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Research article

Age-related susceptibility of naive and memory CD4 T cells to apoptosis induced by IL−2 deprivation or PHA addition

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Abstract

The increased age-associated incidence of infectious and cancer diseases has been related to the alteration of immune functioning found in the elderly (immunosenescence). The reduction of naive T cells, which determine an impaired ability to mount immune responses to new antigens, represents a hallmark of the aging process. The aim of this study was to evaluate the susceptibility to apoptosis of purified naive and memory $CD4^+$ T cells from peripheral blood of healthy people ranging in age from 20 to 98 years. Two mechanisms of T cell elimination by apoptosis have been evaluated: cytokine deprivation and activation-induced cell death. After Interleukin−2 deprivation, the percentage of naive and memory $CD4^+$ apoptotic cells significantly increased with donor age concomitantly with a reduction of Bcl-2 expression and an increase of intracellular content of reactive oxygen species. After phytohemagglutinin addition, the percentage of apoptotic cells, the expression of CD95, and the intracellular reactive oxygen species, were not significantly correlated with age both in naive and memory $CD4^+$ T cells. Our data demonstrate the existence of functional alterations of naive and memory T cell populations during ageing. These alterations are mainly related to the mechanism of the apoptotic event rather than to the type of cell population involved (naive or memory). The alterations of naive and memory T cells may have implications in the age-related susceptibility to diseases.

Introduction

Many studies performed in human subjects and in experimental animals have identified significant changes in the immune system with age. Alterations in T cell and B cell compartments as well as in cell-mediated and humoral responses have been reported (Jackola et al. 1994; Paganelli et al. 1994; Globerson 1995; Wikby et al. 1998; Chakravarti and Abraham 1999). In particular, the age-related decline of immune response has been found primarily associated with changes in T cell repertoire rather than in B cells or antigen-presenting cells (Thoman and Weigle 1989; Murasko and Goonewardene 1990; Miller 1996; Castle et al. 1999). The impaired T cell function seems to play a relevant role in increasing the morbidity and mortality in elderly individuals during infection, autoimmune diseases, and cancer (Nagel et al. 1988; Miller 1996; Aggarwal and Gupta 1998).

The increased susceptibility to age-related diseases has been particularly associated with the impaired ability to mount immune responses to new antigens, whereas immune responses against recall antigens may be still conserved (Weigle 1989; Ahmed and Gray 1996). The immune responses to novel antigens rely on the availability of naive T cells. In this context, immunosenescence has been associated with a numerical change in T cell repertoire, mainly represented by an expansion of memory and a reduction of

naive T lymphocytes (De Paoli et al. 1988; Utsuyama et al. 1992; Cossarizza et al. 1996; Fagnoni et al. 2000; Fahey et al. 2000). Whereas the expansion of memory T cells has been suggested to be the consequence of repeated antigenic stimulations (Sprent and Surh 2001), the reduction of naive T cells has been related to increased apoptosis of this cell population (Chrest et al. 1995; Phelouzat et al. 1996; Herndon et al. 1997; Potestio et al. 1998).

It is known that apoptosis can be considered a physiological mechanism for normal development and for maintenance of cellular homeostasis in multicellular organisms (Wyllie et al. 1980; Cohen 1996). In lymphocytes, apoptosis plays an important role in maintaining T cell repertoire and deletion of autoreactive T and B lymphocytes, thus limiting immune responses (Osborne 1996). Many studies have suggested that T cell elimination by apoptosis can be induced by two major mechanisms. One of these process, known as activation-induced cell death (AICD), requires the re-ligation of T cell receptor (TCR) on activated T cells (Krammer et al. 1994; Lynch et al. 1995; Vaux and Strasser 1996). Differently, a second mechanism of apoptosis can be induced by cytokine deprivation, which determines a decrease in apoptosis-inhibitory proteins (Bcl-2 and Bcl-x_L) (Akbar et al. 1993a,b, 1996; Cohen 1993).

Increased apoptosis of T cells with naive phenotype was found following *in vitro* activation of T cells from aged subjects with PHA and IL-2 (Herndon et al. 1997). In the same study, cells with memory phenotype were found susceptible to activation-induced apoptosis to a similar degree among young and old individuals (Herndon et al. 1997). Other studies, conducted on whole T cells, found that T cells from aged mice or T cells from elderly humans undergo apoptosis more frequently following stimulation with mitogen or anti-CD3 antibody, when compared to T cells from young controls (Chrest et al. 1995; Phelouzat et al. 1996; Potestio et al. 1998). Thus, in the above studies, the age-related alteration of apoptosis of lymphocyte populations have mainly related to the type of population involved (naive or memory) rather than to the mechanism of the apoptotic event. Taking into account the relevant role that apoptosis may play in determining the age-related numerical change in T-cell repertoire, in this study we evaluated the susceptibility to apoptosis, by cytokine deprivation (IL-2 deprivation) or AICD (phytohemagglutinin addition), in purified populations of naive and memory $CD4^+$ T cells from peripheral blood of healthy people ranging in age from 20 to 98 years. Our data demonstrate the existence of functional alterations of both naive and memory T cell populations during ageing. These alterations are mainly related to the mechanism of the apoptotic event (cytokine deprivation *versus* AICD) rather than to the type of cell population involved (naive or memory). These data provide new insights into age-related T-cell-mediated immunodeficiency which may have implications in the increased susceptibility to infections and cancer diseases present in the elderly.

