

## Original article

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L. KASZUBOWSKA<sup>1</sup>, A. DETTLAFF-POKORA<sup>2</sup>, L. HAK<sup>3</sup>, M. SZARYNSKA<sup>1</sup>,  
M. RYBA<sup>3</sup>, J. MYSLIWSKA<sup>3</sup>, A. MYSLIWSKI<sup>1</sup>

### SUCCESSFUL AGEING OF NONAGENARIANS IS RELATED TO THE SENSITIVITY OF NK CELLS TO ACTIVATION

<sup>1</sup> Department of Histology, Medical University of Gdansk, <sup>2</sup> Department of Biochemistry,  
Medical University of Gdansk, <sup>3</sup> Department of Immunology,  
Medical University of Gdansk, Gdansk, Poland

NK cells are a component of innate immunity which activity significantly correlates with health status. The aim of our study was to estimate a status of NK (natural killer) cells in the very old (mean age  $92\pm 2$  ys) and old subjects (mean age  $78\pm 5$  ys) as compared to a control group of young individuals (mean age  $25\pm 4$  ys). NK cells were characterized by measurement of their cytotoxic activity, expression of intracellular interferon  $\gamma$ , telomere length and telomerase activity in resting and activated cells. The results revealed that the oldest seniors did not differ from the other age groups in the number of NK cells and NK cytotoxic activity, however, they displayed the shortest telomeres and the lowest telomerase activity. Surprisingly, activated NK cells of the very old, similarly to the old subjects, were able to significantly increase intracellular level of IFN $\gamma$ . Moreover, activated with IL-2 NK cells of the old and oldest seniors showed increased telomerase activity. The results of our study suggest that the functional status of NK cells and their sensitivity to activation is well preserved until very advanced age and may contribute to longevity and successful ageing.

**Keywords:** *nonagenarians, NK cell function, intracellular IFN $\gamma$ , telomere length, telomerase activity*

#### INTRODUCTION

The process of immunoageing, accompanied by a progressive remodeling of the function of the immune system, is to a great extent regulated by different hormonal immunomodulators such as estrogens, androgens, progesterone,

glucocorticoids and melatonin which blood levels and cellular transduction systems undergo significant changes in the elderly (1-3). Ageing of the immune system relates to both innate and adaptive immunity although the innate one is regarded to be better preserved than adaptive one. The age-related alterations of adaptive immunity include a decline of naive T lymphocytes and accumulation of memory/effector T cells, reduction in the number of B cells, up-regulation of the inflammatory responses and dysregulation of the Th1/Th2 system. The increased number of NK cells with well-preserved cytotoxic function is a characteristic feature of the innate part of ageing immune system (4).

NK cells (CD3<sup>-</sup> CD16<sup>+</sup> CD56<sup>+</sup>) are important components of antiviral and antitumor immunity (5). Morphologically they represent large granular lymphocytes and constitute approximately 10-20% of peripheral blood lymphocyte population. The NK activity well correlates with a health status. Healthy individuals may reveal high cytotoxic activity until late senescence and present cytotoxic capacity similar to the young adult counterparts (6, 7). Contrary to other cells of innate immunity, activated NK cells can proliferate and low telomerase activity has been detected even in non-activated NK cells (8, 9). Mature granulocytes, monocytes and mast cells do not proliferate and do not reveal telomerase activity (10).

Telomere shortening is one of the hallmarks of the ageing process and results from inability of DNA polymerases to replicate a linear DNA molecule (end replication problem) and presence of unrepaired damage of DNA caused mainly by oxidative stress (11). Telomeres are structures located at the ends of eukaryotic chromosomes composed of tandemly repeated short sequences (TTAGGG in vertebrates) associated with specific proteins (12). They progressively shorten with each cell division that results in most somatic cells in chromosomal instability and cellular senescence (11). Similarly to other cells, telomeres of the immune cells including B and T lymphocytes, granulocytes, monocytes and NK (natural killer) cells shorten with age (9, 10). Activated lymphocytes, however, increase the activity of telomerase, a ribonucleoprotein DNA polymerase complex that maintains telomere length. The complex comprises the protein reverse transcriptase (hTERT) and a template RNA (hTR). Telomerase activity is absent in most normal human somatic cells because of the lack of hTERT expression. Normally telomerase activity is detected only in stem cells, germ line cells and activated lymphocytes (13). Telomerase activity is also found in neoplastic cells (14).

