

# Age-related impairment of human T lymphocytes' activation: specific differences between CD4<sup>+</sup> and CD8<sup>+</sup> subsets

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## Abstract

The relevance of physiological immune aging is of great interest with respect to determining disorders with pathologic immune function in aging individuals. In recent years, the relevance of changes in peripheral lymphocytes in age-associated neurologic diseases has become more evident. Due to the lack of immunological studies, covering more than one event after mitogenic activation, we envisaged a new concept in the present study, aiming to investigate several events, starting from T cell receptor (TCR) ligation up to T cell proliferation. In addition, we addressed the question whether changes are present in the subsets (CD4, CD8) with aging. Phosphorylation of tyrosine residues declines with increasing age in CD4<sup>+</sup> cells. Fewer levels of CD69 positive cells after 4 h mitogenic activation, altered expression of cytokines (IL2, IFN- $\gamma$  and TNF- $\alpha$ ; 22 h) and lower proliferation (72 h) were determined in aging. Moreover, it could be shown that CD8<sup>+</sup> lymphocytes react more effectively to mitogenic stimulation with reference to CD69 expression and proliferation in both age groups (< 35 and > 60 years old). These data indicate that T cell activation, mediated by TCR engagement, is significantly impaired in aging and both subsets are affected. However, bypassing the TCR does not fully restore T cell function, indicating that there are more mechanisms involved than impaired signal transduction through TCR only. The results will be discussed in relation to their relevance in neurodegenerative and psychiatric disorders.

*Keywords:* Immunosenescence; Neurodegeneration; T lymphocyte subsets; Tyrosine phosphorylation; CD69 expression; Cytokine expression; Proliferation

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## 1. Introduction

Gerontology, especially immunogerontology, is becoming an increasingly important discipline in medicine, since more people are aging in the industrialized countries. A decline of immune function, termed immunosenescence, has been known for decades, mainly involving specific immunity (Guidi et al., 1998; Pawelec et al., 1999; Castle, 2000; Ginaldi et al., 2001; Linton and Thoman, 2001). However, the causes for immunosenescence remain unclear. Membrane changes taking place in the aging process are involved in altered binding of hormones, antigens and cytokines (Woda et al., 1979), as well as in defective transmission through ion channels (Griffith et al., 2000). Moreover, other theories explain declined cellular activity within aging by increased oxidative stress (Guyton et al., 1998). Previous studies on lymphocytes revealed an increase of reactive oxygen species (ROS) in aged subjects (Schindowski et al., 2000) or elevated by-products caused by ROS (e.g. lipidperoxidation (Hendricks and Heidrick, 1998), oxidized proteins (Stadtman, 1992; Berlett and Stadtman, 1997) and DNA-damage (Wei et al., 1998)). Altered and impaired signal transduction, in general, seems to be a major cause for impaired cellular function in all aged tissues (Eckert et al., 1994; Müller et al., 1996).

The performance of the immune system is closely related to the health situation of the individual. Longevity is associated with appropriate immune function (Sansoni et al., 1992, 1997; Franceschi et al., 1995). Moreover, recent research indicates that the immune system is not autonomous. It interacts with the whole organism in bi-directional ways (Guidi et al., 1998). Close interaction of the central nervous system (CNS) and immune cells has become more evident in recent years. Immune cell function is modulated by neuroendocrines as well as by neurotransmitters and vice versa (Hanisch et al., 1996; Carson and Sutcliffe, 1999). In addition, neurodegenerative diseases, such as dementia of Alzheimer type (DAT (Eckert et al., 1998, 2001; Schindowski et al., 1999)), Down's Syndrome (DS (Park et al., 2000)) and the demyelating disease, multiple scler-

osis (MS (Crucian et al., 1995a)) are accompanied by altered lymphocyte function and apoptosis. Chronic psychosocial and emotional stress is associated with declined and/or impaired immune function (Berkenbosch et al., 1991).

The clinical relevance of immunosenescence is quite high, since impaired function of the immune network contributes to pathologies such as cancer, increased susceptibility to infectious diseases and autoimmune disorders, cardiovascular diseases and the not clearly defined general multimorbidity (Bruunsgaard et al., 2000a). Moreover, a prospective study emphasized the close relation between decreased T cell proliferation after mitogenic activation and increased mortality (Murasko et al., 1987).

The relevance of physiological immune aging is, in addition, of great interest with respect to determine disorders with pathologic immune function in aging individuals. However, there is growing evidence that neurological disorders are often associated with changed immune properties. Immune cells provide good accessibility and are therefore a useful tool to explore the mechanism taking place within these disorders.

Here, we describe the function of T lymphocyte subsets (CD4, T helper cells and CD8, cytotoxic T suppressor cells) in physiological 'healthy' aging. We determined early events as tyrosine phosphorylation and CD69 expression and subsequent events, such as cytokine expression and proliferation after T cell-receptor engagement or by bypassing this receptor with phorbol ester (PMA) and  $Ca^{2+}$  ionophores (Ionomycin).

This study is of great importance and relevance to compare immunological changes taking place in many diseases in elderly patients, for instance, many neuroinflammatory diseases show changes in peripheral immune competent cells.

## 2. Material and methods

### 2.1. Subjects

Blood samples were taken from healthy subjects < 35 years, termed young ( $n = 22$ ; 15 women and seven men; mean age  $27.5 \pm 4.9$  years) and

healthy persons > 60 years, termed old group ( $n = 25$ , 16 women, nine men, mean age  $72.9 \pm 9.2$  years). The remainders were part of the intermediate ages shown in Figs. 2 and 3. Subjects with psychiatric and neurodegenerative disorders or acute infections were excluded. In addition, subjects with pathological distribution of lymphocyte subpopulations in flow cytometric analysis ac-

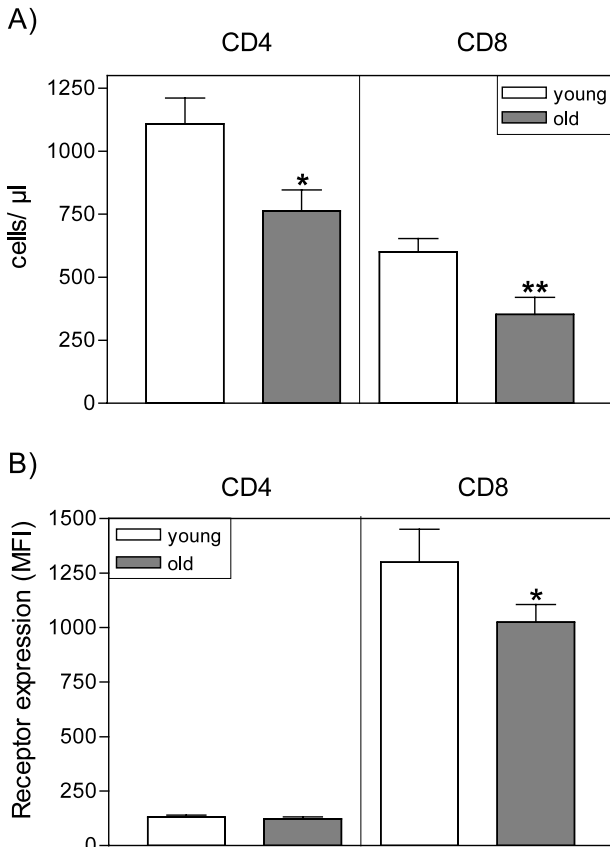


Fig. 1. (A) The total number of CD4 and CD8 lymphocytes is significantly reduced in blood samples from aged donors. Cell counts were determined by using the TrueCount® kit, providing a standardized number of beads in the staining tube. (B) The density of the costimulatory receptors CD4 and CD8 on lymphocytes from young and old subjects. Mean fluorescence intensity (MFI) determined by the mean channel number is proportional to the density of the measured receptor. No changes concerning CD4-receptor density were observed between lymphocytes from young and old donors. In contrast, a significant decrease of CD8-receptors was determined in lymphocytes from aged donors when compared with young donors.

