

# Age-related changes of apoptotic cell death in human lymphocytes

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## Abstract

Apoptosis seems to be involved in immunosenescence associated with aging. Moreover, in lymphocytes (PBL) of patients with Alzheimer's disease, an increased susceptibility to the apoptotic pathway has been described possibly due to impaired protection of oxidative stress. Accordingly, it seemed to be of particular interest to investigate the contribution of normal aging to the susceptibility from human lymphocytes to programmed cell death. We could show that PBL from elderly individuals (>60 years) accumulate apoptosing cells to a significant higher extent in spontaneous and activation-induced cell death compared to younger controls (<35 years). Treatment with the oxidative stressor 2-deoxy-D-ribose or with agonistic-CD95-antibody pronounced this effect even more implicating a higher sensitivity to reactive oxygen species and a higher functional CD95 expression, respectively. In addition, expression of the activation markers HLA-DR and CD95 was significantly increased in CD3<sup>+</sup>-cells of aged subjects, while expression of CD25 did not seem to be affected by age. Expression of Bcl-2 was increased in aging and correlated with the number of apoptotic cells.

*Keywords:* Aging; Apoptosis; Lymphocytes; Oxidative stress; Activation markers; Bcl-2

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## 1. Introduction

Programmed cell death (PCD) or apoptosis is of particular interest in aging, as it is thought to play an important role in various age-related degenerative diseases. Apoptosis of white blood cells could be one reason for the decrease of the total number of leukocytes and the decrease of the immune response with aging related to cancer, infections, and autoimmune disorders [3]. Several changes in lymphocytes have been observed related to aging: diminished synthesis of growth and survive factors [46,51], impaired intracellular calcium regulation [18], different surface molecule expression [43], and defects of signal transduction [36]. Some of these pathological changes are additionally altered in patients with Alzheimer's disease (AD), but are not present in vascular dementia [13–15,17]. In order to differentiate common and divergent mechanisms of an enhanced susceptibility of lymphocytes to apoptosis in aging and sporadic AD, we investigated in detail the molecular basis of enhanced apoptosis in aged human lymphocytes.

Oxidative stress increases with aging and leads to enhanced cellular damage (e.g. lipidperoxidation, protein oxidation, DNA damage (for review see ref. [10]). In addition, reactive oxygen species (ROS) can trigger cells to undergo programmed cell death and can act as second messenger by influencing transcription factors like NF- $\kappa$ B and AP-1. Increased oxidative stress and decreased ability to cope with ROS could amplify apoptotic cell death in aging lymphocytes. Bcl-2 acts in an antioxidative and antiapoptotic way and can interfere with the apoptotic pathway [7,47].

One of the best known and characterized surface receptors related to PCD in lymphocytes is Apo1/Fas (CD95), a member of the tumor necrosis factor (TNF) superfamily. Stimulation of this receptor by binding to its physiological ligand (Fas-L) or an agonistic anti-Fas antibody leads to apoptosis via a distinct pathway [26]. In addition, activated lymphocytes express the  $\alpha$ -chain of the IL2-receptor (CD25) and the MHC class-II related molecule HLA-DR. Lymphocytes bearing these surface molecules or activation markers [4] show enhanced sensitivity to apoptosis upon stimulation of these receptors. Accumulation of chronically activated lymphocytes seems to be one reason for the higher susceptibility to apoptotic cell death probably leading to a lowered leukocyte number in aged individuals. In the

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present study, we evaluated the basal, spontaneous, activation-, and agent-induced apoptosis and ROS of native and activated peripheral human lymphocytes. In addition, basal expression of activation markers (CD25, CD95, and HLA-DR) and Bcl-2 was determined.

## 2. Materials and Methods

### 2.1. Subjects

Blood samples were taken from  $n = 85$  healthy subjects (55 women and 30 men) between 15 and 93 years. There were 20 women and 12 men in the aged group ( $n = 32$ ; aged over 60 years, mean age  $73.8 \pm 8.3$  years) and 25 women and 9 men in the group of young controls ( $n = 34$ ; age under 35 years, mean age  $26.4 \pm 5.1$  years). The remainders were part of the intermediate ages shown in Figs. 3, 4, and 6. Subjects with psychiatric and neurodegenerative disorders or acute infections were excluded. In addition, subjects with pathological distribution of lymphocyte subpopulations in flow-cytometric analysis according to the Senieir protocol [25] were rejected from the study. Most of the elderly patients were taking drugs against cardiovascular disorders. None of the patients received psychotropic medication, drugs with known effects on the immune system, or antioxidative agents.

### 2.2. Cells

Peripheral blood lymphocytes (PBL) were prepared from healthy donors by separating on Ficoll (Biochrom KG, Berlin, Germany) as previously described [16]. Cells were investigated directly (native cells/PBMC) or after short-term culture (activated cells or so called lymphoblasts) as indicated. In concordance with other studies [1,48], the T cell subsets CD4 and CD8 did not differ significantly with aging, as measured in wholeblood by FACS analysis. For short-term culture, cells were seeded at a density of  $0.5 \times 10^6$ /ml in complete medium (RPMI-1640 supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml; all GibcoBRL, Karlsruhe, Germany) supplemented with 5  $\mu$ g/ml PHA-L (Sigma, Deisenhofen, Germany). After 48 h of incubation at 37°C and 5% CO<sub>2</sub>, cells were washed once in PBS (Gibco BRL), seeded in complete medium containing 50 U/ml of human recombinant IL-2 (Boehringer Mannheim), and incubated for 72 h to predispose to apoptosis according to the method of Zheng et al. [53]. Activated lymphocytes consisted to nearly 100% of CD3<sup>+</sup> cells and expressed CD95 to over 96% as determined in FACS analysis.