Materials and methods

Isolation of naive and memory T cell populations

Human peripheral blood was obtained from a population of 47 healthy people ranging in age from 20 to 98 years after informed consent. All study procedures and forms were approved by the Local Ethical Committee. The health status of all people recruited for this study was assessed according to a protocol which included: (1) The assessment of current and past health status by means of a specific questionnaire; (2) the presence of diseases known to affect haematopoiesis (cancer, chronic infections, collagen vascular disease, rheumatoid disease); (3) the measurement of basic laboratory parameters. Peripheral blood mononuclear cells (PBMCs) were fractionated on Lympholyte H (Cederlane, Canada) and separated by density gradient centrifugation at 800 g for 20 min. Cells from the interface of the gradients where washed twice with Ca^{2+}-and Mg^{2+}-free phosphate-buffered saline (PBS, Gibco/Life Technologies, Grand Island, NY), and then counted and suspended in PBS.

$CD4^+$ T-lymphocytes were separated from PBMCs by immunoselection using coated beads (Miltenyi et al. 1990). Briefly, PBMCs (10×10^6) were incubated with CD4 MicroBeads (Miltenyi Biotec, Bergisch, Gladbach, Germany) for

15 min at 4 °C in MACS buffer. MACS was performed according to the manufacturers instructions. Positive selection of $CD4^+$ cells was performed by passing the cell suspension through a mini-MACS separation column. $CD4^+$ cell fractions was collected, counted and labeled with monoclonal antibodies anti-CD95 (PE) and anti-CD62L (FITC) (Becton Dickinson, San Jose, CA) for 15 min at room temperature. Cells were then washed, suspended in PBS (2×10^6/ml), and sorted through fluorescence-activated cell sorting (FACS Vantage, Becton Dickinson, San Jose, CA). The purity of T-cell preparations, assessed by cytofluorimetric analysis, was greater than 95%.

Cell stimulation and induction of apoptosis

Naive and memory $CD4^+$ T cells sorted through flow cytometry were separately cultured at a density of 7×10^5 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) (all from Gibco/Life Technologies, complete medium), and activated for 3 days by addition of 1% Phytohemagglutinin (PHA, Gibco) and Phorbol-12-myristate-13-acetate (2 ng/ml, PMA, Calbiochem, Milan, Italy) at 37 °C, 5% CO_2. After 3 days, stimulated T cells were washed, counted, and cultured at 7×10^5 cells/ml in complete medium supplemented with 30 U/ml Ril-2 (Chiron Italia, Milan, Italy). Each cell population was cultured in this condition for further 7 days, and every 2 or 3 days the culture medium was replaced by fresh medium, maintaining the same cell concentration.

On day ten of culture, apoptosis was induced in the naive and memory $CD4^+$ T cell populations either removing IL-2 (apoptosis induced by cytokine deprivation) or adding PHA. All cultures were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. At the end of the culture cells were checked for viability, by trypan blue exclusion (Sigma, St. Louis, MO, USA), and proliferation. Both before and after apoptosis induction, cells were analyzed for apoptosis, for the rates of intracellular oxidation, and for the expression of Bcl-2 and CD95.

Proliferation assay

For proliferation assay, purified naive and memory $CD4^+$ T cells were cultured with PMA and PHA for 3 days and then with IL-2 for 7 days as above described. Cells were added in 96-well round-bottom microtiter plates (Nunc, Roskilde, Denmark) at a concentration of 7×10^4 cells/well in a total volume of 100 μl of complete medium supplemented with 30 U/ml IL-2 and kept at 37 °C in 5% CO_2. Each well was pulsed with 1 μCi of ^{3}H-TdR for 18 h before the harvesting. Uptake of ^{3}H-TdR was measured in a liquid scintillation counter, and the results were expressed as the mean counts per minute plus or minus standard deviation (cpm $\pm$ s.d.).

Monoclonal antibodies and flow cytometric analysis

To evaluate Bcl-2 expression in naive and memory $CD4^+$ T cells before and after induction of apoptosis by IL-2 deprivation, T cells were washed with a solution of PBS, 5% FBS, and 0.1% NaN_3 (Sigma) and fixed with PBS 2% paraformaldehyde for 15 min at 4 °C. The fixed cells were washed with a permeabilization buffer containing PBS, 1% bovine serum albumin (BSA, Sigma) and 0.05% saponin (Sigma) and then incubated with PE-conjugated Mouse anti-human Bcl-2 (PharMingen, San Diego, CA) for 30 min on ice. Cells were finally washed in PBS, 1% BSA, and 0.01% saponin, resuspended in PBS and immediately analyzed with a Coulter XL flow cytometer. To evaluate CD95 expression before and after induction of apoptosis PHA addition, cells were washed in PBS containing 0.1% NaN_3 plus 5% FBS and labeled with PE-conjugated mouse anti-human CD95 (PharMingen) for 30 min on ice. After that the cells were washed in PBS containing 0.1% NaN_3 and suspended in PBS for flow cytometric analysis. PE-conjugated mouse IgG_1 (PharMingen) was used as isotype control for both monoclonal antibodies staining.

Quantification of apoptosis by flow cytometry

Apoptosis was measured using (1) binding of fluorescein-labeled Annexin V to phosphatidylserine

residues on the outer leaflet of the cell membrane and (2) subdiploid DNA peaks analysis after staining with Propidium Iodide (PI) as previously described (Nicoletti et al. 1991).