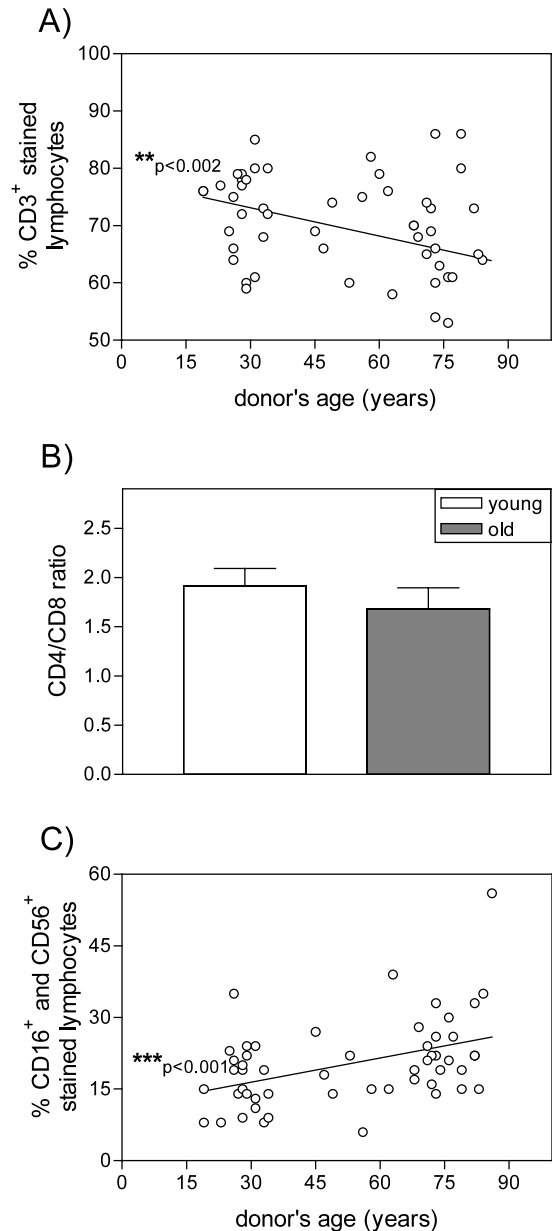


Fig. 2. Lymphocyte subset distribution in aging. The percentage of all FSC-SSC-gated lymphocytes was determined and plotted against donor's age. (A) The percentage of CD3 positive cells significantly decreases with increasing age of the blood donor. (B) The subset distribution of the CD3+ T cells, expressed as CD4/CD8-ratio, did not differ significantly between the two age groups. (C) The percentage of natural killer (NK) lymphocytes significantly rises with increasing donor's age.

cording to the Senieur protocol (Ligthart et al., 1984) 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.

This study was approved by the responsible ethical committee and written informed consent was obtained from all subjects.

## 2.2. Whole blood cultures

Blood was collected in NH<sub>4</sub>-heparin vacuum syringes. One volume of blood was diluted in 9 volume of RPMI supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml; all GibcoBRL, Karlsruhe, Germany). The cell suspension was cultured in 96-well plates (Iwaki/Dunn,

Germany). Cells were stimulated with either PHA-L (10 µg/ml) or 12-*O*-tetradecanoyl 12-phorbol 13-acetate (PMA) and Ionomycin (50 and 200 ng/ml, respectively; all Sigma, Germany) or remained untreated (basal).

## 2.3. Cytometry

Whole blood cultures were transferred into a 1.2 ml 96-well plate, centrifuged at 300 × *g* and the supernatants were discarded. Whole blood was stained with the appropriate antibodies (all were purchased from Pharmingen/Becton Dickinson, Heidelberg, Germany) following the manufacturer's manual. In brief, 1 µl of each antibody was added to blood cell pellets and incubated for 10 min at room temperature. Red blood cells were lysed by adding 500 µl of hypotonic buffer (Cell-Lyse<sup>®</sup>, Becton Dickinson) for 15 min. Since

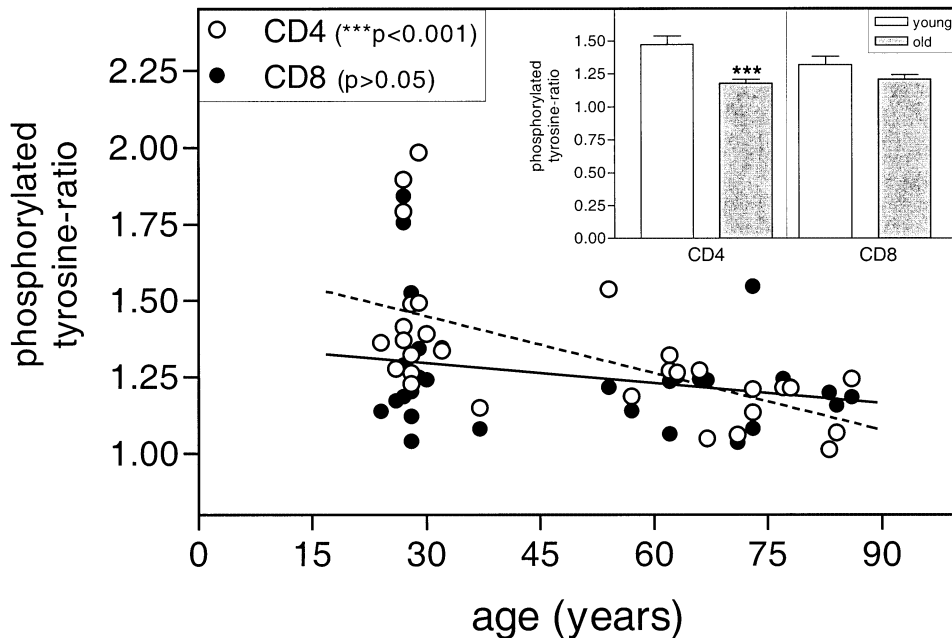


Fig. 3. Phosphorylation of tyrosine residues upon PHA-L activation in aging. Whole blood cultures were stimulated with PHA-L for 3 min at 37 °C and intracellularly stained with an antibody that detects phosphorylated tyrosine residues or an isotype-control. Data are expressed as ratio of PHA-stimulated and unstimulated cells. The ratio of the mean fluorescence intensities (MFI) of phosphorylated tyrosines of PHA-L stimulated and untreated cells correlates significantly with the donor's age in CD4<sup>+</sup> cells (open circles, dashed line). When the groups of young and aged donors were compared, there was a significant decrease of phosphorylated tyrosine residues in the group of old volunteers (inset). In CD8<sup>+</sup> lymphocytes, a tendency towards reduced levels could be observed with aging, however, those changes are not significant.