### 2.3. Induction of apoptosis

Cell death was induced with the following stimuli for 24 h:  $10^{-7}$  M dexamethasone, 10 mM 2-deoxy-D-ribose,

100 nM staurosporine (all purchased from Sigma) or 0.4 ng/ml agonistic CD95-antibody (clone 2R2, Boehringer Mannheim, Mannheim, Germany). Incubation of cells over 24 h with medium alone and in the absence of an additional stimulus is termed “spontaneous in vitro apoptosis.” Spontaneous in vitro apoptosis is defined by the cell death of primary adult cells that occurs after removing them from their natural environment and culturing them under in vitro conditions. Withdrawal of the growth factor IL-2 leads lymphoblasts into the so-called activation-induced cell death (AICD).

### 2.4. Assays of apoptotic cell death

For analyzing the sub-G<sub>1</sub> DNA content, which is defined as percent apoptotic cells, lymphocytes were harvested by centrifugation at various time points and the pellets resuspended in lysis buffer (0.1% sodium citrate and 0.1% Triton X-100) containing 50  $\mu$ g/ml of propidium iodide (all Sigma) ([29]). Samples were stored at 4°C for 1–2 h before flow cytometry analysis (FACSCalibur) using CELLQUEST software (both Becton Dickinson).

### 2.5. Determination of oxidative stress

The production of ROS was detected by flow cytometry using dihydrorhodamine 123 (DHR; Molecular Probes) as described elsewhere [35,38]. Lymphocytes ( $1.25 \times 10^5$ ) were suspended in 1 ml HBSS, and DHR was added to a final concentration of 10  $\mu$ M and incubated for 15 min at 37°C. Then, the conversion of DHR to its fluorescent derivative rhodamine 123 was detected. Control samples containing no stimulus were run in parallel, and all aliquots were stored on ice before analysis (“baseline”). The mean fluorescence intensity (MFI) in FL1 was calculated by determining the mean channel number (MCN) of the peak. This MCN value was converted to a linear value for comparative purposes by calculating the antilog of  $x = \text{sample mean channel}/(\text{total number of channels}/\text{number of log decades}) = \text{sample mean channel}/(1024/4) = \text{sample mean channel}/256$ . All data presented are expressed as  $\Delta$  MFI, representing the increase over baseline.

### 2.6. Examination of surface-expressed receptors

Determination of CD3, CD19, CD4, CD8, HLA-DR, CD56 + CD16, CD95, and CD25 was performed with an aliquot of the heparinized blood sample with the SimulSet staining kit (Becton Dickinson) according to the manufacturer's manual. Samples were assayed with FACSCalibur using SIMULSET software (all Becton Dickinson).

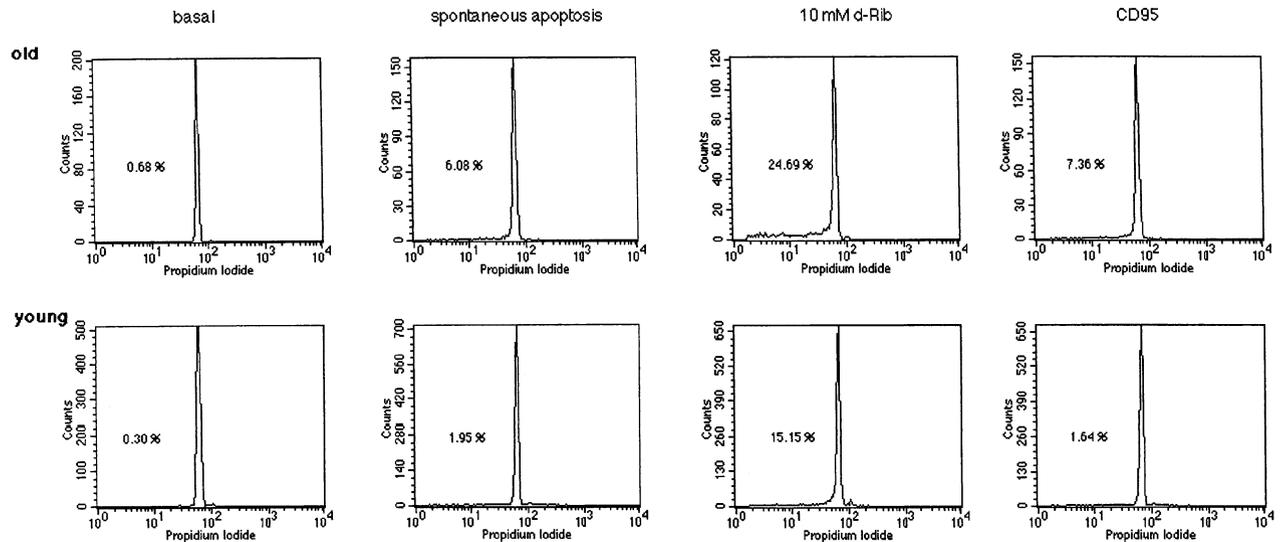


Fig. 1. Histogram plots of propidium iodide stained and lysed PBMC from each an old (upper level) and a young (lower level) individual. Percentage of sub-G<sub>1</sub>-DNA-content (% apoptotic cells) under different conditions is indicated. First column shows basal apoptotic levels of freshly isolated PBMC, the second displays the spontaneous in vitro apoptosis after 24 h in culture, and the last two columns exhibit dRib- and CD95-induced apoptosis after 24 h.

### 2.7. Quantitation of Bcl-2

The content of Bcl-2 was determined from peripheral lymphocytes ( $1 \times 10^6$  cells) with an ELISA assay (Oncogene, USA) according to the manufacturer's manual.

