from extrathymic sites (50, 51). To test these hypotheses, we determined the memory status of this subset. Our results showed that the $CD28^-CD8^+$ subset did not contain any preferential accumulation of $CD45RO^+$ T cells as compared with the $CD28^+CD8^+$ subset and that the proportion of $CD28^-CD45RA^+$ T cells increased with age, representing the majority of $CD28^-CD8^+$ cells in elderly individuals. These findings fit well with a previous report by Okumura et al. (52) showing an age-related increase in the amount of $CD45RA^+$ cells within the $CD8^+$ T cell population. Since reversion from CD45RO to CD45RA has been shown to occur following adoptive transfer in nude rats (53, 54) and has been also suggested in humans (55), we investigated whether these $CD28^-CD45RA^+$ T cells were $CD45RO^+$ reversions or newly generated T cells. We have four lines of evidence indicating that $CD28^-CD45RA^+$ cells may be derived from $CD45RO^+$ T cells. $CD28^-CD45RA^+$ T cells expressed the adhesion molecules CD29 and CD11a with high intensity but did not express CD62L, a characteristic phenotype of memory T cells (52, 56, 57). The $CD28^-CD45RA^+$ subset contained HLA-DR^+ and $CD25^+$ cells, two molecules expressed by recently primed T cells. The $CD28^-CD45RA^+$ subset also produced IFN-γ, IL-4, and IL-10, three cytokines characteristically produced by highly differentiated primed-memory T cells (58–60). Finally, we showed that the telomere DNA content of $CD28^-CD45RA^+$ T cells was reduced to a similar degree to that observed in primed-memory T cells ($CD28^+CD45RA^-$). Thus, our data indicate that irrespective of CD45RA expression, all $CD28^-CD8^+$ T cells were Ag-experienced cells. Expression of CD45RA within the $CD28^-CD8^+$ population appears to reflect a differentiation state rather than immunological memory.

We found that the general concept of aging leading to an increase in the proportion of $CD45RO^+$ T cells (3) may apply for T cells with $CD28^+CD8^+$ phenotype. In contrast, our results suggest that $CD28^-CD8^+$ T cells become $CD45RA^+$ with aging. Additionally, preliminary data from our laboratory suggests that the same age-related patterns of CD45-isoform switch occur among the $CD28^+CD4^+$ and $CD28^-CD4^+$ subsets (data not shown). We have presented evidence suggesting that $CD28^-CD45RA^+$ T cells derive from $CD28^-CD45RO^+$ precursors. We showed that $CD28^-CD45RA^+$ T cells have characteristics of memory-reverting T cells. We found that $CD28^-CD45RO^+$ cells appear before $CD28^-CD45RA^+$ T cells in blood. $CD28^-CD45RA^+$ and $CD28^-CD45RO^+$ T cells shared the same atypical phenotype ($CD57^+$, $CD56^+$, $CD11b^+$) and unique profile of cytokine production (IL-2^-, IL-4^+, IL-10^+, IFN-γ^+), which support a common lineage for both populations. Thus, this new study provides additional evidence in support of an extensive RO-RA reversion, which takes place in human aging. In addition, our findings explain not only the presence and persistence of $CD45RA^+$ T cells in centenarians, but provides a potential mechanism for their higher frequency in the $CD8^+$ vs the $CD4^+$ T cell subset in aged individuals (10).

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Our results on telomere DNA content show that CD28$^-$CD45RA$^-$ T cells had an intermediate telomere content with respect to CD28$^+$CD45RA$^+$ and CD28$^+$CD45RA$^-$ T cells. This finding agrees with the hypothesis that CD28$^-$CD45RA$^-$ T cells derive from CD28$^+$CD45RA$^-$ T cells. It has been suggested that memory T cells show migratory preferences for nonlymphoid tissue (61, 62). Hence, we propose that primed T cells (CD28$^+$CD45RA$^-$) migrate to peripheral sites where they acquire the CD28$^-$CD45RA$^-$ phenotype. Once cells become CD28$^-$, they decrease their proliferation rate and consequently preserve their telomere DNA content. This could explain why CD28$^-$CD45RA$^-$ T cells have more telomere DNA content than CD28$^+$CD45RA$^-$ T cells. Evidence supporting a common origin for CD28$^+$ and CD28$^-$ T cells was provided by previous studies (63, 64), where it was shown that the similitude of Vβ repertoires between peripheral CD28$^-$ and CD28$^+$ T cells can only be attributed to their divergence from a same T cell population.

Telomere length, in addition to providing a historical record of cell replication, appears to play a critical control in regulating cell division. Nevertheless, we have showed that the poor proliferative response of CD28$^-$CD8$^+$ T cells cannot be attributed to their shortened telomeres. CD28$^+$CD45RA$^-$ T cells proliferated considerably more upon mitogenic stimulation than CD28$^-$CD45RA$^+$ T cells, although both subsets held similar telomere DNA content. In addition, telomere shortening was not associated with CD28 loss since the CD28$^+$CD45RA$^-$ showed the lowest telomere content in most donors. Lack of CD28 expression in vivo seems to be the result of differentiation rather than of proliferation.

We have shown that on a per cell basis the vast majority of CD28$^-$CD8$^+$ T cells produced IFN-γ but not IL-2 and that these cells were positive for IL-4 and IL-10 in elderly donors. Moreover, the proportions of IFN-γ^+, IL-4$^+$, and IL-10$^+$ T cells were considerably higher among the CD28$^-$CD8$^+$ than in the CD28$^+$CD8$^+$ subset. Considering the large predominance of CD28$^-$CD8$^+$ T cells in the elderly, it would be reasonable to expect significant alterations in the cytokine network during aging. In fact, several studies have shown that cytokines preferentially secreted by preactivated or memory T cells, such as IFN-γ, IL-4, and IL-10, are produced in increasing concentrations later in life (65–67). This cytokine profile may also explain some of the suppressor effects on T cell response that have been associated with CD28$^-$CD8$^+$ T lymphocytes. In that respect, it was shown that inhibition of the proliferative response of CD4$^+$ T cells by CD8$^+$ T cell clones was mediated by the simultaneous production of IL-10 and IFN-γ (68). In addition, it has been shown that IL-4 is capable of down-regulating the cytotoxic function of CD8$^+$ cells (69) while IL-10 induces T cell anergy (70) and both cytokines act synergistically to inhibit cell mediated immune-responses (65, 71). Further studies will be necessary to establish the significance of these findings in the impairment of the immune response in aging. Moreover, future investigations to determine the conditions that lead to the generation of CD28$^-$ T cells will bring about increased understanding not only on the pathobiology of the aging process but also on normal T cell homeostasis.

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