The process of successful ageing, i.e. ageing in good psychophysical conditions (15) is immunologically characterized by preserved lymphoproliferative responses and NK cytotoxic activity as well as conserved antigen presentation (16). Healthy centenarians show normal number of T lymphocytes, increased production of immunoglobulins, lack of organ-specific autoantibodies, well preserved NK activity and retained proliferative capability of T lymphocytes (17). Studies on human longevity performed by Olshansky *et al.*

revealed that the age of 85 years may be regarded as the average biological limit to life (18). Studies on the phenomena of longevity and successful ageing performed on groups of seniors at the age over 85 are therefore of special interest.

The aim of our study was to compare the function of NK cells, their cytotoxic properties and sensitivity to activation in the group of nonagenarians representing population of selected, long-lived seniors and a group of elderly below the critical age of 85, who belonged to much larger population of seniors. Moreover, we measured telomere length and telomerase activity in NK cells as parameters of cellular ageing.

## MATERIAL AND METHODS

### *Study population*

Peripheral blood mononuclear cells (PBMC) were obtained from 35 young subjects referred to as 'young' (mean age  $25\pm 4$  ys; 19 women and 16 men) and 35 elderly: 28 seniors at the age below 85 referred to as 'old' (mean age  $78\pm 5$  ys; 23 women and 5 men) and 7 seniors at the age over 85 referred to as 'the oldest' (mean age  $92\pm 2$  ys; all women). The elderly volunteers did not suffer from infections, inflammatory or autoimmune diseases for 6 months before and during the study. However, some seniors were afflicted with degenerative joint and spine disease (14), cataract (3), depression (7), circulatory insufficiency (4), ischemic heart disease (10), ischemic brain disease (2), diabetes mellitus (4), osteoporosis (3). Informed consent from volunteers and approval from Ethical Committee of Medical University of Gdansk were obtained.

### *Peripheral blood mononuclear cell separation*

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised peripheral blood by conventional ficoll-uropoline density gradient centrifugation. PBMC were collected at the interphase, washed and resuspended in PBS-1% FBS solution.

### *NK cell separation*

NK cells (CD3-CD16<sup>+</sup>CD56<sup>+</sup> cells) were isolated from PBMC by negative selection with the use of NK isolation kit and MACS magnetic separator (Miltenyi Biotec, USA). PBMC were incubated with Biotin-Antibody Cocktail (suspension of biotin-conjugated monoclonal antibodies against CD3, CD4, CD14, CD15, CD19, CD36, CD123 and Glycophorin A) for 10 min, then washed with washing buffer and incubated with Anti-Biotin Microbeads (microbeads conjugated to monoclonal anti-biotin antibodies) for subsequent 15 min. Then cells were resuspended in 500  $\mu$ l of washing buffer and applied onto the column. Cells eluted from the column in magnetic field represented the enriched NK cell fraction of about 90% purity. The purity of the enriched NK cells was evaluated by flow cytometry. Aliquots of the cell fractions were stained with anti-CD56 PE-conjugated (BD, PharMingen, USA) and anti-CD3 FITC-conjugated (BD PharMingen, USA) monoclonal antibodies (20  $\mu$ g/test).

### *NK cytotoxic activity*

PBMC ( $2\times 10^5$  cells/200  $\mu$ l) were cultured with target K562 cells ( $2\times 10^4$  cells/200  $\mu$ l) mixed in a ratio of 10:1 in RPMI 1640 medium supplemented with 1% FBS for 4 h. Then culture plates were

centrifuged and supernatants were transferred to new plates. Subsequently the amount of lactate dehydrogenase (LDH) released from the lysed cells was estimated with the use of Cytotoxicity Detection Kit (Roche Diagnostics, Germany). LDH present in supernatants reduced tetrazolium salt to formazan salt resulting in a color reaction. The absorbance of samples was measured at 490 nm with microplate reader (Multiscan MCC/340, Labsystems, Finland). Spontaneous LDH release was determined by incubation of target cells with culture medium. The maximum release of LDH was determined by incubation of target cells in the medium with a final concentration of 1% Triton X-100 (Sigma Chemical, USA). The percentage of NK cytotoxicity was calculated according to the formula: NK cytotoxicity (%) = [(experimental value – spontaneous LDH release) / (maximum LDH release – spontaneous LDH release)] x 100%.