### 2.8. Statistics

Statistical analysis was performed by Student's paired/unpaired *t*-test or correlation calculations (PRISM 2.01; GraphPad Software, San Diego, CA, USA). All data presented are means  $\pm$  SEM.

## 3. Results

### 3.1. Basal and spontaneous apoptosis are increased in lymphocytes of elderly subjects

Using PI staining, we quantified sub-G<sub>1</sub>-DNA content in lymphocytes from healthy elderly and young controls. Fig. 1 shows the histogram plots of PI-stained lymphocytes of two representative subjects. Student's *t*-test revealed that the percentage of basal apoptotic nuclei in freshly isolated lymphocytes was significantly elevated in aged subjects compared to young controls (means  $\pm$  SEM: old,  $1.35 \pm 0.20\%$  versus young,  $0.84 \pm 0.11\%$ ; \*,  $P < 0.05$ ; Fig. 2a). In addition, we found a significantly higher content of cells undergoing spontaneous in vitro apoptosis after 24 h in the group of old subjects in relation to young ones (Fig. 2b; \*\*,  $P < 0.01$ ; old,  $8.06 \pm 0.85\%$ , median  $5.90\%$ ; young,  $5.52 \pm 0.43\%$ , median  $4.60\%$ ). Subtracting the background of basal cell death still revealed significantly higher levels of apo-

ptotic cells in lymphocytes from aged controls ( $\Delta$  increase over baseline: old,  $6.56 \pm 0.86\%$  versus young,  $4.33 \pm 0.42\%$ ; \*,  $P = 0.014$ ; Fig. 2c). Moreover, when all investigated subjects were pooled for regression analysis, increasing age was correlated with percentage of apoptotic cells by regression analysis. Spontaneous apoptosis after 24 h increased 2.1-fold (slope =  $0.061 \pm 0.02$ ) between 15 and 93 years of age [Fig. 3, dotted line (- -)]. There was a significant (\*\*\*,  $P < 0.001$ ,  $n = 85$ ) correlation between age and the portion of apoptotic cells. To ascertain that these alterations in apoptotic cell death were not linked to alterations in the distribution of PBMC subpopulations, the same experiments were performed with activated lymphocytes consisting to over 95% of CD3<sup>+</sup> cells. Lymphocytes, which are undergoing proliferation, can be triggered to programmed cell death by withdrawal of IL-2. IL-2 is the most critical determinant in this process [23]. Activated lymphocytes have been shown to be more sensitive to apoptosis than nontreated native cells. Basal apoptotic levels of activated T cells correlated significantly with the donor age (\*,  $P < 0.05$ ,  $n = 52$ ; Fig. 4; young,  $9.99 \pm 1.05\%$ , median  $9.87\%$  versus old,  $13.29 \pm 1.04\%$ , median  $12.17\%$ ). In addition, spontaneous in vitro apoptosis after 24 h (under these conditions also called AICD: activation-induced cell death, that is triggered by IL-2 treatment followed by withdrawal) did as well significantly correlate with age (\*,  $P < 0.05$ ,  $n = 45$ ; Fig. 4; young,  $16.22 \pm 1.29\%$ , median  $15.23\%$  versus old,  $19.58 \pm 1.45\%$ , median  $19.15\%$ ).

### 3.2. Increased basal levels of ROS in aging

The oxidative-sensitive fluorescent dye DHR 123 was used to detect presence of ROS in human lymphocytes.

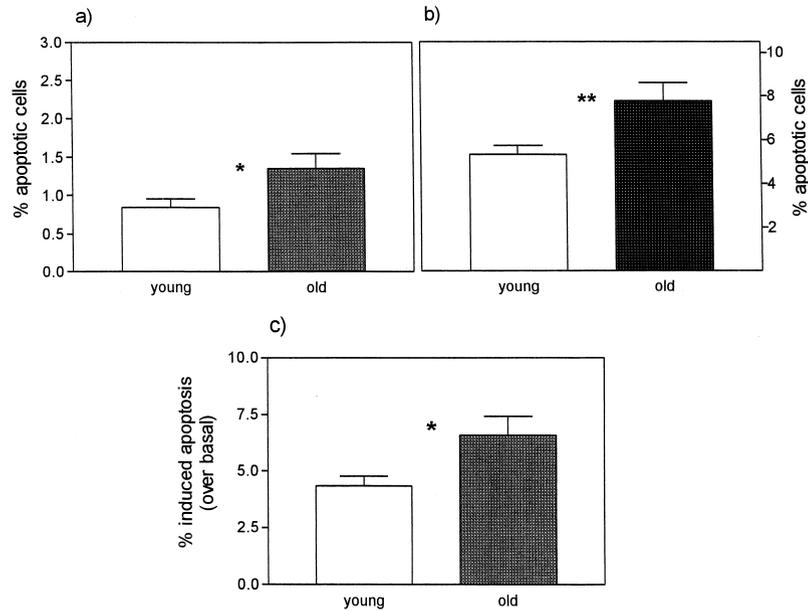


Fig. 2. (a) Basal levels of apoptotic nuclei in freshly isolated PBMC. Apoptosis was determined by propidium iodide staining as described in Section 2. Cells derived from aged humans (over 60 years old) display significantly more basal apoptotic nuclei ( $1.35 \pm 0.196\%$ ,  $n = 34$ ) than younger controls (up to 35 years old;  $0.840 \pm 0.113\%$ ;  $n = 32$ ; \*,  $P < 0.05$ , Student's  $t$ -test). (b) Spontaneous apoptosis in PBMC of young and old humans: cells were cultured for 24 h in the absence of stimuli. In aging, significantly more apoptotic cells accumulate under this condition. PBMC from old subjects displayed  $8.06 \pm 0.85\%$  apoptotic cells while young subjects showed only  $5.52 \pm 0.43\%$  apoptotic nuclei; \*\*,  $P < 0.01$ . (c) The age-related difference in spontaneous apoptosis is still present after subtraction of the individual basal background (young,  $4.33 \pm 0.42\%$  versus old,  $6.56 \pm 0.86\%$ ; \*,  $P = 0.014$ ).