PMA/Ionomycin treatment stabilizes the red blood cell membrane, 1 ml CellLyse was needed to completely lyse the erythrocytes. Cells were pelleted by  $300 \times g$  and additionally washed with 500  $\mu$ l CellWash<sup>®</sup> (Becton Dickinson).

Samples were assayed with FACSCalibur using Simulset software (all Becton Dickinson). Dead cells, granulocytes and monocytes were excluded by forward (FSC) and side (SSC) angle scattered light gating. For each sample, except total numeration with TrueCount<sup>®</sup> beads, 10,000 cells were acquired. All fluorescence channels were acquired on a logarithmic scale.

The lymphocyte subsets were quantified by staining with anti-CD3-FITC (clone SK7), anti-CD19-PE (clone 4G7) and anti-CD16-PE (clone B73.1) + anti-CD56-PE (clone MY31), in order to explore the percentage of T-, B- and NK-lymphocytes, respectively. T lymphocyte subsets were determined by staining with anti-CD4-FITC (clone SK3) and anti-CD8-PR (clone SK1; all Becton Dickinson). Early T cell activation was determined by staining with anti-CD69-FITC (clone L78) or IgG<sub>1</sub>-isotype control (clone X40).

In all experiments concerning activation with PHA-L or PMA/Ionomycin, cells were stained with CD3-PerCp and CD8-PE in order to gate all CD3<sup>+</sup> T cells and to differentiate between CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD8<sup>-</sup> lymphocytes. The findings of Rostaing et al. (1999) and unpublished data of our group revealed that the CD4 antigen is down-regulated after PHA activation, while the CD8 surface density remains unchanged. Therefore, we electronically gated the CD3<sup>+</sup>CD8<sup>+</sup> cells (T cytotoxic/suppressor cells) and the CD3<sup>+</sup>CD8<sup>-</sup> cells (mostly CD3<sup>+</sup>CD4<sup>+</sup> or T helper cells) and assumed that most CD8<sup>-</sup> cells are CD4<sup>+</sup>. In preliminary experiments, no age-related changes in the percentage of double positive (CD4<sup>+</sup>CD8<sup>+</sup>) and double negative (CD4<sup>-</sup>CD8<sup>-</sup>) were found (data not shown).

#### 2.4. Quantification of lymphocyte number

Absolute numbers of lymphocytes were determined with TrueCount<sup>®</sup> beads (Becton Dickinson) and CD3-PerCp, CD8-PE and CD4-FITC, according to the manufacturer's manual. In brief, 50

$\mu$ l of whole blood and 1  $\mu$ l antibody reagent containing fluorochrome conjugated antibodies against anti-CD3, anti-CD8, and anti-CD4 (TriTest<sup>®</sup>, Becton Dickinson) were pipetted into a TrueCount<sup>®</sup> tube containing a specific amount of beads and incubated for 15 min at room temperature. Some 250  $\mu$ l CellLyse<sup>®</sup> were added for an additional 15 min, in order to lyse red blood cells. Samples were measured by flow cytometry with settings appropriate for suspension containing lysed red blood cells without wash. For each sample, 100,000 cells were acquired. Cell population and beads were gated and cell count calculated in relation to the bead's number.

#### 2.5. Detection of cytokine expressing cells

Whole blood cultures were placed in an incubator for 22 h at 37 °C. For cytokine detection, cells were incubated with Brefeldin A (Sigma) at a concentration of 10  $\mu$ g/ml for the last 16 h of stimulation, to inhibit cytokine secretion. Cultures were surface stained as described above, followed by fixation and permeabilization with FACS-Perm<sup>®</sup> (Becton Dickinson). Intracellular cytokine staining was performed with anti-cytokine mAbs specific for IL2, IFN- $\gamma$  and TNF- $\alpha$  or an isotype control (clones 5344.111, 25723.11 or 6401.1111, respectively; Becton Dickinson) in FACSPerm<sup>®</sup> for 30 min at room temperature.

#### 2.6. Determination of proliferation

Whole blood cultures were incubated for 72 h with or without stimulation at 37 °C. Before harvest (24 h), Brom-3'-desoxy-uridine (BrdU; Sigma) was added at a final concentration of 20  $\mu$ M to the cultures. Cells were stained according to Gaines et al. (1996) using an anti-BrdU-antibody or isotype control (Becton Dickinson). In brief, surface receptors were stained as described above. After red blood cell lysis and an additional washing-step, leukocytes were transferred into a 96-microtiter round-bottom plate and fixed with 100  $\mu$ l 4% paraformaldehyde (CellFix<sup>®</sup>, Pharmingen) overnight at 4 °C. Cells were permeabilized by washing with saponin-containing buffer (PermWash<sup>®</sup>). Cells were resuspended in PBS containing 10 mM

MgCl<sub>2</sub> (Sigma) and 50 U DNase-I (Boehringer Mannheim/Roche, Germany) and incubated at 37 °C for 45 min. DNA digest was stopped by centrifugation at 4 °C, cells were washed and resuspended in 50 µl PermWash<sup>®</sup>. Blood cells were stained with 1 µl of an anti-BrdU-antibody (clone B44) or IgG<sub>1</sub>-isotype control (Becton Dickinson) for 30 min at room temperature in the dark. After washing out excessive antibodies, cells were resuspended in CellWash<sup>®</sup> and analyzed by flow cytometry.

### 2.7. Determination of phosphorylated tyrosine residues

T cells were stimulated and samples prepared according to Hubert et al. (1997). In brief, 500 µl diluted whole blood was transferred into micro-centrifuge tubes and brought to 37 °C in a heat-block with gentle agitation. Cells were activated by the addition of PHA-L (10 µg/ml) for 3 min, rapidly chilled on ice and centrifuged at 500 × *g*. Pellets were resuspended in freezing medium, containing 10% DMSO (Sigma) and 50% FCS in RPMI medium (GibcoBRL). Samples were stored at –20 °C until analysis. For sample preparation, cells were rapidly thawed at 37 °C, transferred into a 1.2 ml 96-well plate and washed twice with CellWash<sup>®</sup>. Red cell lysis was not necessary since freezing the cells already destroyed red blood cells' membranes. After surface staining, cells were fixed with 4% paraformaldehyde (CellFix<sup>®</sup>) for 20 min on ice, permeabilized with 1% saponin (PermWash<sup>®</sup>), stained with anti-phosphotyrosine antibody (clone 4G10, Santa Cruz Biotechnology, USA), that detects all kinds of phosphotyrosine containing proteins or isotype control (IgG2b, clone 27–35, Pharmigen) and analyzed by flow cytometry. The ratio between mean fluorescence intensity of the stimulated and unstimulated cells was calculated.

### 2.8. Statistics

Statistical analysis was performed by Student's paired/unpaired *t*-test or correlation calculations (Prism 3.0, GraphPad Software Inc., USA). All data presented are mean ± S.E.M. \*\*\**P* < 0.001, \*\**P* < 0.01 and \**P* < 0.05.

## 3. Results

In order to determine immunosenescence, the total number and percentage of lymphocytes were investigated in freshly collected blood samples. Several events were determined after mitogenic activation, e.g. phosphorylation of tyrosine residues after 3 min, expression of CD69 and cytokines after 4 and 22 h, respectively and proliferation was assessed after 72 h.