For Annexin V binding to the cell membrane, cells were washed in PBS resuspended in Binding Buffer and stained with a FITC-labeled recombinant Annexin V (Alexis Co., Laufelfingen, Switzerland). After staining, cells were washed with PBS and labeled with Propidium Iodide (Sigma) just before flow cytometry analysis. Simultaneous staining of tumor cells with Annexin V (green fluorescence) and with the non-vital dye Propidium Iodide (red fluorescence) allowed the discrimination of intact cells ($FITC^-PI^-$), early apoptotic ($FITC^+PI^-$), and late apoptotic or necrotic cells ($FITC^+PI^+$). Fluorescence was determined using an XL flow cytometer (Coulter, Hialeah, FL). For subdiploid DNA evaluation, cells were washed twice in PBS and resuspended in 0.75 ml hypotonic fluorochrome solution containing 50 μg/ml PI (Calbiochem, La Jolla, CA, USA) in 0.1% sodium citrate plus 0.1% Triton x-100 (Sigma, St. Louis, MO, USA) in polypropylene tubes (Nunc, Roskilde, Denmark). The tubes were placed at 4 °C in the dark overnight before the flow-cytometric analysis. The PI fluorescence of individual nuclei was determined using a Coulter XL flow cytometer and the percentage of apoptotic nuclei was determined on the basis of the number of subdiploid DNA peaks in the fluorescence histogram.

Flow cytometric analysis of rates of intracellular oxidation

Hydroethidine (HE) was obtained from Molecular Probes (Eugene, OR, USA), and stock solution were prepared and stored at −20 °C. As previously reported (Packham et al. 1996), cells were incubated with HE (160 μM) and samples analyzed using a Coulter XL flow cytometer. Debris were excluded from the analysis by electronic gating of forward and side scatter measurements.

Statistical analysis

Data were analyzed for statistical significance by using parametric or nonparametric tests on the basis of the distribution of the data. Comparisons of variables among groups were made by analysis of variance (ANOVA on ranks), and the Pearson Correlation was used to correlate the data with the donor age. Significance was set at the 5% level ($p < 0.05$). Statistical analysis was performed with SigmaStat software version 1.03 (Jadel Scientific, Germany).

Results

Functional parameters of in vitro *activated naive and memory $CD4^+$ T cells*

Naive and memory T cells were sorted among $CD4^+$ T lymphocytes through magnetic beads and then cytofluorimetry. Enriched populations were then *in vitro* activated for three days with PHA and PMA, and further cultured for 7 days with IL-2. On day ten of culture, cells were checked for viability and proliferative capacity. As shown in Figure 1a, naive and memory $CD4^+$ T cells at the end of culture had a cell viability greater than 90%, without statistically significant difference in the three aged-groups considered (20–45, 46–70, and >70 years-old). Similarly, the proliferative capacity of naive and memory enriched $CD4^+$ T cell populations did not show significant changes among donors of different age (Figure 1b).

Apoptosis and Bcl-2 expression of naive and memory $CD4^+$ T cell populations after IL-2 deprivation

To analyze the functional responsiveness of lymphocyte populations to deprivation stimuli, apoptosis of naive and memory $CD4^+$ T cells was studied after overnight deprivation of IL-2. As shown in Figure 2a, b, the apoptosis of both naive and memory $CD4^+$ T cells was positively correlated with the age of subjects after removal of IL-2 from the culture medium ($R = 0.49$, $p = 0.003$, and $R = 0.43$, $p = 0.01$, for naive and memory $CD4^+$ T cells, respectively).

To investigate whether the apoptosis induced by IL-2 deprivation was related to an age-related reduction of Bcl-2 expression, naive and memory $CD4^+$ T cells were analyzed for Bcl-2 protein

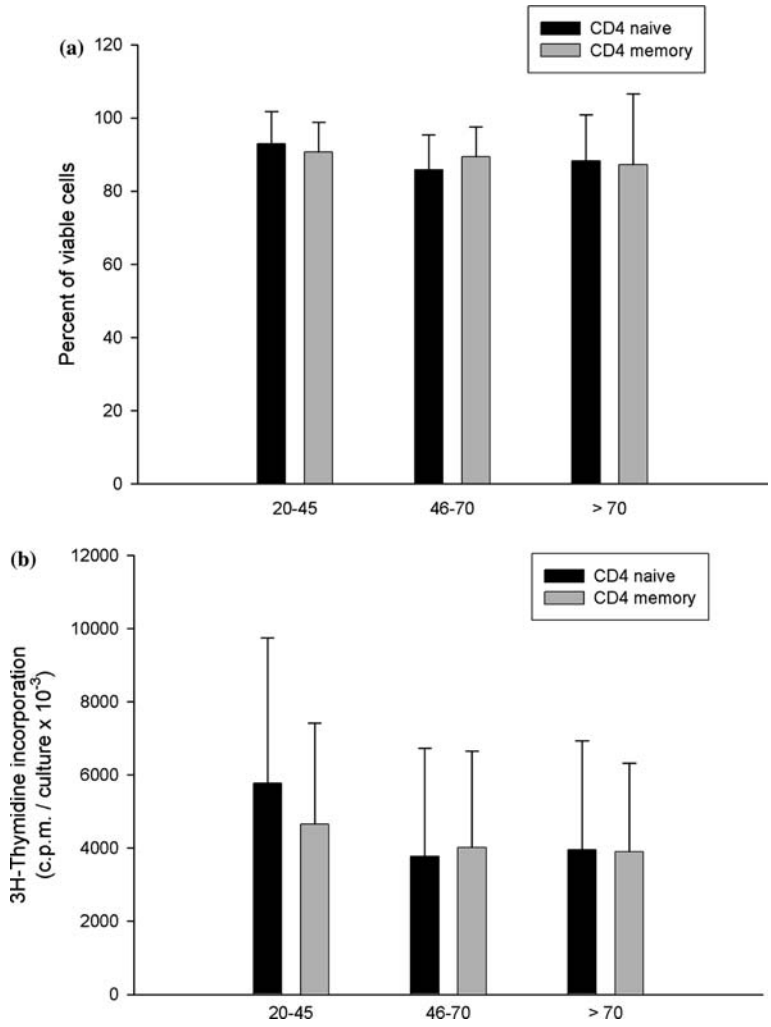

Figure 1. Viability and proliferative capacity of purified naive and memory $CD4^+$ T cells. $CD62L^+CD95^-$ naive and $CD62L^-CD95^+$ memory $CD4^+$ T cells from donors with increasing age were purified and cultured for 10 days. At the end of culture cells were analyzed for viability (a) by trypan blue exclusion, and for proliferative capacity (b) by ^{3}H-TdR uptake. In purified naive and memory $CD4^+$ T cells both the cell viability and the proliferative capacity did not show significant changes among donors of different age.