Basal levels of ROS (expressed as MFI in arbitrary units) accumulate significantly in aged human lymphocytes (Fig. 5; \*,  $P < 0.05$ ;  $n = 26$ –27).

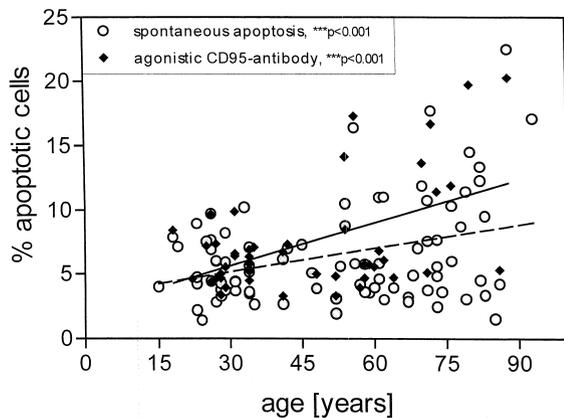


Fig. 3. Spontaneous and CD95-induced apoptosis in PBMC after 24 h incubation. Lymphocyte apoptosis correlates with increasing age. PBL from subjects ranging from 17 to 82 years of age were cultured 24 h in vitro and spontaneous apoptosis (○) was determined by flow cytometry analysis. % Apoptotic cells was correlated with donor age ( $r = 0.34$ ; \*\*\*,  $P < 0.001$ ,  $n = 85$ ). The percent increase per year is  $0.06 \pm 0.03\%$ . ◆ symbolize apoptosis of cells cultured for 24 h with an agonistic CD95-antibody. In addition, correlation with age is also highly significant ( $r = 0.50$ ; \*\*\*,  $P < 0.001$ ,  $n = 46$ ). CD95-induced apoptosis increases even more with age than the spontaneous in vitro apoptosis does (slopes of the linear regression analysis:  $0.122 \pm 0.04$ ;  $0.061 \pm 0.02$ , respectively).

### 3.3. PBMC from aged donors are more vulnerable to induction of apoptosis by oxidative stress

Oxidative stress was induced with 2-deoxy-D-ribose (dRib) [6]. Cells were incubated for 24 h at  $37^\circ\text{C}$ , harvested, and propidium iodide stained and analyzed by flow cytometry. In the presence of 10 mM dRib, the sub  $G_1$ -DNA content was elevated in both groups, but PBMC of elderly subjects react significantly more sensitive to this apoptotic

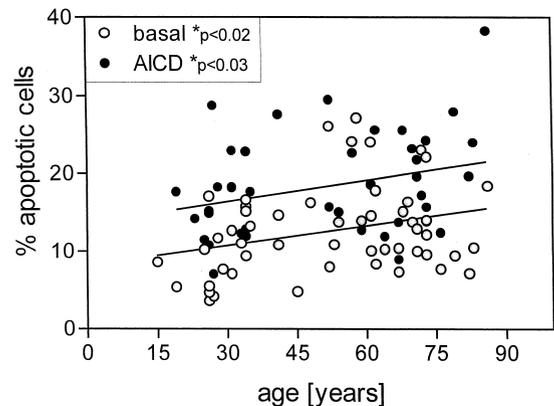


Fig. 4. Basal apoptotic levels and activation-induced cell death (AICD) of activated lymphoblasts correlate significantly with the age of the donor. ○ show basal apoptotic levels after lymphocyte activation as described in Section 2. Correlation with subjects' age is significant (\*,  $P < 0.02$ ;  $n = 52$ ). ● display AICD of activated lymphoblasts after 24 h withdrawal of IL-2 (\*,  $P < 0.03$ ;  $n = 45$ ).

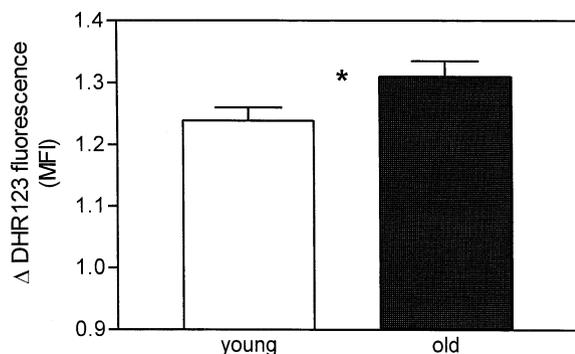


Fig. 5. Basal levels of ROS in human peripheral lymphocytes. Significantly higher levels could be determined in aging (old,  $1.310 \pm 0.025$ ;  $n = 27$ ; versus young,  $1.239 \pm 0.021$ ;  $n = 26$ ; \*,  $P < 0.05$ ).

stimulus than the younger group. After subtraction of spontaneous apoptosis, a significant increase of vulnerability to ROS-induced apoptosis was determined (Fig. 6: correlation \*,  $P < 0.05$  and Fig. 7a: old,  $7.92 \pm 1.8\%$  versus young,  $3.0 \pm 1.2\%$ ; \*,  $P < 0.05$ ), indicating an age-related impairment to cope with oxidative stress. In addition, higher concentrations of 2-deoxy-D-ribose (50 mM) yielded in a higher percentage of apoptotic cells, which was not any different between young and aged lymphocytes (old,  $7.22 \pm 1.20$  versus young,  $6.85 \pm 1.45$ ;  $n = 37$  per group;  $P = 0.42$ ). Moreover, PBMC derived from aged volunteers showed significantly increased levels of ROS, as determined by DHR123 fluorescence, after dRib treatment compared to young subjects (Fig. 7b; \*,  $P < 0.05$ ;  $n = 23$ –24).