### 3.1. Absolute number of total T cells, as well as the number of CD4<sup>+</sup>- and CD8<sup>+</sup>-lymphocytes decreases with aging

To determine the absolute amount of T lymphocytes with respect to their subsets, an aliquot of heparinized blood samples was quantified by flow cytometry using TrueCount<sup>®</sup> beads and antibodies against CD3, CD8 and CD4. Only CD3-PerCp-positive cells were electronically gated and a minimum of 100,000 cells per experiment was acquired. No changes concerning appearance of the forward-side scatter plot could be observed. In both age groups, lymphocytes could be clearly identified and were sharply determined from monocytes and granulocytes. The number of CD3-positive cells significantly decreased in aging (CD3, young: 1888 ± 147 cells/µl; old: 1236 ± 153 cells/µl; *n* = 17–21; *P* < 0.005). The loss of cells observed in aged subjects seemed to be distributed to both subsets (CD4, young: 1109 ± 103 cells/µl; old: 763 ± 84 cells/µl; *n* = 17–21; *P* < 0.012 and CD8, young: 601 ± 53 cells/µl; old: 353 ± 68 cells/µl; *n* = 17–21; *P* < 0.005; Fig. 1A). However, it appeared that the number of CD8<sup>+</sup> cells was more affected by aging (42% less cells per µl compared with young donors) than the T helper lymphocytes (CD4<sup>+</sup>; 32% less cells per µl).

In addition, the mean fluorescence intensity (MFI) that is proportional to receptor surface density was measured from the above determinations. The density of the CD3- and CD4-receptor was not altered between the group of young and old donors (CD3, young: 182 ± 17; old: 160 ± 7, CD4, young: 130 ± 10; old: 122 ± 10; *n* = 11–14; Fig. 1B). Interestingly, the density of CD8-recep-

tor on each single CD8<sup>+</sup> T cell was significantly decreased in the aged group (CD8, young:  $1300 \pm 151$ ; old:  $1025 \pm 81$ ;  $n = 11-14$ ;  $*P < 0.05$ ; Fig. 1B).

To explore the changes of subsets within the whole lymphocyte population, the percentage of T-, B- and NK (natural killer)-lymphocytes were surveyed and plotted against the donors' age. The percentage of CD3-positive cells (within the gated lymphocyte population, as determined by gating in FSC/SSC) decreases with age ( $n = 54$ ;  $**P < 0.002$ ;  $r^2 = 0.15$ ; Fig. 2A). The ratio of T helper to T suppressor-lymphocytes, termed CD4/CD8-ratio, was not significantly changed in old individuals (young:  $1.91 \pm 0.18$ ; old:  $1.68 \pm 0.21$ ;  $n = 20-22$ ; Fig. 2B). The very slight insignificant reduction of the CD4/CD8-ratio in the aged group became evident, looking at the absolute number of CD4<sup>+</sup> and CD8<sup>+</sup> cells. The loss of CD8<sup>+</sup> cells was more dominant than the loss of CD4<sup>+</sup> in aging (Fig. 1A). However, when the CD4/CD8-ratio was correlated with the donor's age, there were no statistically significant changes ( $n = 53$ ;  $r^2 = 0.004$ , data not shown). The percentage of B lymphocytes was not significantly affected by the volunteer's age ( $n = 53$ ,  $r^2 = 0.006$ ; data not shown). Focussing on the NK subset, there was a significant increase of the percentage of CD16<sup>+</sup> and CD56<sup>+</sup> cells within the lymphocyte gate ( $n = 53$ ;  $***P < 0.001$ ;  $r^2 = 0.19$ ; Fig. 2C).

### 3.2. Tyrosine phosphorylation subsequent to TCR engagement declines with the donor's age

After TCR engagement, the number of phosphorylated tyrosines raises as a consequence of the induction of kinase cascades. Whole blood cultures were stimulated with PHA-L (10  $\mu\text{g/ml}$ ) or remained untreated for 3 min at 37 °C with gentle agitation. Cells were transferred on ice in order to stop the reactions. Surface and intracellular staining was performed as described. A ratio was calculated from the mean fluorescence intensity of PHA-L stimulated and the untreated control cells.

The ratio of tyrosine phosphorylation of CD4<sup>+</sup> lymphocytes correlated significantly with the

donor's age ( $***P < 0.001$ ;  $n = 30$ ; open circles in Fig. 3), indicating a lower sensitivity of T helper cells via the TCR in aging. However, no statistically significant correlation was found for the amount of phosphorylated tyrosine residues with aging in the CD8 subset ( $P = 0.083$ ;  $n = 30$ ; open triangles in Fig. 3). A comparison of the two age groups revealed a highly significant reduction of the phosphorylated tyrosine-ratio in T helper lymphocytes (CD4, young:  $1.473 \pm 0.065$ ; old:  $1.180 \pm 0.283$ ;  $***P < 0.001$ ;  $n = 13-14$ ; Fig. 3 inset). In CD8<sup>+</sup> cells, levels of phosphorylated tyrosine were reduced as well, though not statistically significant (CD8, young:  $1.319 \pm 0.063$ ; old:  $1.209 \pm 0.035$ ;  $P = 0.074$ ;  $n = 13-14$ ; Fig. 3 inset).

### 3.3. Expression of early activation marker CD69 upon PHA-L and PMA/Ionomycin stimulation decreases with aging

Whole blood cultures were prepared as described above and stimulated with 10  $\mu\text{g/ml}$  PHA-L or 50 ng/ml PMA and 200 ng/ml Ionomycin. After 4 h incubation, leukocytes were stained with CD3-PerCp, CD8-PE and CD69-FITC and further processed as indicated. Fig. 4 shows a set of density plots 4 h without stimulation (control; Fig. 4, upper panel) and with stimulation (PHA-L, Fig. 4, middle panel and PMA + Ionomycin, Fig. 4, lower panel) from one young and one old individual.

Basal levels of CD69 in untreated control cells were quite low and not different between young and old humans (CD4, young:  $1.16 \pm 0.22\%$ ; old:  $1.14 \pm 0.17\%$ ; CD8, young:  $1.91 \pm 0.52\%$ ; old:  $1.75 \pm 0.38\%$ ;  $n = 19-20$ ). A reduction of CD69 expression after T-cell activation with PHA-L was determined for both T-cell subsets in the aged group compared to the young group (CD4, young:  $49.64 \pm 2.64\%$ ; old:  $39.59 \pm 3.56\%$ ;  $*P < 0.014$ ; CD8, young:  $53.09 \pm 2.86\%$ ; old:  $42.28 \pm 4.30\%$ ;  $*P < 0.02$ ;  $n = 19-20$ ; Fig. 5A).