#### *Detection of NK cells (CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> cells) in peripheral blood*

Samples of venous blood were aliquoted into 12x75 mm plastic tubes (Falcon, Becton Dickinson Company, USA), 100 µl per tube. Cells were stained with anti-CD3 ECD-labeled (Immunotech Beckman Coulter, France), anti-CD56 PE-labeled (BD PharMingen, USA) and anti-CD16 FITC-labeled (BD PharMingen, USA) monoclonal antibodies (20 µg/test). For each set, an appropriate isotype control was prepared. After 30 min of incubation in the dark at room temperature, red blood cells were lysed and samples fixed with the use of one-step Immunoprep reagent supplied by Immunotech, USA with the use of Q-prep Immunology Workstation supplied by Coulter, USA. The level of CD56 expression determines the phenotypic classification of NK cells into CD56<sup>dim</sup> NK cells exhibiting higher natural cytotoxic activity and CD56<sup>bright</sup> NK cells revealing the capacity to produce cytokines and low natural cytotoxicity (19).

#### *Determination of telomere length in NK cells by flow-FISH method*

Telomeres were detected with the use of Telomere PNA Kit/FITC for Flow Cytometry (DAKO, Denmark). Briefly, purified NK cells (1x10<sup>6</sup> cells) were suspended in 300 µl of hybridization mixture containing 70% formamide with (test samples) or without (control samples) FITC-conjugated telomere PNA (peptide nucleic acid) probe (CCCTAA)<sub>3</sub>. Samples were heat-denatured at 82°C for 10 min and hybridised at room temperature in the dark overnight. Then cells were washed twice with Wash Solution and stained with propidium iodide solution (10 µg/ml propidium iodide and 100 µg/ml RN-ase in PBS) for 2 hours. Flow cytometric analysis was performed on Coulter Epics XL cytometer equipped with 488 nm argon laser. The obtained data were used for the determination of a relative telomere length (RTL), i.e. telomere length of sample cells compared to telomere length of control cells (1301 cell line characterized by very long telomeres) (20).

#### *Estimation of telomerase activity in NK cells*

Telomerase activity was measured in NK cell extracts with the use of TRAP<sub>EZE</sub><sup>®</sup> ELISA Telomerase Detection Kit (Chemicon International, USA) following manufacturer's instructions. NK cells freshly isolated or activated with IL-2 (500 U/ml, 96 h) were suspended in lysis buffer containing CHAPS on ice for 30 min. After centrifugation supernatants were transferred into fresh tubes, quick-frozen in liquid nitrogen and stored in -70°C until use. TRAP (Telomeric Repeat Amplification Protocol) reaction consisted of 2 steps: in the first one telomerase present in extracts added a number of telomeric repeats (GGTTAG) to the 3' end of biotinylated Telomerase Substrate oligonucleotide (b-TS) and in the second step the extended products were amplified by PCR. As the TRAP extension/amplification reaction was performed with biotinylated primers and DNP (dinitrophenyl)-labeled dCTP, the TRAP products were tagged

with biotin and DNP residues. The labeled products were immobilized to streptavidin-coated microtiter plates via biotin-streptavidin interactions and then detected by anti-DNP antibodies conjugated to horseradish peroxidase (HRP). The amount of TRAP products was determined by HRP activity with the use of TMB substrate (3,3',5,5' tetramethylbenzidine) and subsequent color development. Absorbance of the samples was measured at 450 nm (with a reference wavelength of 690 nm) using BIORAD microplate reader.