content through staining with a specific monoclonal antibody before and after cytokine deprivation. As shown in Figure 3 (panel A), on day ten of culture, Bcl-2 expression was not correlated with donor age in both naive and memory $CD4^+$ T cells. Differently, a statistically significant negative correlation between Bcl-2 expression and age of subjects was found both in naive ($R = -0.42$, $p = 0.006$) and memory ($R = -0.32$, $p = 0.04$) $CD4^+$ T cells after one day IL-2 deprivation (Figure 3, panel B).

Apoptosis and CD95 expression of naive and memory $CD4^+$ T cell populations after PHA administration

The apoptosis induced by PHA addition in naive and memory $CD4^+$ T cells was then analyzed. As shown in Figure 4a, b, PHA addition significantly increased the percentage of apoptotic naive and memory $CD4^+$ T cells. Differently from the apoptosis induced by IL-2 deprivation, the percentage of apoptotic cells

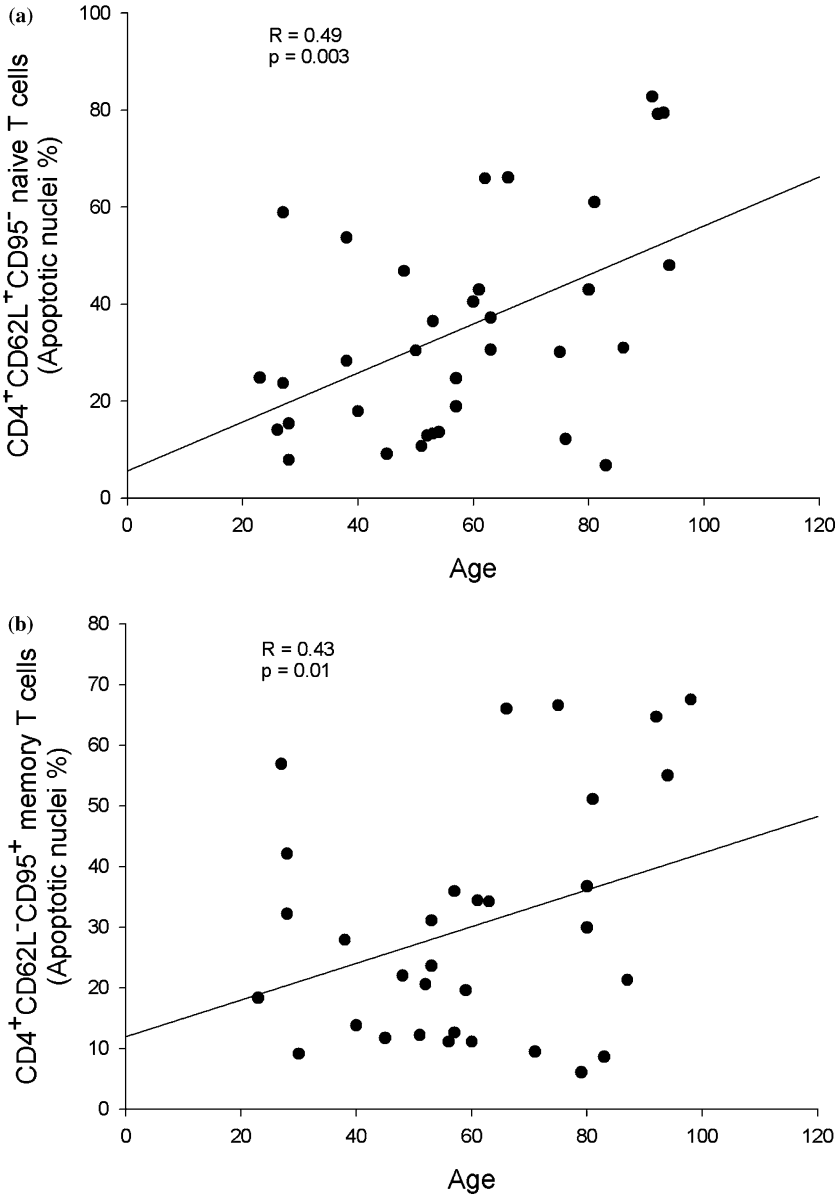

Figure 2. Effect of cytokine deprivation on apoptosis in purified naive and memory $CD4^+$ T cells during ageing. $CD62L^+CD95^-$ naive and $CD62L^-CD95^+$ memory $CD4^+$ T cells from donors with increasing age were purified, cultured for 10 days, and deprived of IL−2 for 24 h. Apoptosis of naive (a) and memory (b) $CD4^+$ T cells was determined through subdiploid DNA peak analysis after staining with Propidium Iodide (PI). The number of apoptotic cells significantly increased with age in purified naive and memory $CD4^+$ T cells. *R* and *p* values were calculated by linear regression analysis.

after PHA addition was not significantly correlated with age, both in naive and memory $CD4^+$ T cells (Figure 4c, d).