#### 3.4. Functional CD95 expression is higher in lymphocytes of elderly subjects

Incubation for 24 h with an agonistic monoclonal anti-CD95 antibody that acts like a Fas-L, showed significant

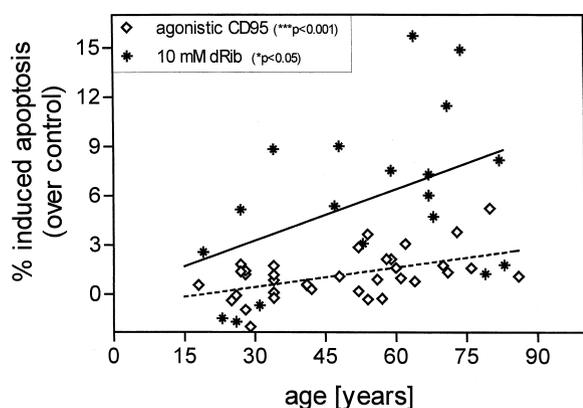


Fig. 6. Spontaneous in vitro apoptosis was subtracted from CD95- and dRib-induced apoptosis resulting in the percentage of apoptosis induced by the agent. This so-called induced apoptosis over control was correlated with age of subjects. \* Represent dRib-induced apoptosis;  $\diamond$  symbolize CD95-induced apoptosis.

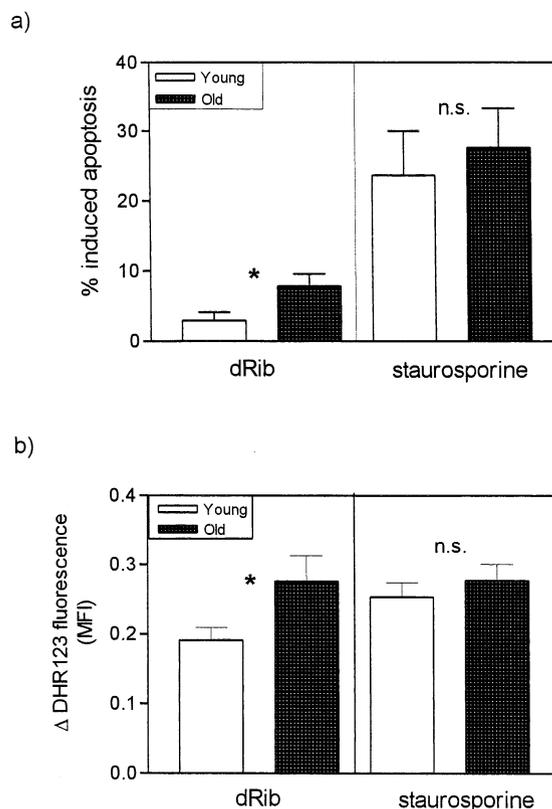


Fig. 7. (a) Induced apoptosis over control after 24 h by dRib (10 mM) and staurosporine (100 nM) in groups of young and old subjects (dRib: young,  $3.00 \pm 1.19$  versus old,  $7.92 \pm 1.74$ ,  $n = 11$ ; \*,  $P < 0.05$ ; staurosporine:  $23.71 \pm 6.33$  versus  $27.65 \pm 5.63$ ; NS;  $n = 11$ , respectively). (b) ROS levels in PBMC cultured for 16 h with dRib and staurosporine. Incubation with the oxidative stressor dRib leads to an age-related increase in DHR123 fluorescence ( $\Delta$  increase over baseline; old,  $0.276 \pm 0.037$  versus young,  $0.191 \pm 0.018$ ;  $n = 24$ ; \*,  $P < 0.05$ ). Treatment with the potent apoptotic agent staurosporine generates increased levels of ROS without any age-related differences (old,  $0.277 \pm 0.023$  versus young,  $0.259 \pm 0.022$ ,  $n = 16$ ).

changes in the induction of apoptosis between cells from young and old done on native cells (young,  $6.19 \pm 0.26\%$ , median 5.74%; versus old,  $11.05 \pm 1.78\%$ , median 9.11%; \*\*\*,  $P \leq 0.001$ ). Apoptotic cells correlated significantly with donor age after incubation with a CD95 antibody (\*\*\*,  $P < 0.001$ ; Fig. 3, black line). However, in young donors, the number of apoptotic cells is not altered after CD95 treatment compared to spontaneous apoptosis (paired  $t$ -test is not significant). In contrast, PBMC from aged donors are far more sensitive to apoptosis with agonistic CD95 antibody (paired  $t$ -test gave: \*\*\*,  $P < 0.001$ ).

By subtracting the spontaneous apoptosis in each subject, the CD95-induced apoptosis is determined that increases significantly with aging (Fig. 6).

