Clin Exp Immunol 2000; **119**:433–440

# Proliferative responses of blood mononuclear cells (BMNC) in a cohort of elderly humans: role of lymphocyte phenotype and cytokine production

H. BRUUNSGAARD, A. N. PEDERSEN*, M. SCHROLL†, P. SKINHØJ & B. K. PEDERSEN

*Department of Infectious Diseases, H:S, Rigshospitalet, University of Copenhagen, Copenhagen, *Centre of Preventive Medicine, Glostrup University Hospital and Ministry of Food, Agriculture and Fisheries, Glostrup, and †Department of Geriatric Medicine, H:S, Bispebjerg Hospital, Denmark*

*(Accepted for publication 3 November 1999)*

## SUMMARY

Age-related impaired T cell function is associated with increased mortality risk. The purpose of the present study was therefore to identify factors associated with the age-related decreased phytohaemagglutinin (PHA)-induced proliferative response of lymphocytes in a cohort of 174 81-year-old humans and in 91 young controls. Decreased proliferation was associated with a reduced number of true naive $CD4^+$ cells ($CD62L^+CD45RO^-$). Furthermore, a low IL-2-stimulated proliferation was correlated with a decreased PHA response in the elderly cohort, whereas reciprocal interactions of IL-10- and IL-2-producing cells were of importance in both elderly and young subjects. Accordingly, a minimum of true naive $CD4^+$ cells was required for a normal proliferative response to PHA, perhaps by providing sufficient IL-2 which is critical for growth of naive as well as memory cells.

**Keywords** ageing proliferation cytokines naive T lymphocytes

## INTRODUCTION

T cell proliferation declines with ageing and this may be important for the ability of cells to undergo clonal expansion and to set up an effective response to antigenic stimulations *in vivo* [1]. Furthermore, age-related increases in the proportion of memory T cells to naive T cells have consistently been reported [1]. Associations between increased mortality and lack of response to mitogens have been demonstrated in healthy, elderly humans [2–4]. Furthermore, age-associated decreases in the proliferative response may be more pronounced when mitogens such as phytohaemagglutinin (PHA) are used compared with well-defined activators such as anti-CD3 [5]. It has been suggested that the age-associated increased pool of memory T cells may represent a potential for immune dysregulation through the overproduction of certain immunoregulatory cytokines [6].

Isoforms of the leucocyte common antigen CD45 have been promoted as potential markers of memory T cells [7]. $CD45RA^+$ cells and $CD45RO^+$ cells are considered as nearly reciprocal subsets in humans and have been used as markers of naive cells and memory cells, respectively [7,8]. However, within the $CD45RA^+$ cell compartment there is also a subset which shows characteristics of activated cells with regard to cytokine production and effector functions [9–11]. Furthermore, $CD45RO^+$ T cells are able to revert to the CD45RA phenotype [8]. Accordingly, 'true' naive T cells are not defined by CD45 isoforms alone. CD62L plays an important role in the generation of primary T cell responses [8]. Thus, 'true' naive T cells co-express CD62L and CD45RA [12,13] and show distinct functions from $CD62L^-$ $CD45RA^+$ cells [9,10]. Cytokine-activated naive T cells express the $CD62L^-CD45RA^+CD45RO^-$ phenotype and show functional features intermediate between naive and memory cells *in vitro* when stimulated by the T cell receptor [10]. A T cell subset with the same phenotype has been described in the blood *in vivo* [10]. $CD62L^-CD45RA^+CD45RO^-CD8^+$ cells isolated from the blood of humans have a cytokine profile similar to antigen-experienced cells, including low IL-2 production, and this subset shows potent cytotoxicity without previous *in vitro* stimulation [9].

The purpose of the present study was to evaluate the importance of T cell subsets and cytokine production for the proliferative response to PHA in a cohort of 81-year-old humans. Associations were investigated between, on one hand, the proliferative response to PHA, and on the other the lymphocyte phenotype, cytokine profile, and proliferative response to different stimulators (IL-2, pokeweed mitogen (PWM) and candida). Naive and memory T cells were identified by their constellation of the CD62L (L-selectin) adhesion molecule and the CD45RO isoform of the CD45 glycoprotein. Naive T lymphocytes were defined as $CD62L^+CD45RO^-$ cells; memory T lymphocytes were defined

Correspondence: Helle Bruunsgaard MD, Department of Infectious Diseases M7641, Rigshospitalet, Tagensvej 20, 2200 Copenhagen N, Denmark.
E-mail: infdishb@rh.dk

as $CD45RO^+$ cells; $CD62L^-CD45RO^-$ cells, which may correspond to $CD62L^-CD45RA^+$ cells, were defined as intermediate cells. Furthermore, the importance of CD28 glycoprotein expression, which has a critical role in the costimulatory events that occur along with engagement of the T cell antigen receptor [14], was investigated. The *in vivo* priming of cytokine production was investigated by cytokine levels in supernatants from short-lived PHA stimulations of whole blood. IL-2 and interferon-gamma (IFN-$\gamma$) represented T helper 1 (Th1) and/or T cytotoxic 1 (Tc1) cytokines and IL-10 represented a Th2/Tc2 cytokine [15]. Ratios of IL-2/IL-10 and IFN-$\gamma$/IL-10 were calculated as measures for the balance between Th1/Tc1 and Th2/Tc2 responses.

## SUBJECTS AND METHODS

*Elderly subjects*

The elderly subjects were from the 1914 cohort in Glostrup, which is a longitudinal study of ageing [16,17]. In 1995, survivors were asked to participate in a survey of octogenarians [18]. One hundred and seventy-four individuals aged 80–81 years (88/86 women/men) accepted an extra visit to the laboratory in the Department of Infectious Diseases, Rigshospitalet, for the collection of blood samples used in the immunological surveys. Immunological data are not complete for all subjects due to technical laboratory problems.

No one had dementia or suffered from acute illness prior to the collection of blood samples. Statistical analyses were done with and without subjects having disorders known or suspected to influence immune function: cancer at present or previously ($n = 25$), acute or chronic inflammatory disorders ($n = 5$); intakes of systemic corticosteroids ($n = 7$), > 100 mg acetyl salicylic acid ($n = 8$), or non-steroidal anti-inflammatory drugs ($n = 20$); and increased leucocyte counts in the blood ($> 15 \times 10^9/l$, $n = 1$). In total, 55 elderly people were separated due to these criteria.

