Age-related differences in phenotype and function of CD4\(^+\) T cells are due to a phenotypic shift from naive to memory effector CD4\(^+\) T cells

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Abstract

Based on the combined expression of CD27 and CD28, a putative model of T cell differentiation has been previously proposed. We used CD27 and CD28 expression in order to comparatively study the size, cytokine production capacity and proliferative response of CD4\(^+\) T cell sub-populations from healthy young and elderly volunteers. Elderly persons had a lower percentage of CD27\(^+\)CD28\(^+\) but a higher percentage of CD27\(^-\)CD28\(^-\) and CD27\(^-\)CD28\(^-\) CD4\(^+\) T cells than the young persons. CD27\(^-\)CD28\(^-\) CD4\(^+\) T cells were present, although at relatively low numbers, in the vast majority of the healthy elderly donors but were only sporadically detected in young persons. Each CD4\(^+\) T cell sub-population exhibited a distinct phenotype and cytokine production profile, which were not affected by age. When purified CD27\(^+\)CD28\(^+\) were stimulated by staphylococcal enterotoxin B, they proliferated to a greater extent than CD27\(^-\)CD28\(^-\) and CD27\(^-\)CD28\(^-\) CD4\(^+\) T cells. However, we did not observe age-related differences in proliferative response of each sub-population. We concluded that although the size of the different sub-populations differed between the young and the old group, the functional characteristics of each sub-population were the same in both age groups. This suggests that on a per cell basis there is no functional impairment of CD4 memory T cells in elderly persons. Consequently, potential differences in the function of the total CD4\(^+\) T cell population are most likely due to different composition of repertoire.

Introduction

As individuals age, a substantial diminution in immune responsiveness is taking place. This leads to an increase in the incidence of severe infections and decrease of the protective effect of vaccination (1). Involution of the thymus and diminished output of T lymphocytes are the most recognized changes in the immune system with age (2). Therefore, in elderly individuals, there is an increase in the proportion of T cells that are highly differentiated, as determined by their surface phenotypes and functional characteristics (1, 3). Additionally, alterations in the production of cytokines, or their recognition by cells of the immune system, are quite likely responsible for at least some of the changes that are observed with aging (4).

The expression of the co-stimulatory molecules CD27 and CD28 has been used for studying the differentiation phenotype of antigen-specific cells during the chronic phase of persistent infection (5, 6). According to a putative model of T cell differentiation, CD27\(^+\)CD28\(^+\) T cells represent naive and early-differentiated cells which progress to CD27\(^-\)CD28\(^-\) T cells, which are believed to be fully differentiated cells (5). Since CD27 and CD28 expression has been associated with different stages of CD4\(^+\) (6) and CD8\(^+\) (5) T cell differentiation, we used this classification in order to compare the number of CD4\(^+\) T cell subsets in healthy young and elderly persons. In order to further verify the differentiation status of CD4\(^+\) T cell subsets, we studied the expression of surface markers, such as CD45RA, CD11a and CCR7, perforin production and the length of telomeres. Based on that, we described the phenotype of each CD4\(^+\) T cell sub-population and provided data supporting the above-mentioned model for T cell differentiation.

Although cytokine production in old age is one of the most controversial topics in immunological research, there is now little doubt that aging is associated with an increase in the...
whole body load of IFNγ (1). IFNγ production by CD8+ T cells has been investigated and correlated with the accumulation of CD28- terminally differentiated effector T cells, the number of which increases profoundly with aging (7). Yet, there is very little information on the cytokine-producing profile of distinct CD4+ T cell sub-populations in the context of aging. Additionally, an important question still to be answered is whether age-related differences in cytokine production are due to a phenotypic shift from naive to memory effector CD4+ T cells or changes in function within individual CD4+ T cell subsets.

CD4+ T cell help is essential for the induction of immunity by any vaccine (8, 9). It has been recently shown that IL-2 production by CD4+ T cells is required for effective immunization (8). Since aging is accompanied by decrease in the protective effect of vaccination, we aimed to monitor and directly compare the IL-2, IL-4- and IFN-γ-producing capacity as well as the proliferative ability of CD4+ T cell sub-populations derived from healthy young and elderly individuals. Taking into account all the above, we believe that broader knowledge regarding CD4+ T cell sub-population function in old age and their functional relevance might result in novel interventions to improve immunity and therefore the overall well-being of elderly people.

**Methods**

**Blood donors and cells**

PBMCs from 42 apparently healthy young (22–34 years, n = 19) and elderly (57–93 years, n = 23) were isolated from heparinized venous blood by density gradient sedimentation using Ficoll–Hypaque (Amersham Biosciences, Uppsala, Sweden). The cells were washed twice in RPMI 1640 (Cambrex, Verviers, Belgium) and re-suspended in RPMI 1640 supplemented with 10% FCS. All participants had given their informed written consent to participate in the study.