#### 3.5. Elevated expression of activation markers in aging

In order to explore the mechanism for the increased susceptibility to apoptosis in PBMC from aged donors, we

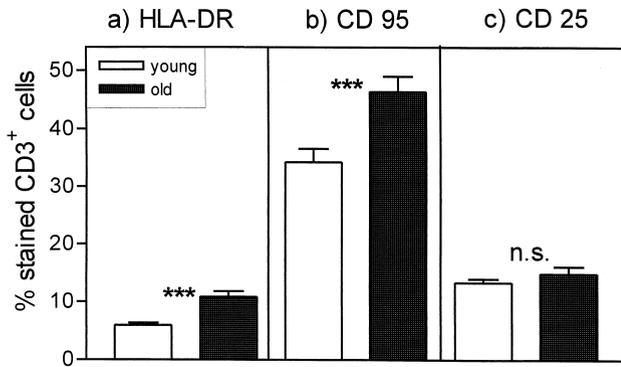


Fig. 8. Determination of surface expressed activation markers of CD3<sup>+</sup>-lymphocytes from young and old genders ( $n = 32-34$ ). T cells from young donors displayed a significantly (\*\*\*,  $P < 0.0001$ ) smaller portion of HLA-DR-positive cells ( $5.9 \pm 0.4\%$ ) than T lymphocytes from older subjects ( $10.8 \pm 1.0\%$ ). CD3<sup>+</sup> cells from young donors expressed significantly less CD95 receptors than elderly subjects (young,  $34.2 \pm 2.3\%$  versus old,  $46.4 \pm 2.6\%$ ). No significant changes with age concerning the expression of CD25 could be determined (young,  $13.3 \pm 0.7\%$  versus old,  $14.8 \pm 1.3\%$ ).

determined the expression of activation markers HLA-DR, CD95 (Apo, Fas), and CD25 (IL-2-receptor). Quantitation of T lymphocytes expressing HLA-DR, CD95, and CD25 (IL-2-R $\alpha$ ) was performed by direct immunofluorescence in wholeblood followed by FACS analysis (Fig. 8). CD3<sup>+</sup> T cells from young donors (up to 35 years) showed a significantly (\*\*\*,  $P < 0.0001$ ) smaller portion of HLA-DR-positive cells ( $5.9 \pm 0.4\%$ ) than CD3<sup>+</sup>-T lymphocytes from aged subjects ( $10.8 \pm 1.0\%$ ). CD3<sup>+</sup> cells from young donors expressed significantly less CD95 receptors than elderly subjects over the age of 60 years (young,  $34.2 \pm 2.3\%$  versus old,  $46.4 \pm 6\%$ ). No significant changes with age concerning the expression of CD25 could be detected (young,  $13.3 \pm 0.7\%$  versus old,  $14.8 \pm 1.3\%$ ).

The portion of cells displaying CD95 correlates, as expected, significantly with the percentage of CD95-agonistic antibody-induced apoptosis (data not shown), confirming the age-related increase in CD95-induced cell death.

The more the peripheral lymphocytes express CD95 and CD25 at basal levels, the higher is the percentage of cells undergoing apoptosis initiated by glutathione depletion (10 mM and 50 mM dRib) as shown by correlation (Fig. 9), indicating that these lymphocytes are more susceptible to oxidative stress.

### 3.6. Apoptosis induced by staurosporine does not correlate with aging

To examine whether the age-related apoptosis is associated with oxidative stress and/or higher expression of activation receptors, we also induced PCD in PBMC by staurosporine. Incubation of PBMC with 100 nM staurosporine showed a dramatic increase in the percentage of apoptotic cells compared with the untreated controls undergoing spontaneous apoptosis (\*\*\*,  $P < 0.001$ ). However, no significant effect could be determined related to aging (Fig. 7a). Staurosporine induces apoptosis independently from the donor's age. Moreover, we investigated the content of ROS in staurosporine-induced apoptosis. The elevated levels of ROS compared to basal levels are in line with the assumption that staurosporine-induced cell death is downstream mediated by ROS, but mainly upstream by caspases [21] in contrast to dRib where ROS act upstream [20]. No age-effect was found for the staurosporine-induced elevation of ROS (Fig. 7b).

### 3.7. Elevated levels of Bcl-2 in lymphocytes from aged humans

Overexpression of Bcl-2 counteracts apoptotic processes [7] probably by activating anti-oxidative mechanisms [47]. To investigate the link between apoptosis and oxidative stress in peripheral human lymphocytes, we determined the basal content of Bcl-2 in freshly isolated PBMC by ELISA. The amount of Bcl-2 increases slightly but not significantly in aging (Fig. 10, inset). Moreover, there was a positive correlation between the content of Bcl-2 and the percentage of basal apoptotic cells (Fig. 10; \*,  $P < 0.05$ ;  $n = 18$ ). In

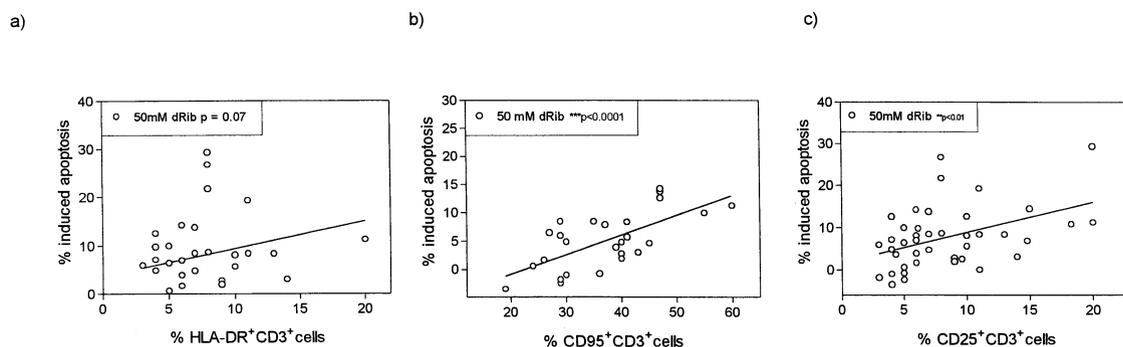


Fig. 9. Correlation of dRib-induced (50 mM) apoptosis and basal expression of activation markers. Correlation with (a) HLA-DR (NS,  $P = 0.07$ ,  $n = 33$ ); (b) with CD95 (\*\*\*,  $P < 0.0001$ ;  $n = 25$ ); and (c) with CD25 (\*\*,  $P < 0.01$ ,  $n = 41$ ).