To determine whether the apoptosis induced by PHA administration was related to the level of CD95 expression, naive and memory $CD4^+$ T cells were analyzed for CD95 protein through staining with a specific monoclonal antibody before and after PHA addition. As shown in Table 1, a low but not significant increase of

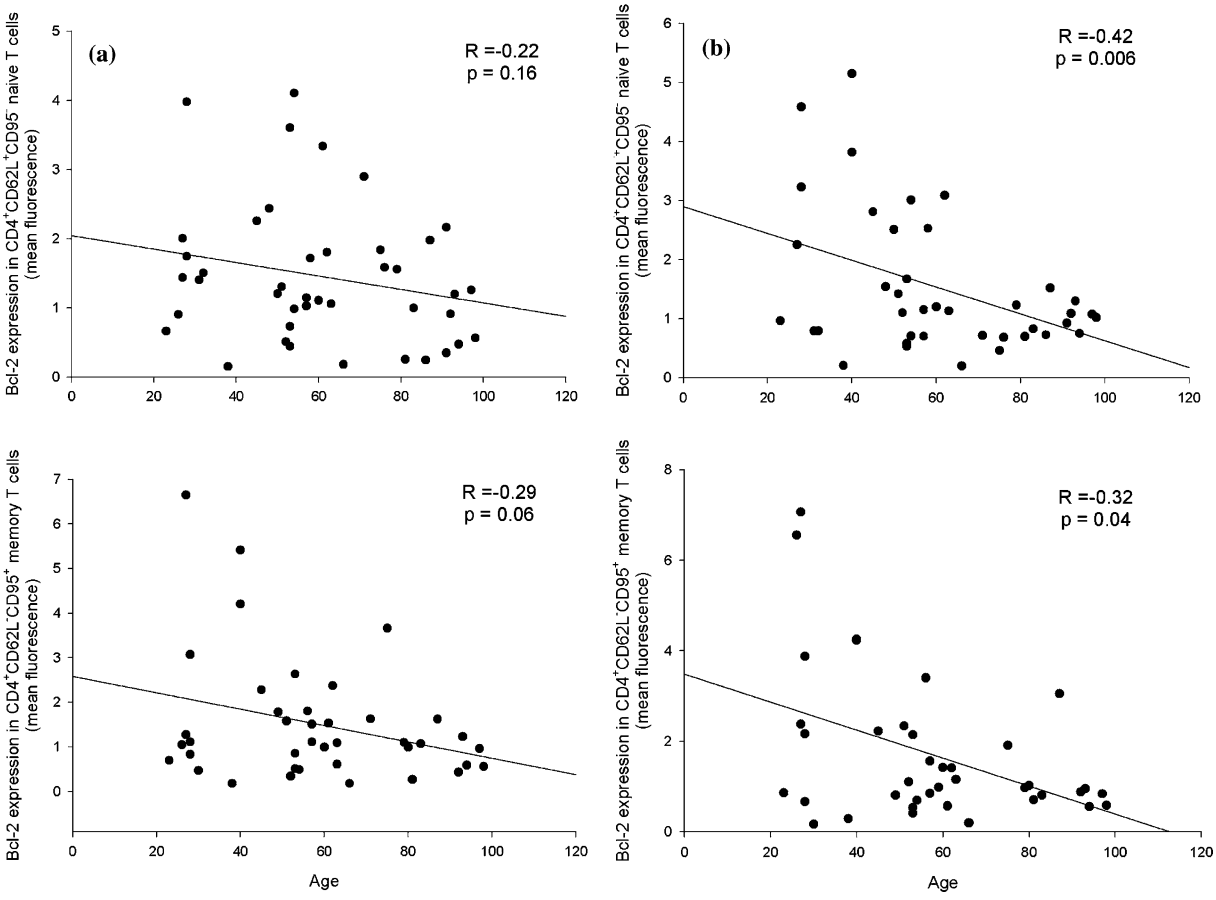

Figure 3. Effect of cytokine deprivation on Bcl-2 expression in purified naive and memory $CD4^+$ T cells during ageing. $CD62L^+CD95^-$ naive and $CD62L^-CD95^+$ memory $CD4^+$ T cells from donors with increasing age were purified, cultured for ten days, and deprived of IL−2 for 24 h. Bcl-2 expression of naive and memory $CD4^+$ T cells was determined through cytofluorimetric analysis by staining with PE-conjugated mouse anti-human Bcl-2, both before and after IL-2 deprivation. Before cytokine deprivation, Bcl-2 expression was not significantly correlated with age in purified naive and memory $CD4^+$ T cells (panel A); after IL−2 deprivation, Bcl-2 expression was significantly reduced with advancing age both in naive and memory $CD4^+$ T cells (panel B). *R* and *p* values were calculated by linear regression analysis.

CD95 expression was observed after PHA administration both in naive and memory $CD4^+$ T cells. CD95 expression was not related to the donor age either before or after PHA addition.

Intracellular ROS in naive and memory $CD4^+$ T cells after IL−2 deprivation or PHA addition

To determine whether apoptosis induced by IL-2 deprivation or PHA addition in naive and memory $CD4^+$ T cells correlated with a modulation of reactive oxygen species (ROS), we quantified the intracellular redox status in T cells by measuring dihydroethidium fluorescence as an indicator of endogenous superoxide levels. As shown in Figure 5, the apoptosis induced by cytokine deprivation was associated with a positive correlation between the intracellular content of ROS and the age of subjects, both in $CD4^+$ naive ($R=0.41$, $p=0.03$) and $CD4^+$ memory T cells ($R=0.48$, $p=0.01$) (Figure 5a, c). A positive but not statistically significant relationship was found between the intracellular content of ROS and donor age in T cell induced apoptosis by PHA, both in naive and memory $CD4^+$ cells (Figure 5b, d).