The phorbol ester PMA and the calcium ionophore Ionomycin bypassed the T-cell receptor by directly activating protein kinase C and by elevating the intracellular levels of Ca<sup>2+</sup>. Levels of activated cells were significantly increased after

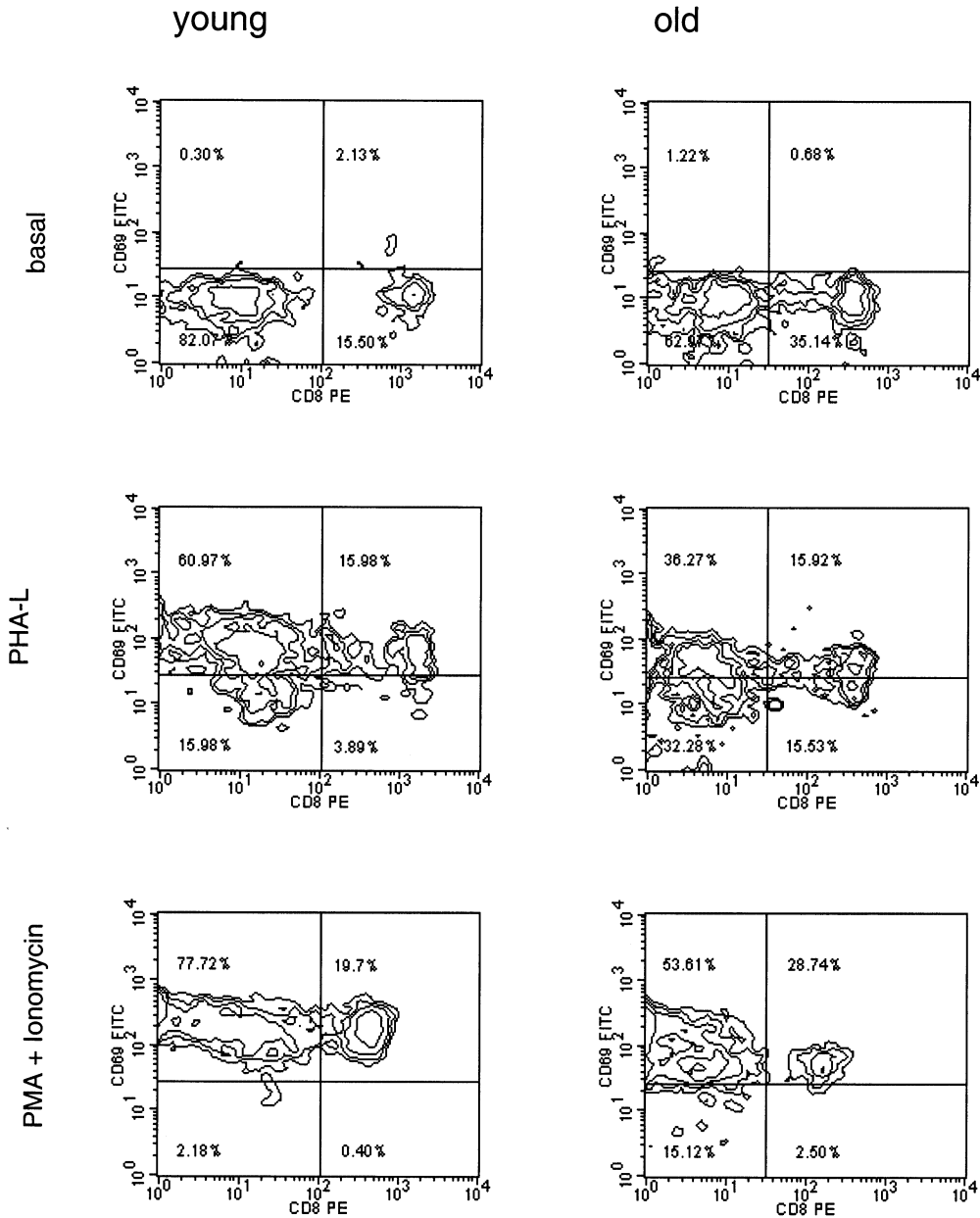


Fig. 4. Density plots of CD69 expression at basal condition (upper panel) and after activation with PHA-L (middle panel) and PMA and Ionomycin (lower panel) for 4 h of a young and an old donor. Only CD3-PerCp-positive gated leukocytes are displayed in the plot. Left quadrants represent CD8<sup>-</sup> cells, e.g. CD4<sup>+</sup> lymphocytes, right quadrants show CD8<sup>+</sup> T cells. The upper quadrants show CD69<sup>+</sup> lymphocytes, those in the left are CD4<sup>+</sup>CD69<sup>+</sup> and the quadrants in the right upper quadrant are CD8<sup>+</sup>CD69<sup>+</sup>.

PMA and Ionomycin treatment in both subsets when compared with the corresponding PHA-L treatment of each group ( $+++P < 0.0001$ ). CD8-

cells derived from young donors showed maximal CD69 expression (nearly 100%) after PMA and Ionomycin treatment. Interestingly, CD4-



lymphocytes were unable to respond to this with the maximal capacity within 4 h. CD69 expression decreased highly significantly on CD8<sup>+</sup> cells and significantly on CD4<sup>+</sup> lymphocytes in aging (CD4, young:  $85.25 \pm 2.40\%$ ; old:  $75.84 \pm 3.3\%$ ;  $*P < 0.025$ ; CD8, young:  $96.94 \pm 0.66\%$ ; old:

$92.46 \pm 0.91\%$ ;  $***P < 0.001$ ;  $n = 19$  in each group; Fig. 5B).

### 3.4. Cytokine expression is altered in lymphocytes derived from aged donors

Whole blood cultures were incubated for 22 h at 37 °C in the absence (basal) or presence of mitogenic activation (PHA-L or PMA/Ionomycin). Before the cells were harvested and stained (16 h), Brefeldin A was added to the cultures in order to block the secretion of the cytokines. Thus, the cytokines were plugged in the Golgi and could therefore be stained with appropriate antibodies (Fig. 8).

The percentages of cytokine expressing cells of both age groups are summarized in Table 1. Lymphocytes derived from aged donors ( $n = 9$ ) showed reduced levels of IL2 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) expression even basal but as well upon activation, when compared with young controls ( $n = 10$ ). In contrast, production of interferon- $\gamma$  (IFN- $\gamma$ ) was significantly elevated in PHA-L activated cells from aged persons. Basal interleukin-2 (IL2) expression was not altered in lymphocytes from aged volunteers. Subsequent to stimulation with PHA-L or PMA/Ionomycin, cells from the elderly tended to display reduced levels of IL2 expressing cells. As a T<sub>H1</sub> cytokine, IL2 was mainly expressed in CD4<sup>+</sup> T lymphocytes. In addition, levels of IFN- $\gamma$  were determined. Here, the percentage of basal IFN- $\gamma$  producing lymphocytes was not altered in lymphocytes from aged donors. Upon stimulation with PHA, cells from old donors displayed significantly more IFN- $\gamma$  positive cells in both T cells subsets when compared with old controls. Bypassing the TCR with PMA/Ionomycin revealed the largest amounts of IFN- $\gamma$ . However, changes between the age groups were not significant. Finally, the percentage of TNF- $\alpha$  positive cells was determined. The T helper subset showed age-related differences in the basal expression of TNF- $\alpha$ . In the CD8<sup>+</sup> population, those changes were not that evident. Mitogenic activation of the cells tends to effect age-related changes more in the CD8 than in the CD4 subset.