*Young subjects*

Forty-five healthy women volunteers and 46 healthy male volunteers aged median 25 years (range 19–31 years) were included as a young control group.

Blood samples were drawn in evacuated blood collection tubes supplemented with 25 IE heparin/ml blood between 7 a.m. and 10 a.m. after an overnight fast.

*Isolation of BMNC*

BMNC were isolated by density gradient centrifugation (Lymphoprep Nyegaard, Oslo, Norway) on LeucoSep tubes (Greiner, Frickenhausen, Germany) and washed three times in medium RPMI 1640 (Gibco, Grand Island, NY). Cells were frozen in freezing medium (50% RPMI 1640, 30% fetal calf serum (FCS; Gibco), and 20% dimethyl sulphoxide (DMSO; Bie Berntsen, Rødovre, Denmark)) and kept in liquid nitrogen until thawed for analysis.

*Proliferative response*

BMNC proliferation assay was performed as previously described [19]. Briefly, cell cultures were performed in triplicates in microtitre plates (Nunc, Roskilde, Denmark), $6 \times 10^5$ BMNC were resuspended in 200 $\mu$l RPMI 1640/10% FCS and incubated with either medium alone, 20 $\mu$g/ml PHA, PWM diluted $1{:}2 \times 10^3$ to induce suboptimal lymphocyte proliferative responses (Life Technologies, Roskilde, Denmark), or 20 U/ml IL-2 (Boehringer, Mannheim, Germany). BMNC were also incubated for 7 days with medium or with ethanol-inactivated candida antigen titrated to the lowest concentration inducing an optimal lymphocyte proliferative response. During the last 24 h cells were exposed to $^3$H-thymidine. Cultures were collected on glassfibre filters with a harvesting machine (Micromate 196; Packard Instruments, Rockville, MD). $^3$H-thymidine incorporation was measured by a direct $\beta$-counter (Matrix 96 direct $\beta$-counter; Packard Instruments). For each triplicate the mean ct/min was calculated. BMNC from both young and elderly subjects were included in each experiment to ensure that the effect of day to day variation would affect the two groups similarly. Stimulation with PHA was chosen as a strong T cell mitogen [14]. In submaximal concentrations PWM stimulates mainly T cells through CD3 [20]. PWM has been shown to be a more sensitive predictor of disease progression than PHA in cohorts of $HIV^+$ patients [21]. Exogenous IL-2 stimulates preferentially proliferation of memory T cells [22] and may compensate for an age-associated defect in the endogenous IL-2 production. Furthermore, IL-2 stimulates proliferation of natural killer (NK) cells. Candida is a recall antigen to which elderly as well as young subjects have been exposed.

*Flow cytometry*

BMNC were washed twice in PBS with 3% FCS. Cells ($1{\cdot}0 \times 10^5$) were resuspended in 100 $\mu$l PBS containing FCS and incubated for 30 min at 4°C with antibodies in volumes recommended by the manufacturer. Labelled cells were washed three times and analysed by a flow cytometer (Epics XL-MCL; Coulter, Hialeah, FL). The subsequent computer analyses were carried out using PC lysis software from Becton Dickinson (Oxnard, CA). The following antibodies were used: FITC-conjugated CD62L (clone FMC46; Dako, Glostrup, Denmark), PE-conjugated CD45R0 (clone UCHL1; Dako) and CD28 PE (clone L293; Becton Dickinson), and peridinin chlorophyll protein (PerCP)-conjugated CD3 (clone SK7; Becton Dickinson), CD4 PerCP (clone SK3; Becton Dickinson), and CD8 PerCP (clone SK1; Becton Dickinson). For determination of background staining, cells were incubated with the relevant mouse isotype antibodies as negative controls. Lymphocytes were distinguished from monocytes on the basis of their forward *versus* right angle light scatters, and controlled by a CD14 Fitch/CD45 PE double-stained sample. A lymphocyte gate was used for all the analyses. Figure 1 shows distinctions between high and low expression of CD62L and CD45RO.

*Whole blood production of IL-2, IL-10 and IFN-$\gamma$*

Whole blood was diluted 1:4 in RPMI 1640 (Kibbutz Beit Haemen, Biological Industries, Israel) and stimulated for 24 h with 5 $\mu$g/ml PHA (Difco, Detroit, MI) at 37°C. After centrifugation, supernatants were harvested and kept at −80°C until analysis in duplicates by commercially available ELISA kits (Quantikine; R&D Systems, Minneapolis, WI); the mean was used in the statistical analysis. Levels in unstimulated blood were below the detection limit of the assays.

*Statistical analysis*

Statistical calculations were performed using SYSTAT statistical software 7.0 (SYSTAT, Evanston, IL). Data are presented as medians and quartiles. Non-normally distributed data were square root (sqr) or $\log_{10}$ transformed in the statistical analyses. Differences between independent groups were tested by a *t*-test. Relationships between the proliferative response to PHA on one hand and the lymphocyte phenotype, cytokine production per lymphocyte, and