**Flow cytometric analysis of T cell-surface markers**

The following antibodies were used in different combinations: anti-CD4 [peridinin chlorophyll protein (PerCP)], anti-CD3 phycocerythrin (PE), anti-CD11a (PE), anti-CD27 [fluorescein (FITC) or PE], anti-CD28 [PE or allophycocyanin (APC)], anti-CD45RA (PE) and anti-CCR7 (PE). All the mAbs used were purchased from BD PharMingen, Schwechat, Austria.

Cell-surface staining was conducted on cryopreserved PBMCs since, in preliminary experiments, no difference in the expression of surface markers between fresh and defrosted PBMCs in individuals was detected. The cells were stained for 25 min at 4°C in the dark. After staining, they were washed and 40 000 events were measured on a FACSCalibur (BD PharMingen).

**Purification of cells**

CD4+ T cells were purified from fresh PBMCs by using anti-CD4-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), using standard procedure recommended by the producer. A purity of ≈95% was obtained.

**Cell stimulation and flow cytometric assessment of intracellular cytokines and perforin production**

Purified CD4+ T cells were stimulated with phorbol myristate acetate (PMA) (30 ng ml⁻¹; Sigma–Aldrich, Vienna, Austria) and ionomycin (30 ng ml⁻¹, Sigma–Aldrich) in the presence of Brefeldin A (10 μg ml⁻¹, Sigma–Aldrich) for 4 h at 37°C. After this stimulation period, they were washed in PBS and stained with anti-CD3 (PerCP), anti-CD27 (FITC) and anti-CD28 (APC) for 25 min at 4°C in the dark. After washing, permeabilization of the cells with CytoFix/CytoPerm (BD PharMingen) solution was performed. Permeabilized cells were stained with anti-IL-2 (PE), anti-IL-4 (PE), anti-IFNγ (PE) or anti-perforin (PE) for 25 min at 4°C in the dark. Finally, they were washed and 40 000 events were measured on a FACSCalibur (BD PharMingen).

**Isolation of CD4+ T cell subsets**

Purified CD4+ T cells were stained with anti-CD27 (FITC), anti-CD3 (PE), anti-CD4 (PE Cy7) and anti-CD28 (APC) for 50 min at 4°C in the dark. After staining, they were washed in PBS and re-suspended in RPMI 1640 supplemented with 10% FCS. The cells remained overnight at 4°C and were sorted into CD28+CD27+, CD27-CD28+ and CD27-CD28- by a FACS Vantage SE (Becton Dickinson, Schwechat, Austria) the next day. An enrichment of >80% of cells of a certain phenotype was obtained in the three sub-populations.

**Cell stimulation and proliferation assays**

CD4+ T cell sub-populations (50 000 cells per well) were stimulated with staphylococcal enterotoxin B (SEB; 5 μg ml⁻¹; Sigma–Aldrich, Schwechat, Austria) for 5 days, in the presence of 50 000 irradiated autologous PBMCs. This incubation period had proven to be optimal in pilot experiments. [3H]thymidine (BIOMEDICA, Vienna, Austria) was added on the last day of stimulation for 8 h. Average counts per minute (c.p.m.) were measured for triplicate, or in the case of low cell number in a certain sub-population duplicate wells, and expressed by subtracting average media c.p.m from average antigen-stimulated c.p.m.

**Telomere length measurement of the CD4+ T cell sub-populations**

The telomere length of the CD4+ T cell sub-populations studied was determined using flow cytometric fluorescent in situ hybridization (ISH) with a fluorescent-labeled peptide nucleic acid (PNA) telomere probe. PNAs are (C₃T₃A₃)₃ nucleic acid analogues that are capable of strongly and specifically interacting with the TTAGGG-targeted DNA sequence and in which the lateral backbone is replaced by a 2-aminoethyl-glycine unit. The PNA telomere probe hybridizes to complementary DNA in low ionic strength solutions that do not favor re-annealing of the target strands, PNA-DNA interactions being more stable than DNA-DNA or DNA-RNA interactions under hybridization conditions (10).