### *Determination of intracellular IFN $\gamma$ in NK cells*

PBMC ( $1 \times 10^6$  cells/1 ml RPMI1640 containing 5% FBS) were activated 5 h with PMA (phorbol 12-myristate 13-acetate) and ionomycin both supplied by Sigma, USA and used at final concentration of 50 ng/ml. Simultaneously 2  $\mu$ l/well of Golgi-Stop reagent (BD PharMingen, USA) was added to PBMC cultures to stop extracellular export of IFN $\gamma$ . Additionally, non-stimulated cultures were performed for the measurement of spontaneous release of intracellular IFN $\gamma$ . After 5 h PBMC were collected, washed with PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free) containing 1% FBS and 0.09% sodium azide and stained for subsequent 30 min with anti-CD3 ECD-labeled (Immunotech Beckman Coulter, France) and anti-CD56 PE-labeled monoclonal antibodies (BD PharMingen, USA). Then, cells were washed and fixed in 4% paraformaldehyde (Sigma, USA) in PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free), washed and permeabilised for 20 min using 0.1% saponin in PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free) containing 1% FBS and 0.09% sodium azide. After permeabilisation cells were washed, stained with anti-IFN $\gamma$  FITC-labeled monoclonal antibodies (1  $\mu$ g/test) (BD PharMingen, USA) and incubated for 30 min. Appropriate isotype controls were also prepared. Then samples were washed, fixed in 2% paraformaldehyde and analyzed by flow cytometry with the use of Coulter Epics XL cytometer equipped with 488 nm argon laser (21).

### *Statistics*

Statistical analysis was performed with the use of STATISTICA 7.1 program. Parametric and non-parametric distributions were calculated by W Shapiro-Wilk test. As the analyzed data revealed no normal distribution they were then analyzed by non-parametric Kruskal-Wallis test to compare three age groups or U-Mann-Whitney test to compare two age groups. The experimental data are presented in the study as median and percentiles (10% / 90%).

## RESULTS

### *Quantitative analysis of NK cells in the senior and young population*

In our studies both young and two elderly groups did not show any differences in the number of peripheral blood leukocytes. The old seniors (<85 ys), however, showed a significant increase in the number of lymphocytes compared to the young subjects ( $p = 0.0467$ ). Percentage of lymphocytes as a fraction of all leukocytes did not differ between the analyzed age groups (*Table 1*).

The number of NK cells ( $\text{CD}3^+16^+56^+$  cells) and the percentage of NK cells in the population of lymphocytes were similar in all compared groups. Subsets of the  $\text{CD}56^+$  cell population also did not display any significant differences between the analyzed groups concerning both cell numbers and percentages in lymphocyte population (*Table 1*).

*Table 1.* Percentages and absolute numbers of NK cells in peripheral blood of the young, seniors under the age of ninety and seniors over the age of ninety. The results are shown as median and percentiles (10% / 90%). Percentage (%) is calculated for NK cells within lymphocyte gate and for lymphocytes within leukocyte gate. Results statistically significant in the table are marked with \*. All differences among three age groups were calculated by the Kruskal-Wallis test. All differences between two age groups were calculated by the U-Mann-Whitney test (presented under the table only for statistically significant differences,  $p < 0.05$ ).

Parameter	Age group			p value (Kruskal-Wallis test)
	Young	Seniors < 85	Seniors > 85	
Leukocytes (number of cells/ $\mu$ l)	5985, 4680/8380	6825, 4900/9810	4940, 2860/11870	$p = 0.2009$
Lymphocytes (number of cells/ $\mu$ l)	1809.38, 1388.41/2536.95	2315.86, 1454.27/3490.40	1717.32, 815.67/2017.90	$p = 0.0443^*$
Lymphocytes (%)	31.50, 19.98/39.46	32.28, 25.30/47.33	31.67, 17.00/36.54	$p = 0.7440$
CD3 <sup>+</sup> 16 <sup>+</sup> 56 <sup>+</sup> cells (number of cells/ $\mu$ l)	184.90, 12.48/477.57	204.33, 82.82/805.69	178.03, 35.51/316.37	$p = 0.3239$
CD3 <sup>+</sup> 16 <sup>+</sup> 56 <sup>+</sup> cells (%)	10.78, 0.9/21.78	8.84, 3.82/32.89	9.08, 4.35/18.03	$p = 0.4335$
CD3 <sup>+</sup> 56 <sup>dim</sup> cells (number of cells/ $\mu$ l)	225.50, 23.86/493.96	271.46, 97.62/790.07	188.53, 47.24/452.24	$p = 0.3819$
CD3 <sup>+</sup> 56 <sup>dim</sup> cells (%)	11.82, 1.97/23.13	10.98, 5.29/34.42	11.22, 5.79/25.77	$p = 0.6827$
CD3 <sup>+</sup> 56 <sup>bright</sup> cells (number of cells/ $\mu$ l)	2.51, 0.48/21.64	4.15, 1.41/23.48	3.70, 2.24/9.14	$p = 0.3770$
CD3 <sup>+</sup> 56 <sup>bright</sup> cells (%)	0.17, 0.03/1.21	0.22, 0.05/1.07	0.28, 0.12/1.13	$p = 0.2473$