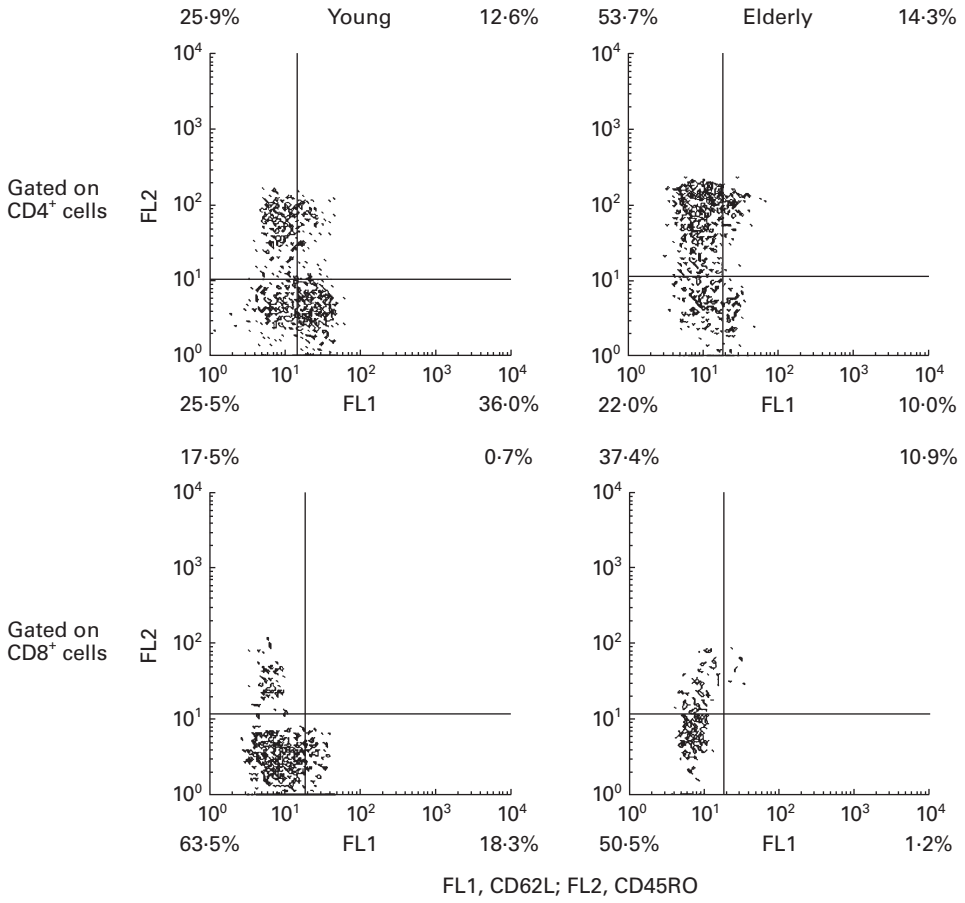


**Fig. 1.** Distinctions between high and low expression of CD62L and CD45RO on $CD4^+$ cells. BMNC were stained by CD62L–Fitch, CD45RO–PE, and CD4–peridinin chlorophyll protein (PerCP) or CD8–PerCP and analysed by flow cytometry. $CD4^+$ or $CD8^+$ lymphocytes were gated based on their forward *versus* right angle light scatters together with a high signal within the emission spectrum of PerCP. Distinctions between high and low expression of CD62L and CD45RO on T lymphocytes were based on mouse isotype antibodies as negative controls. Distributions of the different $CD4^+$ and $CD8^+$ subsets within lymphocytes were subsequently calculated.

proliferative response to different stimulations on the other were investigated by linear regression analysis with independent variables on a sqr or $\log_{10}$ scale. Each independent variable was tested for an interaction with the age group. If an interaction was found ($P < 0{\cdot}05$), separate slopes were calculated for the elderly and the young controls. Analyses were performed univariately and in a multivariate model including naive and intermediate $CD4^+$ cells (%). $P < 0{\cdot}05$ was considered significant.

## RESULTS

*Elderly* versus *young humans*

Proliferative responses to PHA, PWM, IL-2 and candida were reduced in the elderly cohort compared with young controls (Fig. 2). Furthermore, the elderly had reduced percentages of naive $CD4^+$ and $CD8^+$ cells ($CD62L^+CD45RO^-$), percent $CD8^+$ cells (total), percent $CD62L^-CD45RO^-CD8^+$ intermediate cells, and percent $CD28^+CD8^+$ cells compared with young controls, whereas percent $CD62L^-CD45RO^-CD4^+$ intermediate cells were only borderline to be significantly decreased ($P = 0{\cdot}08$). However, elderly people showed increased percentages of memory $CD4^+$ and $CD8^+$ cells ($CD45RO^+$), percent $CD28^-CD8^+$ cells, and percent $CD28^-CD4^+$ cells. Percent $CD4^+$ cells (total) and percent $CD28^+CD4^+$ cells were unaltered (Fig. 2). The unadjusted production of IL-10 was reduced in the elderly cohort, whereas there was no difference in the production of IFN-$\gamma$ and IL-2 or in ratios of cytokines (Table 1).

Blood levels of $CD4^+$ and $CD8^+$ cells were decreased in the elderly cohort due to decreased concentrations of naive $CD4^+$ and $CD8^+$ cells ($CD62L^+CD45RO^-$), intermediate $CD4^+$ and $CD8^+$ cells ($CD62L^-CD45RO^-$), $CD28^+$ cells, and $CD28^-CD4^+$ cells (Table 2). There was no difference with regard to concentrations of $CD45RO^+CD8^+$ memory cells and $CD28^-CD8^+$ cells, whereas the number of $CD45RO^+CD4^+$ memory cells was increased in the elderly subjects (Table 2).

*Linear regression analyses (Table 3)*

In both old and young people the proliferative response to PHA was positively associated with percent $CD4^+$ cells (total), percent $CD62L^-CD45RO^-CD4^+$ intermediate cells and percent $CD28^+$ $CD4^+$ cells. However, in the elderly cohort the proliferative response to PHA showed the strongest correlation to percent $CD62L^+CD45RO^-CD4^+$ naive cells which, on the other hand, did not play a significant part in the response in the young group.