The T cell lymphoblastic leukemia line 1301, which has unusual long telomeres (25 kb) and is tetraploid (11), was used as a standard for each telomere length measurement.
The lymphocytes were fixed and permeabilized using the standard procedure with CytoFix/CytoPerm kit (BD Biosciences, Scwechat, Austria), and then 3 µl of the Cy5-labeled PNA telomere probe (Applied Biosystems, Foster City, CA, USA) or an equivalent quantity of correspondingly labeled control antibody (BD PharMingen) was added in the hybridization buffer. The cells were incubated in a hybridization buffer containing 70% formamide at 82°C for 10 min, and then snapped cool on ice and incubated at room temperature for 90 min. After three rounds of washing in a post-hybridization buffer and three rounds in PBS, cells were analyzed for their FL4 fluorescence on a FACSCalibur flow cytometer equipped with a dual laser system.

The FL4 fluorescence from the Cy5-labeled PNA telomere probes for each T cell subset was collected and plotted on a logarithmic scale, together with the fluorescence of the control lymphoblastic leukemia cell line 1301, which has a known constant telomere length of 25 kb, and the individual telomere length was calculated for each CD4+ T cell subset, by relating the intensity of its fluorescence signal to the one of the standard line.

**Statistical analysis**

Student’s t-test for unpaired data and one-way analysis of variance test followed by post hoc multiple comparisons were performed (SPSS™ for Windows™ v. 11.5, SPSS Inc., Chicago, IL, USA). A two-tailed P value of <0.05 was considered statistically significant.

**Results**

**Aging leads to the accumulation of CD4+ T cells of later differentiation stages, and a decrease of CD27−CD28−CD4+ T cells**

Based on the combined expression of CD27 and CD28 by CD4+ T cells, we defined four CD4+ T cell sub-populations in healthy young and elderly persons: CD27+CD28+, CD27dimCD28+, CD27−CD28* and CD27−CD28− (Fig. 1A). The CD27+CD28+ sub-population was significantly larger in the young cohort. The CD27−CD28− population had the same size in young and old persons, whereas the CD27−CD28* and the CD27+CD28+ populations were significantly larger in the old group (Fig. 1B). A total of 71.4% of the elderly persons (15 persons out of 21) but only 20% of the young persons (3 persons out of 15) had frequencies of CD27−CD28−CD4+ T cells higher than 1% of the CD4+ T cell population. We therefore conclude that there is a shift in the differentiation of CD4+ T cells of elderly persons toward late differentiation stages.

**Phenotypic characterization of CD4+ T cell subsets**

To further characterize these subsets, we used a panel of cell-surface markers, consisting of CD45RA and receptors involved in lymphocyte homing (CCR7) and intercellular adhesion and co-stimulation (CD11a). Additionally, we analyzed the ability of each subset to produce perforin.

Each subset displayed a distinct cell-surface phenotype (Fig. 2): high percentages of CD27+CD28+CD4+ T cells

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**Fig. 1.** CD4+ T cell differentiation pattern in young and old individuals. (A) Representative stainings from one young and one elderly person are shown. Note that, in the majority of young persons, no CD27+CD28+ T cell sub-population was detected. Percentages indicated in the figure refer to CD4+ T cells as 100%. (B) The size of the CD27+CD28+, CD27−CD28* and CD27−CD28− sub-populations differed significantly between young and old donors. Data were analyzed using Student’s t-test for unpaired data, *P < 0.05 and are expressed as means ± SEM (n = 15 in the young and n = 21 in the elderly group).
expressed CD45RA and CCR7 combined with a relatively low CD11a expression. This sub-population did not produce perforin. CD27dimCD28+CD4+ T cells had strongly down-regulated CD45RA and CCR7, C11a expression was enhanced and perforin was not produced. Only a low percentage of CD27–CD28+CD4+ T cells expressed CD45RA and CCR7, but this sub-population expressed relatively high amounts CD11a. A small number of cells in this population was able to produce perforin. A relatively high percentage of CD27–CD28+CD4+ T cell re-expressed CD45RA. This sub-population was CD11a++, did not express CCR7 and produced large amounts of perforin.

Statistically significant age-related changes in the expression of surface markers were found only within the CD27+CD28+CD4+ T sub-population. Elderly persons had lower numbers of CD45RA+ cells than young ones. Additionally, the percentage of CD11a++ cells was elevated among the elderly donors. This suggests that CD27+CD28+CD4+ T cells represent a mostly naive antigen-inexperienced population in the young, but contain larger proportions of antigen-experienced cells of an early differentiation stage in the elderly. The phenotype of the CD27dimCD28+CD4+ T cell subsets was not affected by age.

Each CD4+ T cell sub-population displayed a distinct cytokine production profile, which was not affected by age

We studied the capacity of CD4+ T cell sub-populations to produce IL-2, IL-4 and IFNγ following short-term stimulation by PMA/Ionomycin (Fig. 3).