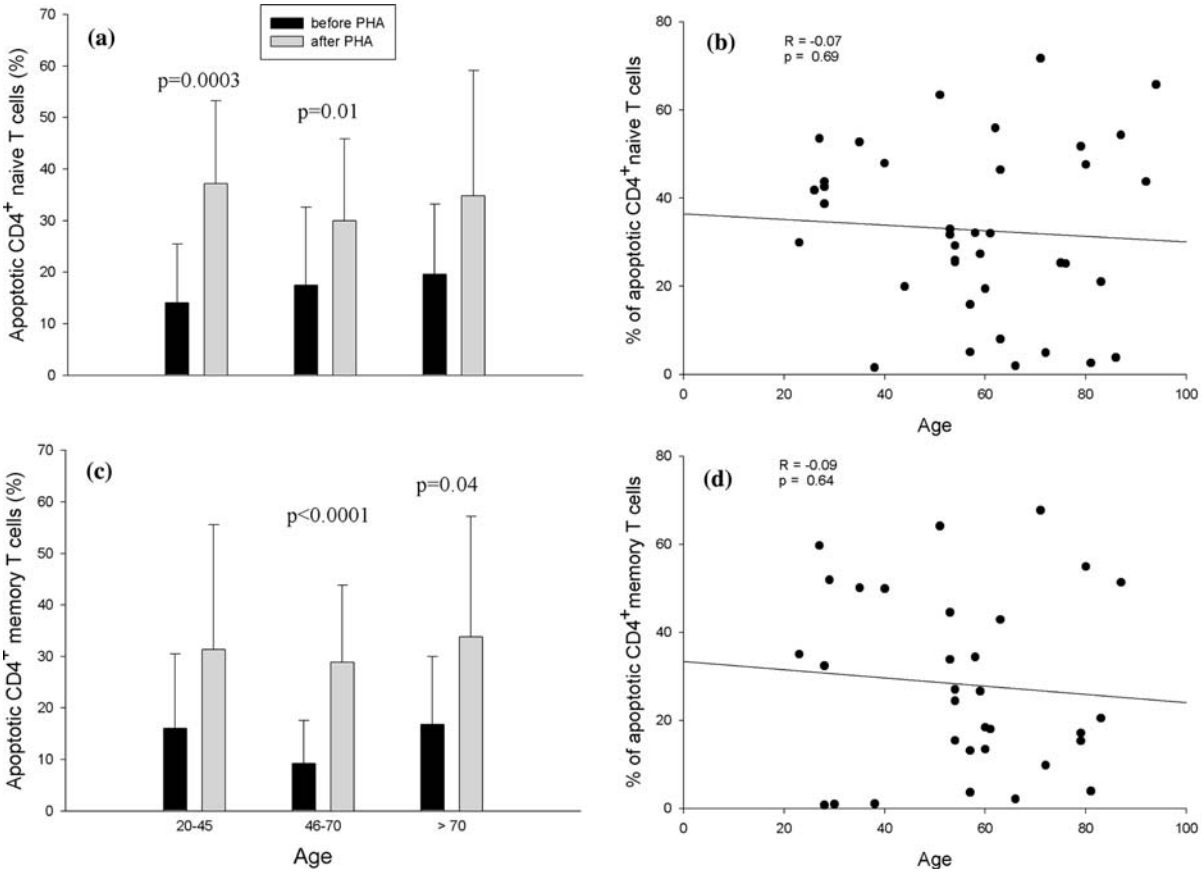

Figure 4. Effect of PHA on apoptosis in purified naive and memory $CD4^+$ T cells during ageing. $CD62L^+CD95^-$ naive and $CD62L^-CD95^+$ memory $CD4^+$ T cells from donors with increasing age were purified, cultured for 10 days, and supplemented with PHA for 24 h. Apoptosis was determined through Annexin V/Propidium iodide staining. (a, c) Apoptosis was increased by PHA supplementation both in naive and memory $CD4^+$ T cells. (b, d) The number of apoptotic cells, calculated as the number of Annexin V ($FITC^+$) and PI^- cells, was not significantly correlated with age either in naive and memory $CD4^+$ T cells. *R* and *p* values were calculated by linear regression analysis.

Discussion

Immunosenescence has been associated with a numerical change in T cell repertoire, mainly represented by an expansion of memory and a reduction of naive T lymphocytes (De Paoli et al. 1988; Utsuyama et al. 1992; Cossarizza et al. 1996; Fagnoni et al. 2000; Fahey et al. 2000). However, no definitive data has been reported on the functional characteristics of these T cell

Table 1. CD95 expression in naive and memory $CD4^+$ T lymphocytes before and after PHA addition.

Age of donors	$CD4^+CD62L^+CD95^-$ naive T cells		$CD4^+CD62L^-CD95^+$ memory T cells	
	Before PHA	After PHA	Before PHA	After PHA
20–45	6.7 ± 3.4	8.3 ± 3.3	5.3 ± 3.5	6.4 ± 3.2
46–70	5.3 ± 2.4	7.0 ± 3.5	5.5 ± 2.1	6.2 ± 1.9
> 70	4.8 ± 2.0	5.9 ± 1.6	6.2 ± 1.2	6.3 ± 1.4

Results are expressed as Mean Fluorescence ± S.D.