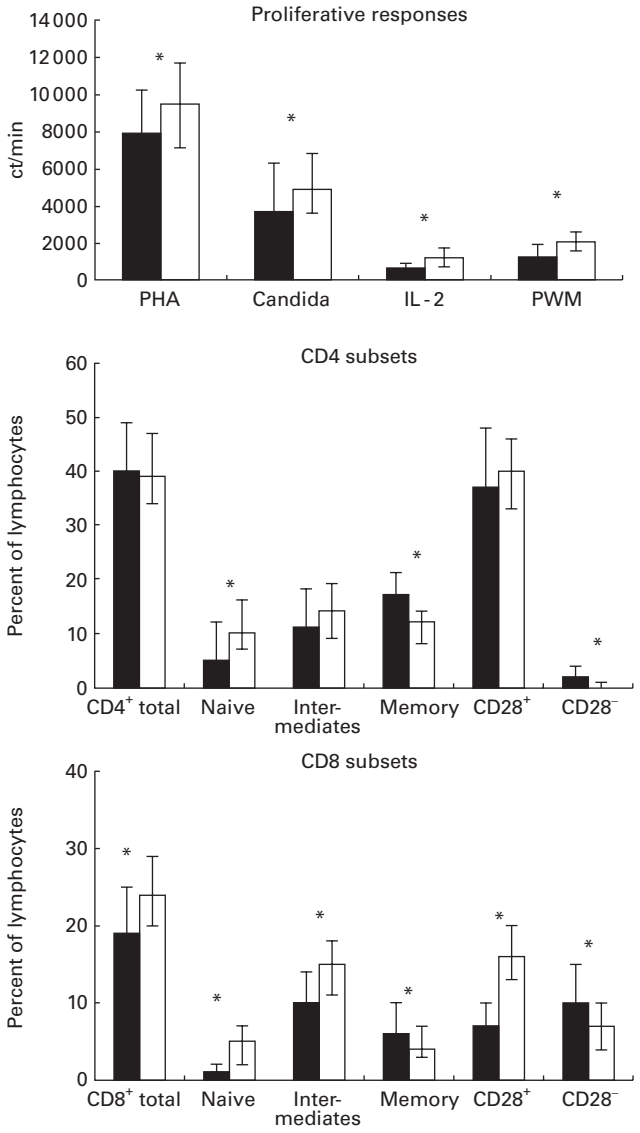


**Fig. 2.** Proliferative responses, CD4 and CD8 subsets in elderly humans (■) and in young controls (□). Medians and quartiles are shown. $*P < 0{\cdot}05$. Proliferative responses to phytohaemagglutinin (PHA): elderly, $n = 161$ *versus* young, $n = 87$; candida: elderly, $n = 118$ *versus* young, $n = 68$; IL-2: elderly, $n = 154$ *versus* young, $n = 87$; pokeweed mitogen (PWM): elderly, $n = 159$ *versus* young, $n = 87$. CD4 subsets, CD4 total: elderly, $n = 169$ *versus* young, $n = 90$; naive, intermediates and memory subsets: elderly, $n = 159$ *versus* young, $n = 86$; CD28 subsets: elderly, $n = 154$ *versus* young, $n = 79$. CD8 subsets, CD8 total: elderly, $n = 168$ *versus* young, $n = 91$; naive, intermediates and memory subsets: elderly, $n = 160$ *versus* young, $n = 86$; CD28 subsets: elderly, $n = 150$ *versus* young, $n = 79$. Naive, $CD62L^+CD45RO^-$ cells; intermediates, $CD62L^-CD45RO^-$ cells; memory cells, $CD45RO^+$ cells.

Furthermore, multiple linear regression analyses showed that the effects of total percent $CD4^+$ cells and percent $CD28^+CD4^+$ cells were not significant when adjusting for percent $CD62L^+$ $CD45RO^-CD4^+$ naive cells and percent $CD62L^-CD45RO^-$ $CD4^+$ intermediate cells. In the following, associations between PHA-induced proliferation and other parameters were analysed with and without adjusting for the effect of naive and intermediate $CD4^+$ cells (multiple linear regression analysis) because these two subsets together explained 17% of the variability in the proliferative response in both age groups.

The proliferative response to PHA was negatively correlated with percent $CD8^+$ cells in a univariate analysis, but not when adjusting for the effect of naive and intermediate $CD4^+$ cells. No associations were found to any particular subsets within the $CD8^+$ cell compartment or to $CD28^-CD4^+$ cells and $CD45RO^+CD4^+$ memory cells (data not shown).

IL-2 production per lymphocyte in response to PHA was positively correlated with the proliferative response in the elderly but not in the young group. However, IL-2 production per lymphocyte showed no significant effect when adjusting for the effect of naive and intermediate $CD4^+$ cells. IL-10 production per lymphocyte in response to PHA was not correlated to the proliferative response in the elderly, whereas it was negatively correlated to the proliferative response in the young group in both univariate and multiple linear regression analyses. IFN-$\gamma$ production was negatively correlated to the proliferative response in both young and elderly subjects but the slope was significantly steeper in the young group. The effect was only significant in univariate regression analysis in the old cohort, whereas it was also significant in multiple linear regression analysis in young controls. The IL-2/IL-10 ratio was positively correlated to the proliferative response in both age groups in univariate as well as in multiple linear regression analyses. The IFN-$\gamma$/IL-10 ratio was only negatively correlated with PHA-induced proliferation in univariate linear regression analysis.

The proliferative response to PHA was correlated to proliferative responses following stimulation by candida or PWM. The effect was still significant when adjusting for the effect of naive and intermediate $CD4^+$ cells (no age-associated differences in the slopes). The proliferative response to IL-2 was only correlated to the PHA response in the old group.

Conclusions were the same when elderly individuals with medical intakes or medical conditions known or suspected to influence the immune system were excluded (data not shown).

## DISCUSSION

The major findings in the present study were that in 81-year-old people the PHA-induced proliferative response of BMNC was associated with: (i) percent $CD62L^+CD45RO^-CD4^+$ naive cells; (ii) PHA-induced IL-2 production; (iii) the proliferative response to exogenous IL-2. However, these associations were not found in young controls. In both elderly and young people PHA-induced BMNC proliferation was correlated with the IL-2/IL-10 ratio and the proliferative response to PWM and candida.

With regard to linear interrelations between proliferative responses and phenotypes of lymphocytes, PHA-induced proliferation in the elderly cohort showed the strongest correlation to true naive $CD4^+$ cells, whereas the association to the intermediate subtype was weak. In contrast, PHA-induced proliferation was only correlated with the intermediate $CD4^+$ subset in the young group. Thus, the decreased presence of true naive $CD4^+$ cells appeared to be a limiting factor in the elderly group, whereas the number might be far above the threshold that limits BMNC proliferation in the young group. Consistent with the present findings, PHA has been described mainly to stimulate proliferation of $CD45RA^+CD4^+$ cells [7], and CD62L has been shown to be crucial in primary T cell responses in CD62L-deficient mice [23]. Negative associations between percent $CD8^+$ cells and PHA-induced