CD27+CD28+CD4+ T cells produced IL-2, but only a low percentage of cells within this subset produced IL-4 or IFNγ.

The CD27dimCD28+CD4+ T cell subset contained larger numbers of IL-2-producing cells. IL-4-producing as well as IFNγ-producing cells were also detected within this subset. CD27+CD28+ cells were the main cytokine-producing sub-population within CD4+ T cells, as they contained relatively high proportions of IL-2, IL-4- and IFNγ-producing cells. CD27–CD28 cells contained high numbers of IFNγ-producing cells, ranging between 73.8 and 99.07%. They produced more IFNγ than any other CD4+ T cell sub-population. These cells also produced IL-2, but no IL-4.

CD4+ T cell subsets displayed identical IL-2, IL-4 and IFNγ production patterns in young and old persons in response to stimulation with PMA and Ionomycin. We therefore conclude that the cytokine production potential of each CD4+ T cell subset is not affected by age.

CD4+ T cell sub-populations proliferate in response to SEB in an age-independent manner

To evaluate the proliferative response of each sub-population, we sorted CD4+ T cells into CD27+CD28+, CD27dimCD28+ and CD27–CD28 (Fig. 4A) and stimulated them with SEB. All CD4+ T cell subsets proliferated after stimulation with SEB, albeit to a different extent. In both young and old cohorts, CD27+CD28+ exhibited significantly higher levels of proliferation than CD27–CD28+ and CD27–CD28– T cells. We did not observe significant differences in the proliferative response of CD27+CD28+ and CD27–CD28–CD4+ T cells. However, CD27–CD28+ T cells tended to proliferate more than the CD27–CD28–CD4+ T cell subset. CD4+ T cells from young persons displayed a slightly, but not significantly, higher proliferative response than CD4+ T cells from elderly donors, within each cell subset (Fig. 4B).
CD4+ T cell subsets function is not affected by age

CD27+CD28+ and CD27+CD28−CD4+ T cells have significantly shorter telomeres than the CD27+CD28+ indicating different replicative histories.

The telomere length of the three CD4+ T cell sub-populations studied was determined using flow cytometric fluorescent ISH with a fluorescent-labeled PNA telomere probe.

A representative experiment shown in Fig. 5(A) demonstrates that telomeres were longer in CD27+CD28+ cells and shorter in the two other subsets.

Statistics were performed considering telomere length of CD27+CD28+CD4+ T cells as 100% and calculating its percent reduction within the other two subsets (Fig. 5B). Using this approach, we found that the telomere length of CD27+CD28+ and CD27−CD28−CD4+ T cells was significantly reduced when compared with that of CD27+CD28+CD4+ T cells. Additionally, CD27−CD28−CD4+ T cells had significantly shorter telomeres than their CD27+CD28+ counterparts. Figure 5 shows data from elderly persons. No significant differences were found in the telomere length of the different sub-populations isolated from young and elderly persons (data not shown).

Discussion

It has previously been suggested that the differential expression of the co-stimulatory receptors CD27 and CD28 is associated with T cell differentiation (5). CD4+ T cells differentiate from CD27+CD28+ cells to CD27−CD28−, with CD27dimCD28+ and CD27−CD28+ cells representing intermediate stages. We show that PBMCs from elderly persons contain lower percentages of CD27+CD28+CD4+ T cells but more CD27−CD28+ and CD27−CD28−CD4+ T cells than...
PBMCs from young donors. CD27+CD28−CD4+ T cells were common, in low percentages, among healthy elderly persons but extremely rare in the young. This finding implies that there may be one or more factors driving the accumulation of CD4+ T cells of later differentiation stages in the elderly.

CD28−CD4+ T cells in relatively high numbers have been detected in patients with rheumatoid arthritis (12) and are believed to play a role in the disease process due to their inflammatory properties. Loss of CD28 expression in CD4+ T cells of healthy individuals has been reported by Colombatti et al. (13), Vallejo et al. (14) and Weyand et al. (15) and shown to be age related since the numbers of those cells exhibited a continuous increase with advancing age (15). We used the combined expression of CD27 and CD28 in order to further characterize the CD4+ T cell sub-populations in different age groups. CD27+CD28−CD4+ T cells, which occurred at relatively low numbers in healthy elderly persons (Fig. 1B), displayed a phenotype consistent with that of terminally differentiated cells. They were able to produce extremely high amounts of IFNγ and perforin. Whether these pro-inflammatory properties are of functional significance in healthy elderly persons is presently not known. In view of the fact that CD27+CD28−CD4+ T cell sub-population is present in low numbers in healthy elderly persons, we suggest that the functional relevance of this population for healthy aging is probably negligible.