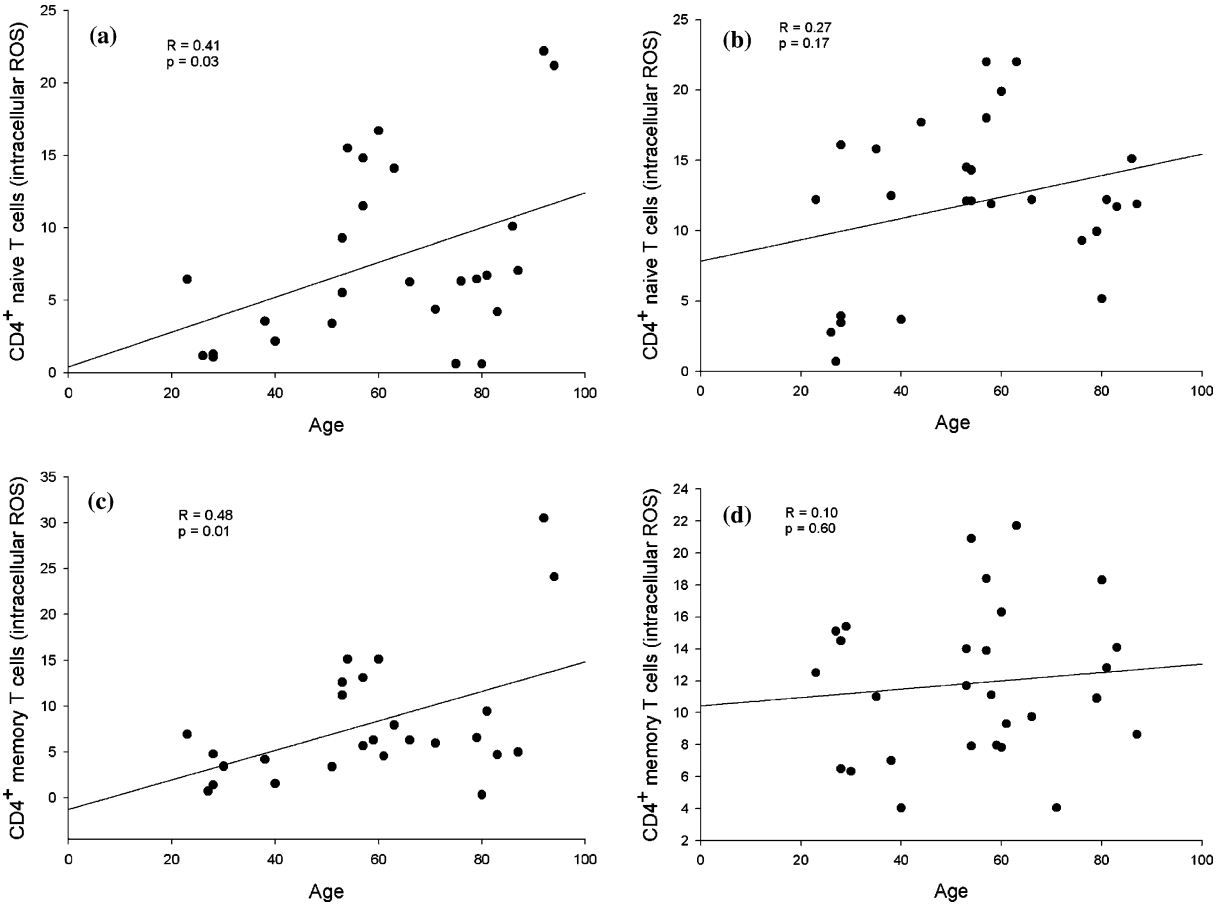

Figure 5. Effect of age on intracellular reactive oxygen species (ROS) in purified naive and memory $CD4^+$ T cells. $CD62L^+CD95^-$ naive and $CD62L^-CD95^+$ memory $CD4^+$ T cells from donors with increasing age were purified, cultured for ten days, and deprived of IL−2 (a, c) or supplemented with PHA (b, d) for 24 h. Intracellular ROS were determined through cytofluorimetry. *R* and *p* values were calculated by linear regression analysis. While the intracellular ROS significantly increased with age in purified naive and memory $CD4^+$ T cells after IL-2 deprivation (a, c), they were not significantly correlated with age after PHA supplementation both in naive and memory $CD4^+$ T cells (b, d).

populations, and on the mechanisms involved in their expansion or reduction, and in particular on their susceptibility to apoptosis.

The main results of this study demonstrate that both naive and memory $CD4^+$ T cells show an age-related increased susceptibility to apoptosis induced by cytokine deprivation but not of apoptotic mechanisms which reproduce activation-induced cell death (AICD).

It is known that apoptosis can be considered a physiological mechanism for normal development and for maintenance of cellular homeostasis in multicellular organisms (Wyllie et al. 1980; Cohen 1996). In lymphocytes, apoptosis plays an important role in maintaining T cell repertoire and deletion of autoreactive T and B lymphocytes, thus limiting immune responses (Osborne 1996). Many reports indicated a different degree of apoptosis in lymphocytes from old individuals, with enhanced or reduced apoptosis in comparison with younger subjects (Chrest et al. 1995; Cossarizza et al. 1996; Phelouzat et al. 1996; Fahey et al. 2000; Yen et al. 2000; Zanni et al. 2003). In order to investigate the mechanisms involved in the age-related numerical alterations of naive and memory T cells, we have evaluated the susceptibility of purified naive and memory $CD4^+$ T cells from donors of different age to