U-Mann Whitney test:

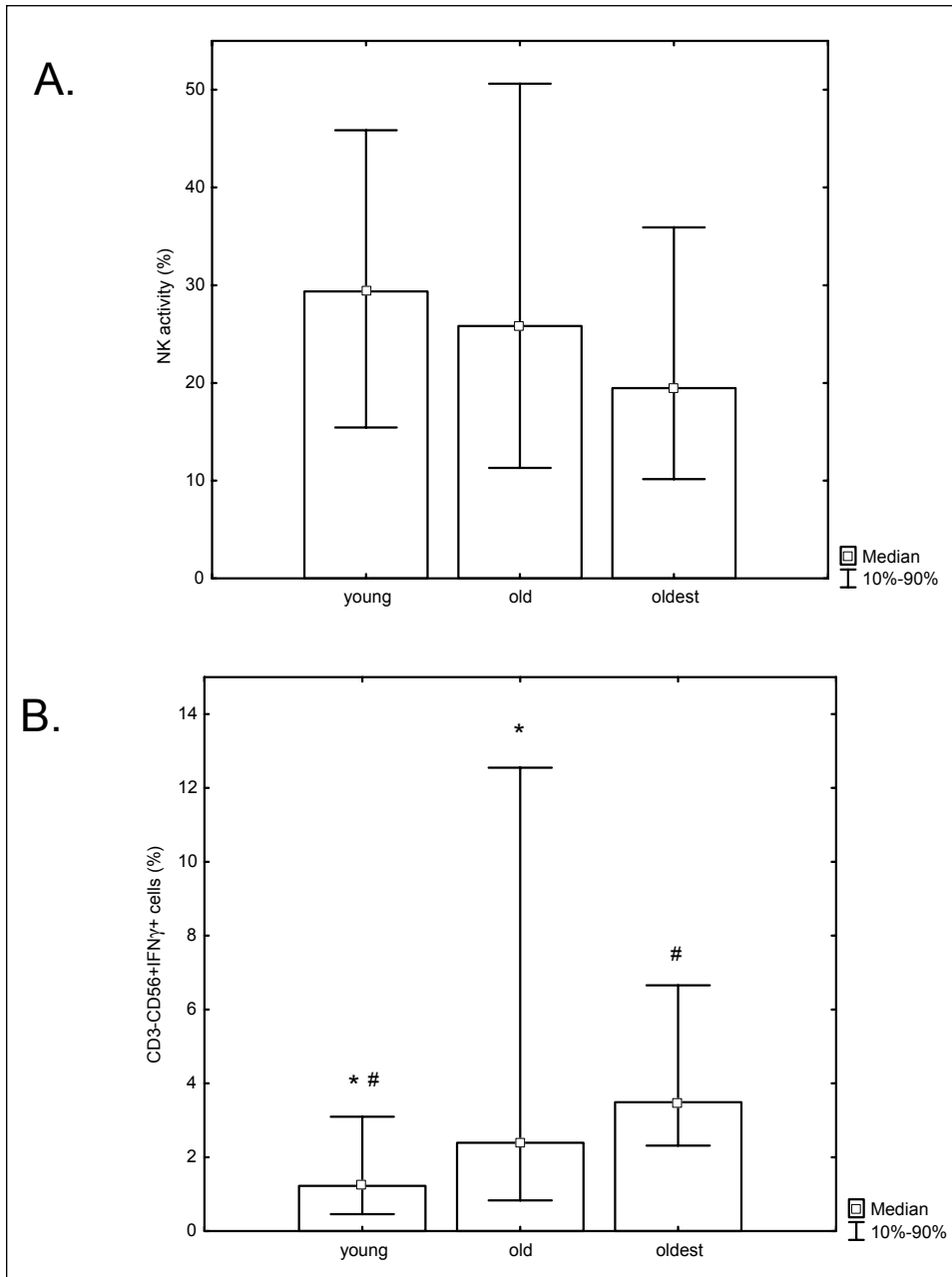
Lymphocytes: young vs seniors < 85,  $p = 0.0467$  ( $p < 0.05$ )