**Table 1.** Levels of cytokines (ng/ml) in whole blood supernatants after phytohaemagglutinin stimulation for 24 h in young and elderly humans

| | Young subjects | Elderly subjects |
|---|---|---|
| IL-2 total (ng/ml) | 1·36 (0·92–2·19), $n = 89$ | 1·32 (0·74–2·11), $n = 162$ |
| IL-2/$10^6$ lymphocytes | 0·74 (0·54–1·19), $n = 87$ | 0·88 (0·524–1·541), $n = 160$ |
| IFN-$\gamma$ total (ng/ml) | 4·45 (2·52–8·16), $n = 83$ | 3·55 (1·17–11·07), $n = 144$ |
| IFN-$\gamma$/$10^6$ lymphocytes | 2·68 (1·33–4·84), $n = 82$ | 2·59 (1·03–8·17), $n = 143$ |
| IL-10 total (ng/ml) | 1·48 (1·10–1·99), $n = 91$ | 1·14 (0·86–1·70)*, $n = 161$ |
| IL-10/$10^6$ lymphocytes | 0·80 (0·65–1·08), $n = 89$ | 0·80 (0·60–1·13), $n = 159$ |
| IL-2/IL-10 ratio | 0·97 (0·66–1·36), $n = 89$ | 1·09 (0·72–1·67), $n = 161$ |
| IFN-$\gamma$/IL-10 ratio | 3·28 (1·83–5·32), $n = 83$ | 3·12 (1·27–8·70), $n = 143$ |

Medians (quartiles) are shown.
*$P = 0·004$.

BMNC proliferation seemed to be secondary to low percentages of $CD4^+$ subsets (multiple linear regression analysis). Thus, a low percentage of $CD4^+$ cells will often be accompanied by a high percentage of $CD8^+$ cells.

In the elderly cohort IL-2 production showed a significant correlation with proliferation to PHA in univariate regression analysis, but not when the analysis was adjusted for the effect of naive and intermediate $CD4^+$ cells. IL-2 production was also correlated with the number of true naive $CD4^+$ cells (data not shown). This finding indicated that one major purpose of naive $CD4^+$ cells might be to provide sufficient IL-2, which again was a limiting factor in the elderly but not in young controls. Whereas production of cytokines such as IFN-$\gamma$ and IL-10 contributed negatively to BMNC proliferation in the young group, it was only of secondary importance to the reduced number of naive $CD4^+$ cells in the elderly cohort. In contrast, a low IL-2/IL-10 ratio was still associated with a decreased proliferative response in multiple linear regression analysis. However, this effect did not seem to be age-dependent, as the slopes were the same for the two age groups. Thus, no evidence was found for an age-related decline in the proliferative response due to dysregulation of cytokines produced by memory cells or due to a shift from Th1/Tc1 to Th2/Tc2 cytokine patterns. The last conclusion is limited by the fact that synthesis of IL-2 and IL-10 in humans is not as tightly restricted to a single subset as in mouse T cells [15], e.g. IL-10 is produced by human Th1 as well as Th2 clones, but Th2 clones show higher IL-10 mRNA levels [24]. Despite these considerations, we chose to let IL-10 represent an approximation to the Th2/Tc2 arm of the immune system because this cytokine, but not the classical Th2/Tc2 cytokine IL-4, can be detected following PHA stimulation. Furthermore, IL-10 is important to consider in the present context, due to the strong anti-proliferative effects. Consistent with the findings in the present study, the age-associated decrease in the proliferative response of BMNC has often been connected to decreased IL-2 production, but data are controversial and there is no consensus [25]. In accordance with the present study, IL-2 production by naive T cells were crucial for the proliferative response of naive as well as memory cells from mice, suggesting that low IL-2 production by memory cells limited their growth in cultures [26]. In addition to the finding that the IL-2/IL-10 ratio was important for the proliferative response in the present study, IL-10 blocked proliferation of isolated naive cells in mice [26]. Naive $CD4^+$ cells from aged mice have been reported to show decreased [27–29] as well as unaltered [30,31] IL-2 production.

No age-associated decline in IL-2 production was found in the

**Table 2.** Blood concentrations of $CD4^+$ and $CD8^+$ subsets in elderly and young humans

| | Young subjects | Elderly subjects |
|---|---|---|
| $CD4^+$ cells | 759 (629–1006), $n = 87$ | 657 (481–849)*, $n = 165$ |
| $CD62L^+CD45RO^-CD4^+$ naive cells ($10^6/l$) | 183 (105–306), $n = 76$ | 91 (36–170)*, $n = 144$ |
| $CD62L^-CD45RO^-CD4^+$ intermediate cells ($10^6/l$) | 248 (133–374), $n = 76$ | 170 (100–269)*, $n = 144$ |
| $CD45R0^+CD4^+$ memory cells ($10^6/l$) | 173 (128–274), $n = 76$ | 245 (154–357)*, $n = 144$ |
| $CD28^+CD4^+$cells ($10^6/l$) | 649 (481–895), $n = 79$ | 513 (391–740)*, $n = 147$ |
| $CD28^-CD4^+$cells ($10^6/l$) | 25 (8–53), $n = 79$ | 19 (5–43)*, $n = 147$ |
| $CD8^+$cells ($10^6/l$) | 402 (309–523), $n = 87$ | 256 (177–353)*, $n = 165$ |
| $CD62L^+CD45RO^-CD8^+$ naive cells ($10^6/l$) | 64 (31–117), $n = 80$ | 17 (8–39)*, $n = 144$ |
| $CD62L^-CD45RO^-CD8^+$ intermediate cells ($10^6/l$) | 250 (168–368), $n = 80$ | 140 (85–225)*, $n = 144$ |
| $CD45RO^+CD8^+$ memory cells ($10^6/l$) | 79 (51–116), $n = 80$ | 80 (45–152), $n = 144$ |
| $CD28^+CD8^+$cells ($10^6/l$) | 254 (125–325), $n = 78$ | 97 (62–145)*, $n = 148$ |
| $CD28^-CD8^+$cells ($10^6/l$) | 109 (68–181), $n = 78$ | 134 (72–230), $n = 148$ |

Medians (quartiles are shown).
*Significant difference ($P < 0·05$) from young controls.