undergo apoptosis. We have studied, in both naive and memory $CD4^+$ T cells, two major mechanisms involved in T cell elimination. One of these mechanisms involves the re-legation of T cell receptor (TCR) on activated T cells, leading to apoptosis, a process known as activation-induced cell death (AICD) (Krammer et al. 1994; Lynch et al. 1995; Vaux and Strasser 1996). A second mechanism of T cell elimination by apoptosis can result from cytokine deprivation, this process being at least as important as AICD in down regulating activated T cell populations (Akbar 1993a,b, 1996; Cohen 1993). AICD requires interaction between CD95 (Fas/APO-1) and its ligand (FasL); CD95 ligand is up-regulated following TCR stimulation when large quantities of antigen are present. This Fas/FasL interaction, in overstimulated T cells, determines death rather than proliferation. AICD protects the host from excessive immune stimulation, where large numbers of T cells and considerable quantities of antigen are present (Abbas 1996). Conversely, the apoptosis induced by cytokine deprivation is correlated with a decrease in apoptosis-inhibitory proteins (Bcl-2 and Bcl-x_L). In particular, activated T cells require the continuous presence of interleukin 2 (IL-2) for clonal expansion and prevention of apoptosis (Cohen 1993). IL-2 prevents activated T cell apoptosis by up-regulating the expression of Bcl-2 and Bcl-x_L (Strasser et al. 1991); Akbar et al. 1993b; Broome et al. 1995) whereas the removal of IL-2 from activated T cells leads to reduced Bcl-2 expression and apoptosis (Akbar et al. 1993a). At the end of an immune response, when cytokine synthesis decreases, activated T cells down regulate expression of anti-apoptotic proteins such as Bcl-2 and Bcl-x_L, undergoing to cell death.

In our work we have studied the two mechanisms of apoptosis in purified naive and memory $CD4^+$ T cells and we have found an age-related difference in apoptosis induced by IL-2 deprivation but not in AICD determined by PHA. In particular, after cytokine deprivation, the percentage of naive and memory $CD4^+$ apoptotic cells significantly increased with age of subjects, concomitantly with an age-related reduction of Bcl-2 expression. With regards to this last point, our results agree with those reported in other papers, showing a decreased Bcl-2 expression in both $CD4^+$ and $CD8^+$ unpurified T cells from aged subjects as compared to young controls (Aggarwal and Gupta 1998; Gupta 2000). Furthermore, our data extend previous findings, firstly reporting a correlation between the increase of apoptosis after cytokine deprivation and the reduction of the apoptosis-inhibiting factor Bcl-2 in purified T cells from subjects with increasing age.

Fas (CD95) and FasL (CD95L) system is mainly involved in T lymphocyte AICD (Russel 1995). In our work AICD was studied in naive and memory $CD4^+$ T cells after overnight addition of PHA; in these conditions we have observed in both naive and memory cell populations a little augment of CD95 expression which correlated with increased apoptosis. Differently with the apoptosis induced by cytokine deprivation, either the percentage of apoptotic cells and the CD95 expression in AICD were not significantly correlated with age, both in naive and memory $CD4^+$ T cells. Herndon et al. (1997), studying a very little number of young and old subjects (seven donors for each age group), reported an increased apoptosis of T cells with naive phenotype following *in vitro* activation of unpurified T cells from aged subjects with PHA and IL-2. Differently, T cells with memory phenotype were susceptible to activation-induced apoptosis to a similar degree among young and old individuals (Herndon et al. 1997). These data do not completely agree with our findings, because of evident differences between the two studies, mainly related to the different number of subjects examined in our and Herndon's studies, and to the fact that we examined purified naive and memory cell populations rather than peripheral blood mononuclear cells. Aggarwal and Gupta (1998) reported an increased expression of Fas and Fas ligand in both $CD4^+$ and $CD8^+$ T cell subsets from ageing subjects. Furthermore, they observed an increased apoptosis in $CD4^+$ and $CD8^+$ T cells from ageing donors, after anti-Fas Ab treatments, as compared with the same lymphocytes from young controls. Also, other papers have suggested an increased expression of CD95 in lymphocytes from old subjects but in these studies the CD95 analysis was performed in fresh or in *ex vivo* human PBMCs

(Phelouzat et al. 1996; Potestio et al. 1998). The lack of a significant correlation between CD95 expression and age of subjects that we have described might be related to an increase of CD95 expression in cells from young donors during activation to the levels found in old subjects. In support of this possibility are the data from Phelouzat et al. (1996), which have reported an age-related difference in Fas receptor expression among young and old donors until 24 h after activation, which disappeared after 48 h of lymphocyte culture.

To determine whether apoptosis induced by IL-2 deprivation or PHA addition in naive and memory $CD4^+$ T cells correlated with an increase in reactive oxygen species (ROS), we quantified the intracellular redox status in T cells. Our results suggested a positive correlation between the intracellular content of ROS and the age of subjects, both in naive and memory $CD4^+$ T cells after cytokine deprivation. Conversely, we have found that the apoptosis induced by PHA addition in different aged donors was not significantly correlated with the intracellular content of ROS. It has been observed that Bcl-2 is localized to intracellular sites of oxygen free radical generation including mitochondria, endoplasmic reticula, and nuclear membranes, and that Bcl-2 prevents cell death by decreasing the net cellular generation of ROS (Hockenbery et al. 1993; Kane et al. 1993). These observations agree with our results clearly showing the strict relationship between Bcl-2 and ROS in regulating apoptosis and suggesting that alterations in these regulatory mechanisms may occur with advancing age.

In conclusion, our data demonstrate the existence of functional alterations of naive and memory T cell populations during ageing. These alterations are mainly related to the mechanism of the apoptotic event (cytokine deprivation *versus* AICD) rather than to the type of cell population involved (naive or memory). The reduced IL-2 levels found in aged subjects (Rea et al. 1996) emphasize the relevance of our findings in providing new insights into the mechanisms implicated in age-related T-cell-mediated immunodeficiency, which are associated with the increased susceptibility to infections and cancer diseases present in the elderly.

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