### *NK cytotoxic activity*

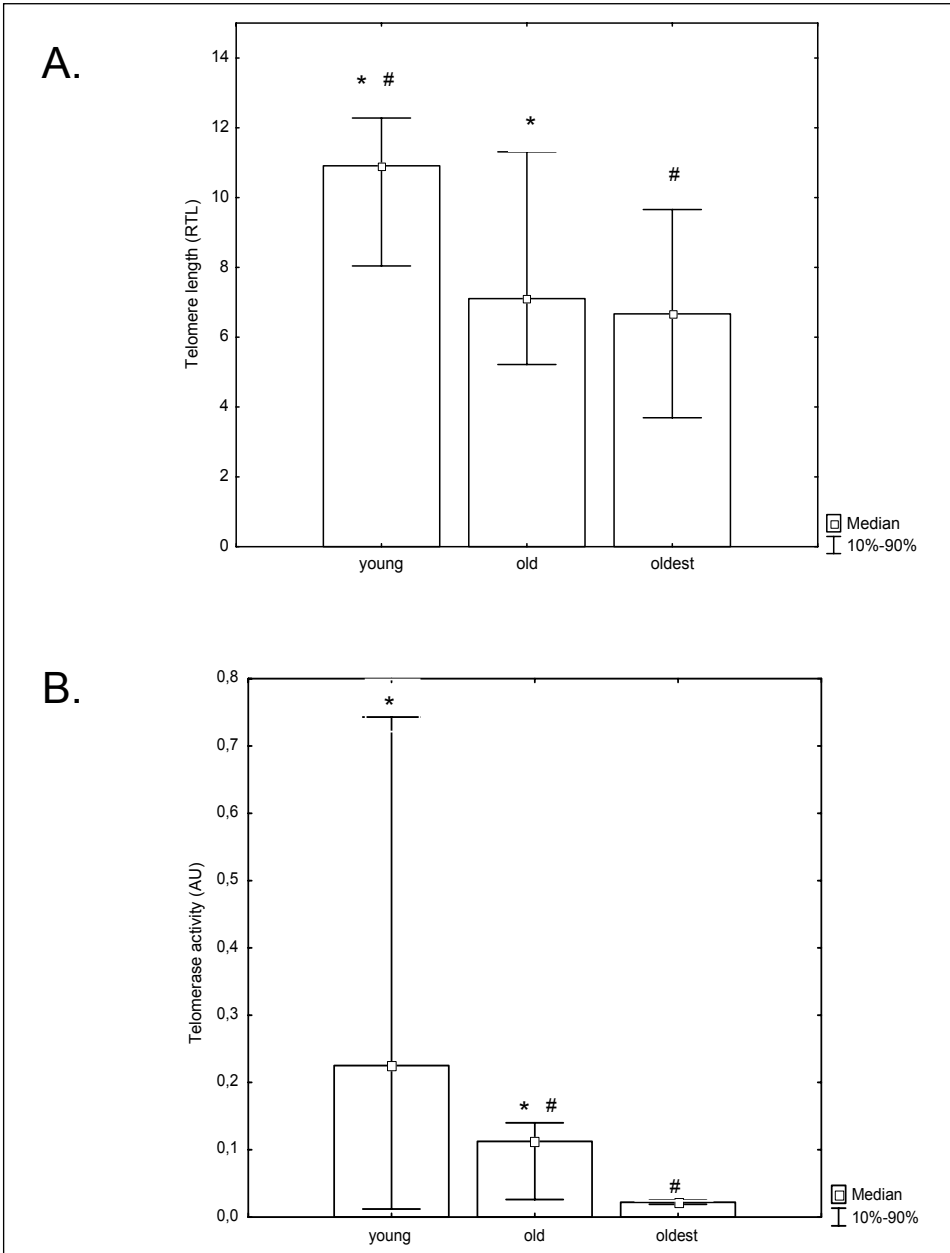
Spontaneous NK cytotoxic activity in freshly isolated cells did not differ significantly between the compared age groups although some age-dependent decrease was observed in the old and the oldest subjects (*Fig. 1a*). When PBMC from all age groups were stimulated 5 h with PMA and ionomycin to estimate their ability to produce intracellular IFN $\gamma$  it appeared that both the old ( $p=0.0329$ ) and the oldest seniors ( $p=0.0051$ ) showed significant increase in the intracellular IFN $\gamma$  expression as compared to the young subjects (*Fig. 1b*).