**Table 3.** Linear regression analysis of the lymphocyte phenotype, cytokine profile, and proliferation after different stimulations on the proliferative response to phytohaemagglutinin (PHA) in elderly and young subjects

| Dependent variable | Independent variable | Univariate linear regression analysis | | | Multiple linear regression analysis | | Adjusted, $r^2$ |
|---|---|---|---|---|---|---|---|
| | | Coefficients (s.e.m.) | $P$ | $r$ | Coefficients (s.e.m.) | $P$ | |
| Proliferative response to PHA | sqr(%$CD4^+$ cells) (elderly + young) | **1144 (239), $n = 245$** | **< 0·0005** | **0·41** | 339 (371), $n = 230$ | 0·4 | 0·16 |
| | sqr(%naive $CD4^+$ cells) (elderly) | **1052 (196), $n = 149$** | **< 0·0005** | **0·40** | **1008 (196), $n = 149$** | **< 0·0005** | **0·17** |
| | sqr(%naive $CD4^+$ cells) (young) | 83 (357), $n = 82$ | 0·8 | 0·02 | 451 (374), $n = 82$ | 0·2 | 0·05 |
| | sqr(%intermediate $CD4^+$ cells) (elderly + young) | **600 (185), $n = 231$** | **0·001** | **0·34** | **646 (179), $n = 231$** | **< 0·0005** | **0·17** |
| | sqr($CD28^+CD4^+$ cells) (elderly + young) | **1087 (222), $n = 222$** | **< 0·0005** | **0·43** | 483 (304), $n = 210$ | 0·1 | 0·19 |
| | sqr(%$CD8^+$ cells) (elderly + young) | **− 529 (252), $n = 245$** | **0·04** | **− 0·32** | − 183 (257), $n = 230$ | 0·5 | 0·17 |
| | sqr(IL-2/$10^6$ lymphocytes) (elderly) | **1770 (743), $n = 154$** | **0·02** | **0·19** | 1045 (743), $n = 141$ | 0·2 | 0·16 |
| | sqr(IL-2/$10^6$ lymphocytes) (young) | − 2253 (1549), $n = 83$ | 0·2 | 0·16 | − 2422 (1538), $n = 78$ | 0·1 | 0·07 |
| | $\log_{10}$(IFN-γ/$10^6$ lymphocytes) (elderly) | **− 1041 (481), $n = 135$** | **0·03** | **0·19** | − 478 (474), $n = 126$ | 0·3 | 0·17 |
| | $\log_{10}$(IFN-γ/$10^6$ lymphocytes) (young) | **− 3512 (1012), $n = 79$** | **0·001** | **0·37** | **− 3494 (1005), $n = 75$** | **0·001** | **0·17** |
| | sqr(IL-10/$10^6$ lymphocytes) (elderly) | − 1436 (1192), $n = 153$ | 0·2 | 0·10 | − 1406 (1144), $n = 140$ | 0·2 | 0·15 |
| | sqr(IL-10/$10^6$ lymphocytes) (young) | **− 7934 (2505), $n = 85$** | **0·002** | **0·33** | **− 7853 (2488), $n = 80$** | **0·002** | **0·16** |
| | $\log_{10}$(IFN-γ/IL-10) (elderly + young) | **− 1117 (456), $n = 215$** | **0·015** | **0·38** | − 680 (457), $n = 202$ | 0·1 | 0·19 |
| | $\log_{10}$(IL-2/IL-10) (elderly + young) | **2337 (796), $n = 240$** | **0·004** | **0·36** | **1847 (819), $n = 222$** | **0·03** | **0·18** |
| | sqr(proliferative response to IL-2) (elderly) | **110 (28), $n = 154$** | **< 0·0005** | **0·30** | **139 (26), $n = 142$** | **< 0·0005** | **0·32** |
| | sqr(proliferative response to IL-2) (young) | − 19 (42), $n = 87$ | 0·6 | 0·05 | 3 (43), $n = 82$ | 0·9 | 0·04 |
| | sqr(proliferative response to candida) (elderly + young) | **53 (9), $n = 182$** | **< 0·0005** | **0·46** | **46 (10), $n = 177$** | **< 0·0005** | **0·23** |
| | sqr(proliferative response to PWM) (elderly + young) | **163 (16), $n = 247$** | **< 0·0005** | **0·60** | **156 (16), $n = 229$** | **< 0·0005** | **0·41** |

Each independent variable was tested for an interaction with age group—if an interaction was found ($P < 0·05$) separate slopes were calculated for elderly and young subjects. Multiple linear regression analysis refers to the model including percent $CD62L^+CD45RO^-CD4^+$ naive cells and percent $CD62L^-CD45RO^-CD4^+$ intermediate cells. Independent variables are square root (sqr) transformed or $\log_{10}$ transformed.

Naive: $CD62L^+CD45RO-$; intermediates: $CD62L^-CD45RO^-$; memory: $CD45RO^+$.

present study. This is in accordance with a study by Sindermann *et al*. [32], in which a similar whole blood culture system was used, although the incubation time was 48 h. IFN-γ and IL-10 are preferentially produced by memory cells. There is no consensus about IFN-γ production in human ageing [25]. Production of IL-10 by T cells has been reported increased in old humans [33,34] in contrast to the lack of age-associated changes in IL-10 and IFN-γ production in the present study. Discrepancies may be due to different incubation times. In this study, short-term stimulations were chosen, attempting to measure *in vivo* priming of cytokine production instead of stimulus-driven T cell differentiation *in vitro*. The former may be important for the proliferative response whereas the latter may have an impact on more differentiated effector functions. Whole blood culture stimulations were chosen because they mimic the *in vivo* environment and are the most reproducible condition of culture [35].

The correlation between proliferative responses to PHA and exogenous IL-2 in the elderly cohort but not in young controls suggested that a part of the age-related decrease in proliferative responses was associated with IL-2 insensitivity, and this could be isolated from the effect of decreased percentages of naive $CD4^+$ cells. Thus, the multiple linear regression model including IL-2-induced BMNC proliferation and the percentage of naive and intermediate $CD4^+$ cells explained 32% ($R^2$) of the variability in PHA-induced proliferation in the elderly cohort. It is consistent with reports of an age-related deficit in the proliferative response

even when endogenous IL-2 is high, lack of restoration by exogenous IL-2, and decreased IL-2 receptor expression [25]. However, it should be taken into account that proliferation of NK cells may also contribute to the IL-2 response in the present study. The strong correlations between proliferative responses to PHA on one hand and responses to PWM and candida on the other in both young and elderly people do not support the idea of a preserved T cell proliferation to recall antigens or stimuli mainly involving the CD3 receptor in ageing [36].