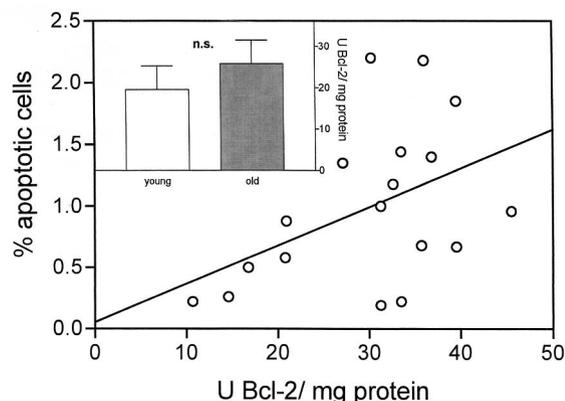


Fig. 10. Basal expression of Bcl-2 increases with aging (inset) in human PBMC. Peripheral cells of young subjects displayed  $19.6 \pm 5.7$  U Bcl-2/mg protein, PBMC of aged volunteers expressed  $25.8 \pm 5.7$  U Bcl-2/mg ( $n = 9$ ). Content of Bcl-2 correlates significantly (\*,  $P < 0.03$ ;  $n = 18$ ) with basal percentage of apoptotic cells in freshly isolated PMBC from young and old donors.

PHA-L and IL-2-activated lymphoblasts, the expression of Bcl-2 correlates significantly with donor age (\*,  $P < 0.05$ ), in activated cells from younger donors more Bcl-2 could be detected than in activated cells derived from aged subjects (old,  $32.71 \pm 4.83$  U Bcl-2/mg protein versus young,  $61.07 \pm 15.85$  U Bcl-2/mg protein,  $n = 7$  per group,  $P = 0.056$ ), indicating an important role of Bcl-2 in lymphocyte activation.

#### 4. Discussion

Previous results of our group reported an increased content of basal and dRib-induced apoptotic cells in PBMC from patients with Alzheimer's disease relative to age-matched controls [13,40]. This effect is probably not related to the physiological process of immunosenescence. In addition, apoptosis is a hallmark in brains derived from Alzheimer's patients, possibly associated with states of increased oxidative stress [8,45,49,52]. Similar alterations could also be detected in peripheral cells of AD patients [27,31]. In order to understand the specific contribution of AD to the enhanced susceptibility of lymphocytes to apoptosis, it is of particular importance to know first the role of normal aging without dementia. In this study, we explored therefore the age-related apoptotic behavior of peripheral human lymphocytes and the role of oxidative stress.

In the present study, we showed age-related differences in the vulnerability of cell death. PBMC and activated CD3<sup>+</sup> lymphoblasts from elderly subjects have an enhanced susceptibility to spontaneous and activation-induced in vitro apoptosis. We found some overlap of apoptotic features for spontaneous or activation-induced in vitro apoptosis between young and old controls. Hereby, few old control subjects showed low levels of apoptotic cells, whereas others exhibited very high apoptotic features. We can not

completely rule out that these findings are confounded by some case of subclinical Alzheimer's disease. At the time point of collecting the blood samples, none of our donors showed any clinical signs of dementia. In addition, a similar increase and distribution of apoptotic features have been detected in lymphocytes of old mice when compared to young ones (unpublished findings). Thus, our results very likely reflect differences of aging. Basal levels of apoptotic nuclei in freshly isolated PBMC are significantly elevated in the group of aged donors compared to younger ones. In addition, basal levels of ROS are similarly increased in aging. This enhanced vulnerability is probably not related to a decline in IL-2 production [32] but must be correlated with an increased basal expression of activation markers (e.g. CD95, CD25, CD69, HLA-DR) and higher levels of ROS. Usually, apoptotic cells are eliminated quite fast after generation. A higher percentage of basal apoptotic levels in aged human PBMC could indicate an impaired phagocytosis of these cells in the aging organism.

The basal apoptotic content has been measured after the isolation procedure of PMBC by density centrifugation. We can not exclude that during the preparation of PBMC within 2 h cells were damaged or were triggered to undergo cell death. However, another study using lysed whole blood technique found similar results in regard to significantly elevated basal apoptosis in lymphocytes from old humans compared to young controls, but even about 5-fold higher basal apoptotic levels compared to our study [34]. Therefore, our isolation procedure seems to be rather gentle and the detected basal apoptosis may at least partly reflect already existing apoptotic cells.

The content of Bcl-2 raises slightly in peripheral cells with aging. Moreover, we and others could show that resting peripheral blood cells do not express large amounts of Bcl-2 at all, but the content of Bcl-2 increases significantly after activation with PHA-L and IL-2 [37]. Basal amounts of Bcl-2 correlate with the content of basal apoptotic cells in aging. Since Bcl-2 is a physiological apoptosis inhibitor, it could be possible that cells prone to undergo apoptosis express higher amounts of Bcl-2 as a defending mechanism. Therefore, it appears not to be surprising that apoptotic cells display more Bcl-2 at basal levels. Activated lymphoblasts displayed the more Bcl-2 the younger the donor was. These data implicate a strong correlation between lymphocyte's functionality concerning activation and the donor age. It seems that the decreased amount of Bcl-2 in activated lymphoblasts in aging might be one factor contributing to immunosenescence.