### *Telomere length and telomerase activity*

Both telomere length and telomerase activity in NK cells decreased significantly with age. Telomeres of both the old seniors ( $p=0.0007$ ) and the oldest seniors ( $p=0.0039$ ) were significantly shorter than telomeres of the young persons. There was, however, no difference between telomere length of the old and the oldest subjects (*Fig. 2a*).



*Fig. 1.* NK cytotoxic activity in the population of the young and seniors. A. NK activity in the population of the young, old and the oldest. Data are presented as median, 10th percentile / 90th percentile. B. Percentage of NK cells with expression of intracellular IFN $\gamma$  (CD3 $\cdot$ 56 $\cdot$ IFN $\gamma$  $\cdot$  cells) after 5 h of activation with PMA and ionomycin in the population of the young, old and the oldest. Data are presented as median 10th percentile / 90th percentile (\*  $p = 0.0329$ ; #  $p = 0.0051$ ).



*Fig. 2.* Telomere length and telomerase activity in NK cells of the young and senior population. A. Telomere length in the population of the young, old and the oldest presented in Relative Telomere Length units (RTL). Data are shown as median 10th percentile / 90th percentile (\*  $p = 0.0007$ ; #  $p = 0.0039$ ). B. Telomerase activity in NK cells isolated from the young, old and the oldest (\*  $p = 0.0464$ ; #  $p = 0.0444$ ) measured at 450 nm ( $A_{450}$ ) presented in arbitrary units (AU). Data are shown as median 10th percentile / 90th percentile.

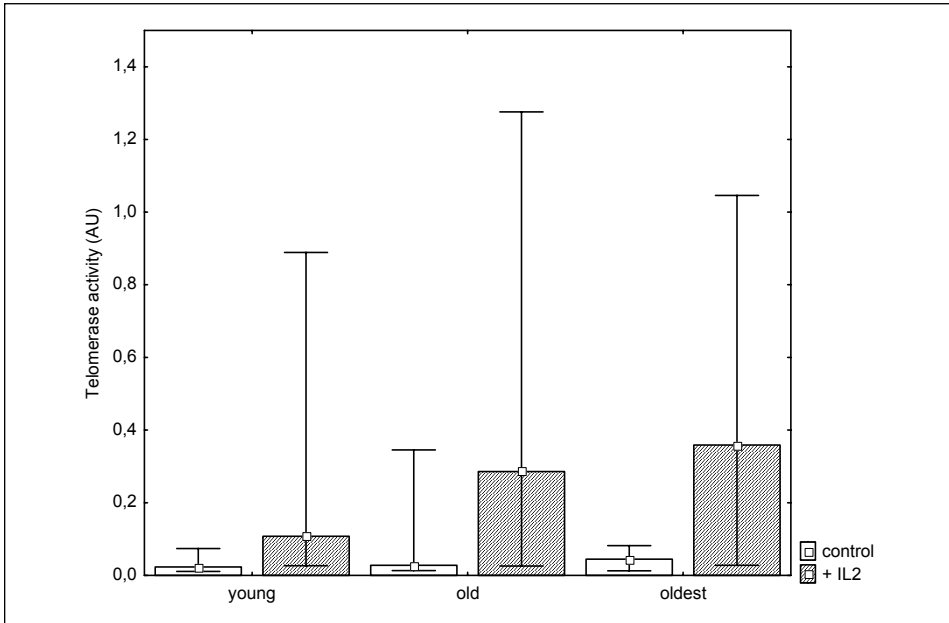


Fig. 3. Telomerase activity in NK cells of the young, old and the oldest after 96 h of stimulation with IL-2 (500 U/ml) measured at 450 nm ( $A_{450}$ ) presented in arbitrary units (AU). Control cells were cultivated simultaneously for 96 h without stimulation. Data are presented as median 10th percentile / 90th percentile.