The expression of CD28 on $CD8^+$ and $CD4^+$ cells was decreased, as reported by others [37–39]. However, lack of CD28 expression was not associated with declined proliferative responses to PHA.

The elderly cohort in the present study represents an approximation to a normal population. Relatively few immunogerontological studies have based their investigations on well-described cohorts. Accordingly, it is poorly described to what extent normal elderly humans with and without chronic and/or age-associated diseases suffer from cellular immunosenescence. Furthermore, we attempted to investigate associations between various immune parameters, which are known to change with ageing, across and within two different age groups. Accordingly, it was important to include a large number of elderly subjects representing a wide spectrum of 'physiological' ageing but with the same chronological age. Exclusions of subjects with severe medical diseases did not influence the conclusions of the study. This is in accordance with the findings by others using even more strict exclusion criteria [40]. However, we chose not to use 'The SENIEUR protocol' which attempts to separate the influence of disease from physiological ageing by strict selection criteria [41]. This was based on the following considerations. With increasing age a growing proportion of individuals do not fulfil the SENIEUR protocol. Thus, only about 10% of the residents of a home for aged could be admitted in accordance with this protocol [42]. This leads to the question if the SENIEUR Protocol selects exceptional individuals instead of normal aged individuals. Furthermore, the protocol may exclude the subjects who have suffered most from the process of ageing, although these individuals may constitute the very population in which it is important to assess dysregulated immune function for its clinical relevance [43]. Additionally, changes in immune parameters may reflect ongoing pathological processes (e.g. atherosclerosis and dementia) which develop over decades. However, clinical symptoms may first show up late in the process, and in the meantime these immunological changes will be recognized as representing 'physiological' and/or 'successful' ageing, although they may be pathological. We thought it was questionable if physiological ageing could be separated from age-associated diseases. Furthermore, we found it would be bizarre to exclude 70–90% of the population we wanted to study. We chose to solve these problems by selecting our elderly individuals on epidemiological instead of biological criteria.

In conclusion, the present study demonstrated that in a cohort of elderly humans the decreased proliferative response to PHA was associated with a reduced number of true naive $CD4^+$ cells. This indicates that a minimum of true naive $CD4^+$ cells is required for a normal proliferative response to PHA, perhaps by providing sufficient IL-2 which is critical for growth of naive as well as memory cells. Furthermore, low IL-2 responsiveness was associated with decreased PHA response in the elderly cohort but not in young controls. The age-associated decreased proliferative response to PHA and lack of naive $CD4^+$ cells may have clinical implications by inducing an immunodeficiency similar to what is seen in $HIV^+$ subjects [44].

## ACKNOWLEDGMENTS

The excellent assistance of Hanne Willumsen, Ruth Rousing, Leila Jacobsen, Birgit Jensen and Gitte Petersen is acknowledged. The research was supported by the Velux Foundation and The Danish Foundation for the Advancement of Medical Science. H.B. was supported by a grant from The Danish Research Council 9503346.

## REFERENCES

1 Miller RA. The aging immune system: primer and prospectus. Science 1996; **273**:70–4.
2 Murasko DM, Weiner P, Kaye D. Decline in mitogen induced proliferation of lymphocytes with increasing age. Clin Exp Immunol 1987; **70**:440–8.
3 Roberts TI, Whittingham S, Youngchaiyud U, Mackay IR. Ageing, immune response, and mortality. Lancet 1974; **2**:368–70.
4 Ferguson FG, Wikby A, Maxson P, Olsson J, Johansson B. Immune parameters in a longitudinal study of a very old population of Swedish people: a comparison between survivors and nonsurvivors. J Gerontol Biol Sci 1995; **50**:B378–B382.
5 Sansoni P, Brianti V, Fagnoni F *et al*. NK cell activity and T-lymphocyte proliferation in healthy centenarians. Ann NY Acad Sci 1992; **663**:505–7.
6 Ernst DN, Weigle WO, Hobbs MV. Aging and lymphokine production by T cell subsets. Stem Cells 1993; **11**:487–98.
7 Akbar AN, Salmon M, Janossy G. The synergy between naive and memory T cells during activation. Immunol Today 1991; **12**:184–8.
8 Bell EB, Spartshott S, Bunce C. CD4+ T-cell memory, CD45R subsets and the persistence of antigen—a unifying concept. Immunol Today 1998; **19**:60–64.
9 Hamann D, Baars PA, Rep MH *et al*. Phenotypic and functional separation of memory and effector human CD8+ T cells. J Exp Med 1997; **186**:1407–18.
10 Unutmaz D, Baldoni F, Abrignani S. Human naive T cells activated by cytokines differentiate into a split phenotype with functional features intermediate between naive and memory T cells. Int Immunol 1995; **7**:1417–24.
11 Rothstein DM, Sohen S, Daley JF, Schlossman SF, Morimoto C. CD4+CD45RA+ and CD4+CD45RA− T cell subsets in man maintain distinct function and CD45RA expression persists on a subpopulation of CD45RA+ cells after activation with Con A. Cell Immunol 1990; **129**:449–67.
12 Hengel RL, Jones BM, Kennedy MS, Hubbard MR, McDougal JS. Lymphocyte kinetics and precursor frequency-dependent recovery of CD4 (+) CD45RA (+) CD62L (+) naive T cells following triple-drug therapy for HIV type 1 infection. AIDS Res Hum Retrovir 1999; **15**:435–43.
13 Lieberman J, Trimble LA, Friedman RS *et al*. Expansion of CD57 and CD62L−CD45RA+ CD8 T lymphocytes correlates with reduced viral plasma RNA after primary HIV infection. AIDS 1999; **13**:891–9.
14 Weis A. T lymphocyte activation. In: Paul WE, ed. Fundamental immunology. New York: Raven Press, 1993:467–504.
15 Mosmann TR, Sad S. The expanding universe of T cell subsets: Th1, Th2 and more. Immunol Today 1996; **17**:138–46.
16 Schroll M, Jorgensen T, Ingerslev J. The Glostrup Population Studies, 1964–92. Dan Med Bull 1992; **39**:204–7.
17 Schroll M. A ten-year prospective study, 1964–74, of cardiovascular risk factors in men and women from the Glostrup population born in 1914. Dan Med Bull 1982; **29**:213–52.
18 Schroll M, Avlund K, Davidsen M. Predictors of five-year functional ability in a longitudinal survey of men and women aged 75–80. The 1914-population in Glostrup, Denmark. Aging Milano 1997; **9**:143–52.