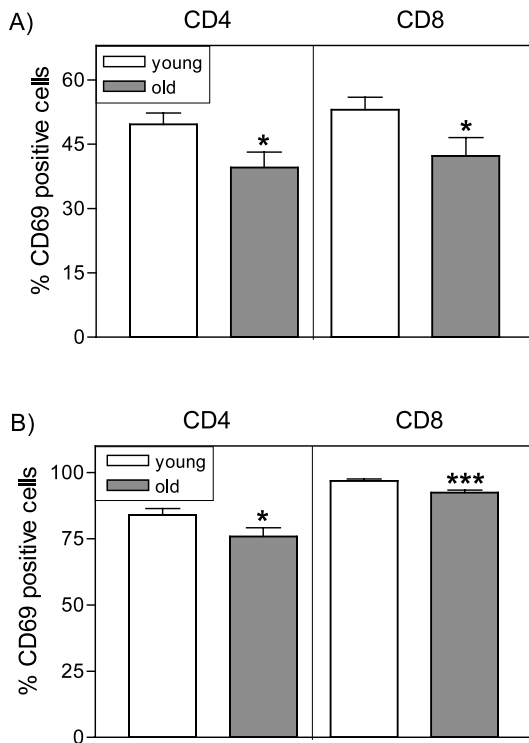


Fig. 5. Expression of the early activation marker CD69 is reduced in aging. Whole blood cultures were activated with either PHA-L or PMA/Ionomycin for 4 h at 37 °C. Subsequently, cells were surface stained for CD3, CD8 and CD69. Only CD3 positive gated cells were analyzed for CD69 expression. (A) TCR engagement triggered by PHA treatment revealed significantly reduced levels of CD69 expressing cells in aged donors in both T cell subsets. (B) This pattern remained when the TCR complex was bypassed with PMA/Ionomycin, though the age-related differences were smaller. ( $n = 19$  per group,  $*P < 0.05$ ;  $***P < 0.001$  compared to young controls;  $+++P < 0.001$  compared to the corresponding PHA-L stimulation in CD4 and CD8 cells of young individuals;  $§§§P < 0.001$  compared to the corresponding PHA-L stimulation in CD4 and CD8 cells of old individuals).

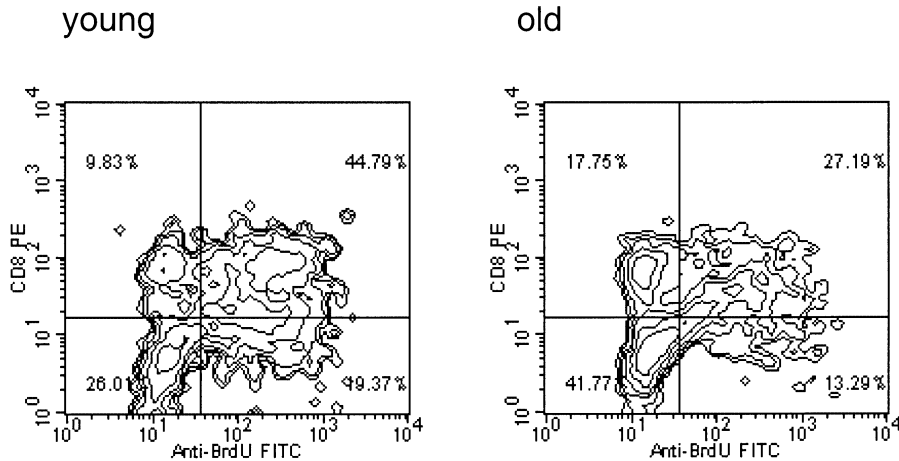


Fig. 6. Dot plots of BrdU-incorporation upon stimulation with PHA-L taken from a young and old subject. Cells were stained with an isotype antibody as control or with an antibody raised against BrdU to explore the content of proliferating cells. Only CD3-PercP-positive gated leukocytes are displayed in the dot plot. Left quadrants represent CD8<sup>-</sup> cells, e.g. CD4<sup>+</sup> lymphocytes, right quadrants show CD8<sup>+</sup> T cells. The upper quadrants show BrdU<sup>+</sup> lymphocytes, those on the left are CD4<sup>+</sup>BrdU<sup>+</sup> and those in the right upper quadrant are CD8<sup>+</sup>BrdU<sup>+</sup>. After 72 h PHA-L activation, the T lymphocytes from the young individual clearly shows more cells that are positive for anti-BrdU staining than the T cells from the old person.

### 3.5. Lymphocyte proliferation is significantly reduced in aging

Seventy-two hours after preparation of the whole blood cultures and mitogenic stimulation, leukocytes were stained as indicated. Preliminary experiments showed that stimulation for 3 days and BrdU-pulse for 24 h are optimal conditions for our purpose. Fig. 6 shows density plots from one young and one old individual after PHA-L stimulation (10 µg/ml).

When proliferation was induced by activation with PHA-L via TCR ligation, T cells derived from young donors incorporated significantly more BrdU within 24 h than cells from elderly persons, indicating a higher proliferation (CD4, young:  $24.75 \pm 1.98\%$ ; old:  $16.07 \pm 1.92\%$ ;  $**P < 0.005$ ; CD8, young:  $42.21 \pm 3.81\%$ ; old:  $19.86 \pm 1.76\%$ ;  $***P < 0.0001$ ;  $n = 22-25$ ; Fig. 7A). When the percentage of BrdU<sup>+</sup> cells was plotted against increasing age, there was significant negative correlation (CD4,  $**P < 0.002$ ; CD8,  $***P < 0.0001$ ). By circumventing the TCR complex with PMA and Ionomycin, the significant decrease of BrdU-positive cells in aging was still evident (CD4, young:  $33.43 \pm 2.84\%$ ; old:  $24.35 \pm 2.99\%$ ;  $*P <$

$0.023$ ; CD8, young:  $50.70 \pm 5.05\%$ ; old:  $27.85 \pm 2.86\%$ ;  $***P < 0.001$ ;  $n = 22-25$ ; Fig. 7B). Levels of proliferating lymphocytes from aged donors were significantly increased after PMA and Ionomycin treatment in both subsets when compared with the corresponding PHA-L treatment of each group ( $^{\$}P < 0.05$ ). In cells derived from young volunteers, only CD4<sup>+</sup> cells showed increased percentage of BrdU<sup>+</sup> lymphocytes ( $+P < 0.05$ ). Correlation with age revealed only in CD4<sup>+</sup> lymphocytes a significant correlation with age ( $*P < 0.05$ ). It is noteworthy that in young cells the proliferative activity of the T lymphocytes was mainly triggered by the CD8 subsets.

## 4. Discussion

The decline of immune function with aging has been a subject in various studies, reviews and comments. However, most studies focus on only one event after mitogenic activation. To our knowledge, there is no study determining several chronological aspects of lymphocyte activation in aging with respect to subsets. In addition, we carried out the lymphocyte experiments in whole

blood cultures in order to maintain their physiological environment and to avoid cellular activation or cell death due to isolation procedure. Moreover, endocrine factors and cytokines, which have a great influence on immunosenescence, remained in the culture vessel and were not washed out during isolation. Lymphocyte activation with lectin (PHA-L) is believed to be quite similar to that taking place in the organism.