This higher susceptibility to apoptosis in aging might be due to an enhanced production and/or not satisfactory elimination of reactive oxygen species in aging, thereby leading to apoptosis. Mitochondria are the major site, where free oxygen radicals are produced. In aging, mutations accumulate in mtDNA, due to its vicinity to radical formation [28]. The mutant mtDNA leads to mitochondria with impaired respiratory function [2]. In addition, ROS act as intracellular

messenger and mediate lymphocyte activation [9] as well as apoptosis [5] by interacting with redox-sensitive transcription factors like NF $\kappa$ B.

Reduced glutathione (GSH) is an important antioxidant factor in the cell. Depletion of intracellular GSH inhibits T cell function [12]. Recently, it could be shown that the levels of GSH decrease in aging in human lymphocytes [50]. The sugar 2-deoxy-D-ribose provokes oxidative stress in cells probably by depleting the intracellular levels of reduced glutathione [20] and it is involved in the formation of free radicals [6], as it significantly elevates the level of ROS in lymphocytes compared to untreated controls [24]. The apoptotic process initiated with dRib can be blocked with N-acetyl-L-cysteine (NAC) a potent radical scavenger [39]. Therefore dRib is a useful tool to simulate oxidative stress in human lymphocytes as shown by [13]. We found that with increasing donor age, peripheral blood cells can cope worse with oxidative stress induced by incubation with 10 mM dRib. Compared to spontaneous apoptosis, dRib-induced apoptosis increases more with age. A possible cause for this finding could be the decreased levels of GSH found in aging lymphocytes [50]. It can be concluded that the less GSH a cell contains, the higher is the susceptibility to apoptosis induced by 10 mM dRib. Moreover, dRib induces significantly higher levels of ROS in PBMC derived from aged donors compared to younger ones. However, as the increase of ROS in aged lymphocytes induced by dRib is less than the increase of apoptosis, other parameters might be additionally relevant. Under these conditions, only little (<1%) necrosis was found as determined with another staining method using 7-aminoactinomycin D [41]. Higher dRib concentrations (>50 mM dRib) were necessary to induce higher levels of necrosis (unpublished data, see also ref. 14). The content of apoptotic cells induced by 50 mM dRib did not correlate with age. Interestingly, the percentage of 50 mM dRib induced apoptotic cells correlates significantly with the content of lymphocytes basally expressing the activation markers CD25, CD95, and HLA-DR. Apoptotic cells induced with a lower concentration of dRib (10 mM) did correlate as well with the expression of CD25. Correlation of 10 mM dRib with CD95 and HLA-DR could not be done as their expression is age-related as well and is therefore linearly related. It seems that dRib leads activated cells more into apoptosis than resting cells, maybe by a different mechanism, providing a powerful tool to explore lymphocyte apoptosis.

It is important to note that the age-related increased susceptibility of PBMC to dRib was not found for staurosporine. Staurosporine-induced elevation of ROS and staurosporine-induced apoptosis were not different in young or aged lymphocytes. Staurosporine induced comparable levels of ROS as dRib did. However, due to the involvement of caspases in staurosporine-induced death signaling upstream of ROS [21], higher levels of apoptotic cells were reached in young as well as in old subjects when compared to

dRib-induced apoptosis. Staurosporine-mediated ROS serve rather as a second messenger [5] and GSH is primarily not affected. Obviously, only certain specific mechanisms become mechanisms disturbed in aged lymphocytes.

In concordance to other groups, we found that the basal expression of functional CD95 increases with age [22,32]. We determined expression of CD95 surface molecule as well as its function with an agonistic antibody leading to apoptosis-specific DNA fragmentation. T lymphocytes from young donors expressed about 12% CD95 less compared to elderly individuals, while only 0.3% of PBMC of young subjects displayed apoptotic morphology after 24 h of incubation with anti-CD95. In the group of aged genders, apoptosis could be induced in about 2.3% of the PBMC. These data implicate that most of the expressed CD95-molecules on CD3<sup>+</sup> cells are not fully functional, maybe due to a lack of the FADD-protein (Fas-associated death domain), since Aggarwal and colleagues could demonstrate for another receptor of the same death receptor super family that TNF- $\alpha$ -induced apoptosis in aged human lymphocytes is associated with an altered expression of the corresponding death domain (TRADD) and death factor (TRAF-2) [1]. In aging, the number of functional CD95 expression raises. Our data of expression of CD95 of native CD3<sup>+</sup> lymphocytes are consistent with the findings of Phelouzat et al. [32], who reported that enhanced susceptibility to apoptosis of T lymphocytes from elderly done is associated with increased expression of the CD95-receptor. Moreover Seishima et al. [42] reported that soluble Fas receptor (sFas) is elevated in serum of aged persons.

Our lab and others have previously provided evidence that the susceptibility to apoptosis of peripheral cells is impaired in Alzheimer's disease: basal levels of apoptotic PBMC are significantly increased in AD compared to age-matched not demented controls [40], in addition dRib-induced apoptosis is altered [13] while spontaneous apoptosis is quite not affected indicating a higher vulnerability to oxidative stress in cells of demented patients. Further, CD95-induced apoptosis is not altered in AD, indicating that mechanisms are evident here that do not contribute to accelerated aging in AD. Moreover, different but not typical age-related expression of activation markers and members of the Bcl-2 family has been reported in dementia of the Alzheimer's type in brain tissue [11,19,30] and as well in AD lymphocytes [33,44]. Therefore, it seems that the pathological changes that take place in the brain of AD patients are not limited to neuronal brain tissue and can be detected in peripheral cells as well. This study will help to further explore the mechanisms taking place in AD and whether they are related to the physiological aging-process.

In conclusion, aging leads to quite specific changes of the susceptibility of human lymphocytes to apoptotic cell death. This knowledge will help to elucidate additional changes taking place in AD.

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