19 Tvede N, Pedersen BK, Hansen FR *et al*. Effect of physical exercise on blood mononuclear cell subpopulations and in vitro proliferative responses. Scand J Immunol 1989; **29**:383–9.
20 Hofmann B, Ødum N, Platz P, Ryder LP, Svejgaard A. Immunological studies in acquired immunodeficiency syndrome. Scand J Immunol 1985; **21**:235–43.
21 Hofmann B, Lindhardt BO, Gerstoft J *et al*. Lymphocyte transformation response to pokeweed mitogen as a predictive marker for development of AIDS and AIDS related symptoms in homosexual men with HIV antibodies. Br Med J Clin Res Ed 1987; **295**:293–6.
22 Taga K, Kasahara Y, Yachie A, Miyawaki T, Taniguchi N. Preferential expression of IL-2 receptor subunits on memory populations within CD4+ and CD8+ T cells. Immunology 1991; **72**:15–19.
23 Xu J, Grewal IS, Geba GP, Flavell RA. Impaired primary T cell responses in L-selectin-deficient mice. J Exp Med 1996; **183**:589–98.
24 Del PG, De CM, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. J Immunol 1993; **150**:353–60.
25 Hobbs MV, Ernst DN. The role of cytokines in aging. In: Aggarwal BB, Puri RK, eds. Human cytokines: their role in disease and therapy. Cambridge, MA: Blackwell Science, 1996:353–63.
26 Dozmorov IM, Miller RA. Regulatory interactions between virgin and memory CD4 T lymphocytes. Cell Immunol 1996; **172**:141–8.
27 Haynes L, Linton PJ, Swain SL. Age-related changes in CD4 T cells of T cell receptor transgenic mice. Mech Ageing Dev 1997; **93**:95–105.
28 Nagelkerken L, Hertogh HA, Dobber R, Drager A. Age-related changes in lymphokine production related to a decreased number of CD45RBhi CD4+ T cells. Eur J Immunol 1991; **21**:273–81.
29 Kurashima C, Utsuyama M. Age-related changes of cytokine production by murine helper T cell subpopulations. Pathobiology 1997; **65**:155–62.
30 Lerner A, Yamada T, Miller RA. Pgp-1hi T lymphocytes accumulate with age in mice and respond poorly to concanavalin A. Eur J Immunol 1989; **19**:977–82.
31 Flurkey K, Stadecker M, Miller RA. Memory T lymphocyte hyporesponsiveness to non-cognate stimuli: a key factor in age-related immunodeficiency. Eur J Immunol 1992; **22**:931–5.
32 Sindermann J, Kruse A, Frercks HJ, Schutz RM, Kirchner H. Investigations of the lymphokine system in elderly individuals. Mech Ageing Dev 1993; **70**:149–59.
33 Castle S, Uyemura K, Wong W, Modlin R, Effros R. Evidence of enhanced type 2 immune response and impaired upregulation of a type 1 response in frail elderly nursing home residents. Mech Ageing Dev 1997; **94**:7–16.
34 Cakman I, Rohwer J, Schutz RM, Kirchner H, Rink L. Dysregulation between TH1 and TH2 T cell subpopulations in the elderly. Mech Ageing Dev 1996; **87**:197–209.
35 De GD, Zangerle PF, Gevaert Y *et al*. Direct stimulation of cytokines (IL-1 beta, TNF-alpha, IL-6, IL-2, IFN-gamma and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation. Cytokine 1992; **4**:239–48.
36 Sansoni P, Fagnoni F, Vescovini R *et al*. T lymphocyte proliferative capability to defined stimuli and costimulatory CD28 pathway is not impaired in healthy centenarians. Mech Ageing Dev 1997; **96**:127–36.
37 Fagnoni F, Vescovini R, Mazzola M *et al*. Expansion of cytotoxic CD8 (+) CD28 (−) T cells in healthy ageing people, including centenarians. Immunology 1996; **88**:501–7.
38 Effros RB, Walford RL, Porter V *et al*. Decline in CD28+ T cells in centenarians and in long-term T cell cultures: a possible cause for both in vivo and in vitro immunosenescence. Exp Gerontol 1994; **29**:601–9.
39 Weyand CM, Brandes JC, Schmidt D, Fulbright JW, Goronzy JJ. Functional properties of CD4 (+) CD28 (−) T cells in the aging immune system. Mech Ageing Dev 1998; **102**:131–47.
40 Traill KN, Schonitzer D, Jurgens G *et al*. Age-related changes in lymphocyte subset proportions, surface differentiation antigen density and plasma membrane fluidity: application of the eurage senieur protocol admission criteria. Mech Ageing Dev 1985; **33**:39–66.
41 Ligthart GJ, Corberand JX, Fournier C *et al*. Admission criteria for immunogerontological studies in man: the SENIEUR protocol. Mech Ageing Dev 1984; **28**:47–55.
42 Ligthart GJ, Corberand JX, Geertzen HG, Meinders AE, Knook DL, Hijmans W. Necessity of the assessment of health status in human immunogerontological studies: evaluation of the SENIEUR protocol. Mech Ageing Dev 1990; **55**:89–105.
43 Pawelec G, Adibzadeh M, Pohla H, Schaudt K. Immunosenescence: ageing of the immune system. Immunol Today 1995; **16**:420–2.
44 Ullum H, Lepri AC, Victor J, Skinhoj P, Phillips AN, Pedersen BK. Increased losses of CD4+CD45RA+ cells in late stages of HIV infection is related to increased risk of death: evidence from a cohort of 347 HIV-infected individuals. AIDS 1997; **11**:1479–85.