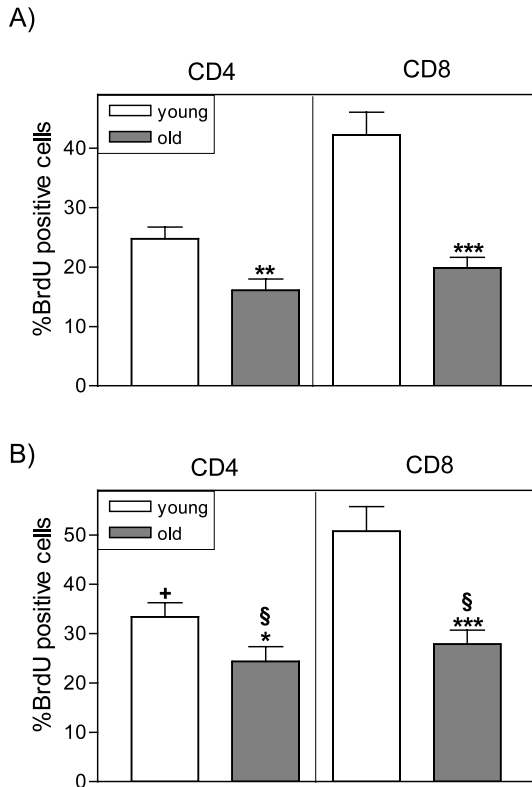


Fig. 7. Proliferation according to activation decreases in the lymphocytes of young and aged donors. (A) PHA-L stimulated cells show a markedly increased proliferation in the CD8 subset compared to CD4 positive lymphocytes. In aging, the number of BrdU-incorporating cells is significantly reduced in both subsets. (B) Activation subsequent to PMA/Ionomycin treatment showed, in addition, a higher percentage of proliferating cells within the CD8 subset. Moreover, lymphocytes from young donors revealed significantly elevated levels of BrdU positive cells when compared with old controls ( $n = 22-25$ ,  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$  compared to young controls;  $+P < 0.05$  compared to the corresponding PHA-L stimulation in CD4 and CD8 cells of young individuals;  $§P < 0.05$  compared to the corresponding PHA-L stimulation in CD4 and CD8 cells of old individuals).

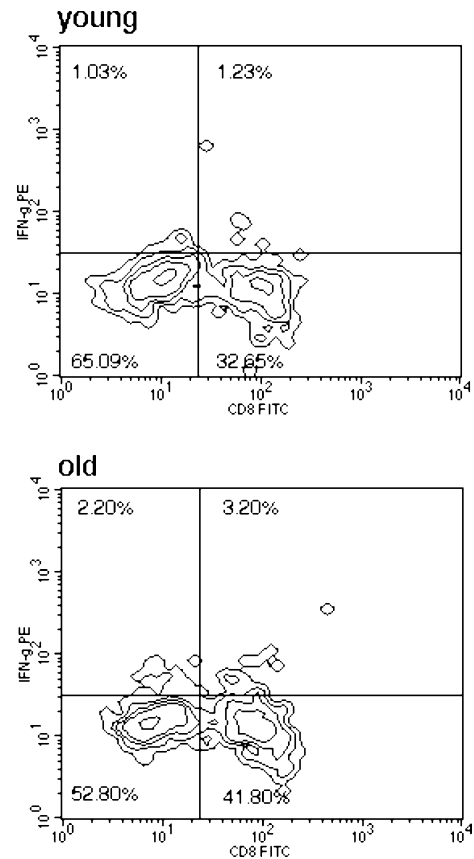


Fig. 8. Plots of cells intracellularly stained for IFN- $\gamma$  after stimulation with PHA for 22 h. Secretion of cytokines was blocked by Brefeldin A treatment for 16 h. Only CD3-PerCP-positive gated leukocytes are displayed in the dot plot. Left quadrants represent CD8<sup>-</sup> cells, e.g. mainly CD4<sup>+</sup> lymphocytes, right quadrants show CD8<sup>+</sup> T cells. The upper quadrants show IFN- $\gamma$ <sup>+</sup> lymphocytes, those in the left are CD8<sup>-</sup> IFN- $\gamma$ <sup>+</sup> and those in the right upper quadrant are CD8<sup>+</sup>.

TCR engagement, that was mimicked in this study by the lectin PHA-L, resulted in phosphorylation of tyrosine-residues mediated by Src and Zap-70-family members (Weiss and Littman, 1994), followed by activation of Ras and Raf/MAP kinase cascade and PLC- $\gamma$  (phospholipase- $\gamma$ ), which interacted with PKC (protein kinase C). Gene expression was induced by the rapid inactivation of I $\kappa$ B- $\alpha$  and subsequent nuclear migration of active NF- $\kappa$ B dimers (Kuo and Leiden, 1999). Ras/Raf/MAP kinase activation led to oligomer-

ization of Fos and Jun that formed the transcription factor AP-1. In addition,  $IP_3$  concentration was raised due to PLC activity and proceeded to release  $Ca^{2+}$  from intracellular stores. Elevated  $Ca^{2+}$  levels initiated the phosphatase calcineurin, which dephosphorylated the transcription factor NF-AT, thereby becoming active. Treatment of lymphocytes with phorbol ester (PMA) plus Ionomycin bypassed the TCR engagement by direct interaction of the PMA with PKC and by increasing the intracellular  $Ca^{2+}$  levels due to ionophore Ionomycin. Altered T cell functionality due to impaired TCR function and/or decreased TCR-complex expression should be avoided by PMA plus Ionomycin regimen.

We observed a significantly decreased number of total T lymphocytes in CD3 and in the CD4 and CD8 subsets in aged humans. However, the CD4/CD8-ratio remained unchanged with aging. In an earlier study, we found increased basal apoptosis of lymphocytes derived from aged individuals (Schindowski et al., 2000). The enhanced incidence of apoptosis might be associated with the loss of CD3<sup>+</sup> cells. A lack of T cells may be one of the numerous causes of immunosenescence, since fewer cells providing immune function are available to maintain immune homeostasis. In agreement with our findings, a

dramatic decrease of lymphocyte count have been observed during the last 3 years before death in a prospective study (Bender et al., 1986).

Soluble CD8 receptor is a marker for T suppressor cell activation (Lenkei et al., 1998). Reduced density of CD8 receptor on T suppressor cells from the elderly could be related to increased levels of soluble CD8. However, Ginaldi et al. (2001) present no changes in the density of CD8 antigen, but it should be noted that they were investigating a subgroup of T cells (CD3<sup>+</sup>CD7<sup>-</sup>) while here, we determined all CD3<sup>+</sup>. Interestingly, in neurological disorders like DAT, increased levels of soluble CD8 are involved as well (Singh, 1994).

Due to thymic involution, however, the number of untouched CD4 and CD8-cells declines with increasing age and with the number of infections during life. Unprimed cells carry the CD45RA isoform of the CD45-receptor, while memory cells express the CD45R0 epitope (Vitetta et al., 1991). We and others (O'Leary et al., 1988; Yen et al., 2000) found no significant changes of the CD4/CD8 ratio with aging. This is an interesting fact as some neurological disorders (DAT and MS, for instance) show increased CD4/CD8-ratios (Shalit et al., 1995; Crucian et al., 1995b; Schindowski et al., 1999).

Table 1  
Altered cytokine production in aging

		CD4			CD8		
		Basal	PHA-L	PMA/Ionomycin	Basal	PHA-L	PMA/Ionomycin
IL-2	Young	1.43 ± 0.88	11.48 ± 5.95	30.80 ± 4.51	0.30 ± 0.20	4.95 ± 3.29	15.67 ± 7.21
	Old	1.29 ± 0.64	5.17 ± 1.36	22.74 ± 4.33	0.41 ± 0.35	0.51 ± 0.90	10.13 ± 5.72
IFN- $\gamma$	Young	0.70 ± 0.34	0.89 ± 0.35	6.50 ± 1.35	1.40 ± 0.90	3.56 ± 1.24	16.38 ± 4.25
	Old	0.25 ± 0.12	2.21 ± 0.35**	11.91 ± 5.31	0.47 ± 0.33	8.76 ± 1.30**	19.79 ± 3.04
TNF- $\alpha$	Young	3.22 ± 1.76	4.27 ± 1.68	31.91 ± 6.49	3.80 ± 1.91	8.05 ± 3.10	38.88 ± 12.20
	Old	0.14 ± 0.07*	5.52 ± 3.01	25.50 ± 14.21	1.15 ± 0.71	4.17 ± 1.52	19.42 ± 7.86

\*  $P < 0.05$  compared to young controls.