While CD28−CD4+ T cells are unable to proliferate and do not produce IL-2 (16), CD28−CD4+ T cells were still able to produce moderate amounts of IL-2 and they can be induced to proliferate after stimulation with SEB. CD27+CD28+ and CD27−CD28− had significantly shorter telomere lengths than their CD27+CD28+ counterparts, indicating that they have undergone more rounds of cell division, consistent with a progressive state of effector T cell differentiation, accompanied by the loss of CD27 and CD28. Considering an average loss of telomere length of 51 bp per year in the adult age in the
CD4+ memory cells (17), the determined telomere length difference found between the investigated CD4+ lymphocyte subsets was equivalent to a 20- to 25-year replicative history difference.

Except for the difference in the size of CD4 subsets, no other age-related difference regarding the composition or functional properties of these subsets was detected. Age-related differences were only detected within the CD27+CD28+CD4+ T cell subset. CD27+CD28+CD4+ T cells from elderly persons expressed lower levels of CD45RA and higher levels of CD11a when compared with young. They included more IL-2-producing cells. Additionally, after in vitro activation by SEB they tended to proliferate less. Taken together, these data suggest that although the CD27+CD28+CD4+ T cell sub-population contains mostly naive cells in the young, in the elderly it contains a mixture of naive and early-differentiated cells.

Experiments in transgenic mice models have shown that naive CD4+ T cells from aged mice produce less IL-2 and proliferate poorly (18), whereas recent findings from our laboratory have demonstrated that after primary stimulation with neoantigens the capacity of T cells from healthy elderly persons to produce IL-2 is not impaired in comparison with young controls (19). Since the CD27-CD28-CD4+ T cell sub-population does not exclusively contain naive cells, we suggest that the increased IL-2 production observed in this population from elderly persons is due to an increased IL-2 production by antigen-experienced cells present in this population than intrinsic changes of aged naive T cells.

Old age is characteristically associated with an overproduction of type I cytokines (20) and high serum neopterin concentrations, which suggest an increased whole body load of IFNγ (21). This is of major clinical significance, as high IFNγ stimulates inflammatory processes, which are known to support the development of age-related diseases such as Alzheimer's disease (22) and atherosclerosis (23).

Whereas many human studies aimed at estimating the amount of cytokines secreted from mixed cell populations, only the work of Yen et al. (24) and Sakata-Kaneko et al. (25) was focused on cytokine production by highly purified CD4+ T cells and demonstrated a generalized up-regulation of IFNγ in old age. Weyand et al. (15) measured cytokine secretion by CD28-CD4+ T cell clones derived from healthy elderly persons, after 24 h stimulation with anti-CD3 and anti-CD28 and clearly showed that CD28-CD4+ T cell clones secreted high concentrations of IL-2 and IFNγ. To our knowledge, our study is the first one that addresses the specific cytokine production pattern of freshly isolated CD4+ T cell subsets in healthy elderly persons. We show that each CD4 subset displays a distinct cytokine production profile following short-term stimulation by PMA/ionomycin. With the exception of IL-2 production by CD27+CD28+CD4+ T cells, no age-related differences in the capacity of the other subsets to produce cytokines were detected. Since the size of the CD4+ T cell subsets was affected by age, we suggest that potential age-related changes in cytokine production by the total CD4+ T cell population are most likely due to a shift in the differentiation of CD4+ T cells of elderly persons toward late differentiation stages. However, age-related changes in cytokine production by CD4+ T cells were not as pronounced as those described for CD8+ T cells (7), and may therefore be less significant for the development of pathology. The fact that no functional defects on a per cell basis were found in the different CD4+ T memory cell sub-populations in the elderly is quite important since it demonstrates that immunization of younger individuals induces long-lived CD4+ memory T cells that function well into old age. This is in agreement with results derived from mouse models that show that CD4+ T cell memory that is generated during youth functions well in old animals (26, 27).

In conclusion, we demonstrate that the size of the different CD4+ T cell sub-populations differs between the young and the old group. However, the functional characteristics of
each sub-population are the same in both age groups, implying that there is no functional impairment on a per cell basis in elderly persons. We therefore suggest that age-related changes within the total CD4+ T cell population are not due to qualitative alterations within the different sub-populations, but are most likely due to a numerical shift toward more differentiated cells.

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Abbreviations

- APC: aliphycocyanin
- c.p.m.: counts per minute
- FITC: fluorescein
- ISH: in situ hybridization
- PE: phycoerythrin
- PerCP: peridinin chlorophyll protein
- PMA: phorbol myristate acetate
- PNA: peptide nucleic acid
- SEB: staphylococcal enterotoxin B

References