\*\*  $P < 0.01$  compared to young controls.

IL2, IFN- $\gamma$  and TNF- $\alpha$  cytokine expression in lymphocytes from young and old donors. Data is expressed in percent of cytokine expressing cells (means ± S.E.M.). Whole blood cultures were stimulated either with PHA-L or PMA/Ionomycin as described or remained untreated (basal) for 22 h. Brefeldin A was added 16 h prior to harvesting the cells. Leukocytes were surface-stained for CD3 and CD8, fixed and permeabilized. Staining with fluorochrome-coupled antibodies against IL2, IFN- $\gamma$ , TNF- $\alpha$  or isotype-control identified cytokine expressing cells.

Mitogenic activation of T cells leads to the phosphorylation and activation of p56<sup>lck</sup> kinase. p56<sup>lck</sup> associates with CD4 and CD8 costimulatory receptor molecules (Abraham et al., 1991). Here, we could show that tyrosine phosphorylation was age-related decreased in CD4<sup>+</sup> cells. CD8<sup>+</sup> lymphocytes display insignificantly reduced phosphorylation ratios. Recently, an altered association of p56<sup>lck</sup> with coreceptors such as CD4 was found in the elderly (Tinkle et al., 1998), suggesting that alterations in p56<sup>lck</sup> tyrosine kinase and its association with CD4 may underlie lowered T cell function during aging.

Moreover, in earlier studies we could show lowered Ca<sup>2+</sup> mobilization after mitogenic stimulation with PHA-L in lymphocytes derived from aged individuals (Eckert et al., 1997, 1998).

CD69 was formerly known as activation inducer molecule (AIM) and is a 60-kDa glycosylated costimulatory molecule for T lymphocyte proliferation, which is expressed after ligation of CD3/T cell receptor complex (Hamann et al., 1998). Although the physiological role of CD69 is still unknown, crosslinking of CD69 induces proliferation. Expression of CD69 in T cells is dependent on the appropriate activation of several intracellular signaling pathways including IP<sub>3</sub> production, increases in intracellular Ca<sup>2+</sup> and the activation of Ras/Raf-1/MAP kinase pathway (Perfetto et al., 1997). In concordance with Rutella et al. (1999), we could show that CD8<sup>+</sup> cells display more CD69-expression after PHA-L activation for 4 h than CD4<sup>+</sup>. CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes derived from elderly persons had reduced CD69 surface expression compared to young persons. After treatment with phorbol ester and Ca<sup>2+</sup> ionophore, the expressed amount of CD69 was significantly higher than after TCR engagement in both age groups. Moreover, a significant age-related difference in expression of CD69 was evident when bypassing the TCR complex with PMA and Ionomycin. These data indicate that impaired expression of the activation marker CD69 in aging by engagement and even by bypassing the TCR. However, reduced activation of lymphocytes from elderly humans seems, therefore, not only to be due to lower expression of CD3 and/or followed by reduced signal trans-

duction (Fulop et al., 1995) after TCR ligation, but also age-related diminished transcription might be involved in our findings (Whisler et al., 1996).

Many earlier reports using different techniques to determine serum levels of cytokines indicate that the lymphocyte's response to mitogens is impaired in elderly subjects (Murasko et al., 1986; Nagel et al., 1988; Bruunsgaard et al., 2000a). In this study, we could only detect a tendency of lymphocytes from young donors to produce more IL-2. However, due to large S.E.M. we could not show significant age-related differences with this method. The relevance of decreased production of IL2 (Rabinowich et al., 1985; Barcellini et al., 1988) in aging, the major growth factor for T cells, remains unclear. However, a prior study revealed no changes in CD25 ( $\alpha$ -chain of the IL2 receptor) expression with aging (Schindowski et al., 2000). The role of interferons in aging is not yet clear. Decreased levels of interferons (IFN- $\alpha$  and - $\gamma$  (Miller, 1980; Lio et al., 2000)) were found by some laboratories while other investigators determined increased levels (Fahey et al., 2000; Sakata-Kaneko et al., 2000; Yen et al., 2000). In addition, our data are in concordance with the latter showing that activated T cells of both subsets from elderly individuals produced more IFN- $\gamma$  than from young controls. The serum concentration of the inflammatory cytokine TNF- $\alpha$  increases with aging (Bruunsgaard et al., 2000b). However, it remains to be elucidated which cells secrete more TNF- $\alpha$  in the elderly. An increased activity of monocytes and macrophages (Han et al., 1995; Sadeghi et al., 1999) or NK cells (Solana and Mariani, 2000), observed in aged individuals, could be responsible for elevated levels of TNF- $\alpha$  as well. Moreover, in neurological disorders that are related to neuroinflammation and increased activity of glial cells, elevated levels of TNF- $\alpha$  and IL6 are detected as in DAT (Singh, 1994) and in parkinsonism (Dobbs et al., 1999).

A decreased amount of transcription factors (AP-1, NF $\kappa$ B and NFAT) have been observed in aged individuals (Trebilcock and Ponnappan, 1996), resulting in fewer transcription of genes after mitogenic activation. Since not only protein levels but mRNA levels of IL2 as well are de-

creased in aging (Barcellini et al., 1988; Nagel et al., 1988), reduced activation of transcription factors surely is another aspect in immunosenescence.

Decline of immune function with increasing age is not restricted to T lymphocytes: several groups reported impaired B cells function (Hijmans et al., 1984; Antonaci et al., 1985; Paganelli et al., 1992; Burns et al., 1993). However, we could show that aging affects CD4<sup>+</sup> cells that interact with B lymphocytes. A defective T helper activity might contribute to an altered B cell functionality and could account for one aspect of the altered humoral activity associated with aging (Guidi et al., 1998). Natural killer lymphocytes are supposed to be involved in immunosurveillance against tumors and in viral defense. Their age-related changes in activity are still controversially discussed through literature (Murasko et al., 1986), but most studies show increased activation of NK cells with advancing age. In the present study, a significant correlation with donor's age of the percentage of NK cells within the lymphocyte population was determined.

Depressive illness and affective disorders are associated with immune and cytokine alterations (Irwin et al., 1987; Zaharia et al., 1993). Reductions of lymphocyte response to PHA and in vitro IL2 production are effects of repeated stress in rats (Batuman et al., 1990). The neurodegenerative disorder itself has proven effects on central and peripheral immune competent cells as well (Garibaldi and Zhang, 1999; Eckert et al., 2001). Recently, we could show that the CD4/CD8-ratio of Alzheimer's disease patients significantly correlates with the disease's severity (measured as cognitive decline). Since there is no change of the ratio of T helper to T suppressor cells associated with aging, the impact of the pathological increase of CD4<sup>+</sup> cells and the decrease of CD8<sup>+</sup> cells seen in DAT should be further explored.

In conclusion, the age-related modifications of human peripheral lymphocytes are important in order to elucidate additional changes taking place in neurological diseases of geronto-psychiatric patients.

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