Our studies revealed also that telomerase activity of the old was significantly lower than activity of the young ( $p=0.0464$ ) and telomerase activity of the oldest was significantly lower than activity presented by the old ( $p=0.0444$ ) (Fig. 2b).

#### *Telomerase activity in resting and activated NK cells*

After stimulation with IL-2 of NK cells isolated from PBMC of the young, old and the oldest, all three age groups revealed an increase in telomerase activity. No significant differences were observed between the analyzed groups but the oldest seniors revealed tendency to present the highest increase in enzyme activity relatively to control cells (Fig. 3).

## DISCUSSION

Ageing of the immune system is characterized by the deterioration of both adaptive and innate immune response. Adaptive immune response is also affected by the proceeding process of thymic involution. Despite some age-related changes the innate immune system is considered to be less sensitive to ageing than adaptive response. Therefore it was of a great interest to compare one of the

less investigated albeit important component of the innate immune response, i.e. NK cells and their contribution to successful ageing.

The number of lymphocytes is a very general determinant of the ageing of the immune system, however, the literature data vary in this respect. Similarly to us also other authors (16, 22) found no differences between lymphocyte number of the young and elderly. However, there are studies which demonstrate that total number of lymphocytes may be increased in the elderly (23) or maintained at the middle-aged level up to the age of 95 and then decreased (24). It is thought that various factors including genetic ones may contribute to these discrepancies (17). The old population, however, presented in our studies a significant increase in the number of lymphocytes relatively to the young.

The studies related to the effect of ageing on the number/percentage of NK cells reveal conflicting data. Similarly to other authors (25, 27) we found that the oldest population of seniors did not differ significantly from the other age groups in the number and percentage of NK cells and that there were no differences both in cell numbers and percentages of CD56<sup>+</sup> subpopulations between the compared groups. Other authors found a significant increase in the number of NK cells as well as an increase in the percentage of more cytotoxic CD56<sup>dim</sup> cells of the NK cell population in aged people (25, 26). On the contrary, the number of CD56<sup>bright</sup> cells, the primary source of NK cell-derived IFN $\gamma$  appeared to decline with age (27). These differences may be partially attributed to the health status of the elderly. Both the oldest and old subjects in our study were generally healthy and had not shown any symptoms for longer time before the investigation.

The cytotoxic activity of the NK cells is thought to be an important component of the ageing immune system. The NK activity of the oldest seniors in our study did not differ significantly from the other age groups. These data corresponded to the previous studies performed by Sansoni and coworkers who found that even very old seniors presented well preserved natural killer activity and this parameter could be interpreted as a factor of longevity (28). In earlier studies, however, NK cytotoxic activity was reported to be unaffected (29), decreased (30) or increased (31) in the elderly.

In normal physiology functioning of the immune system is continuously affected by many activating and inhibiting factors that modulate its activity. Unexpectedly, when we stimulated PBMC with nonspecific activators of lymphocytes, PMA and ionomycin, we found that both groups of seniors displayed significantly higher percentages of cells expressing intracellular IFN $\gamma$  as compared to young subjects. Moreover, we showed that NK cells of very old seniors may effectively produce IFN $\gamma$  after antigenic stimulation. Interferon  $\gamma$  strongly stimulates macrophages (32), T<sub>H</sub>1 lymphocytes (33) and cytotoxic T lymphocytes (34). It also activates neutrophils and stimulates the cytotoxic activity of NK cells (35). Our data are corroborated by study that showed significantly higher rates of IFN $\gamma$  production in seniors attending moderate physical training program (36). The ability of NK cells to produce IFN $\gamma$  can

compensate age-related decrease in the adaptive immunity and help to preserve the function of the immune system. However, some authors described diminished secretion of IFN $\gamma$  after stimulation of NK cells of the elderly (8, 37). Again, the inherent differences in the characteristics of the elderly subjects may be responsible for various results between studies.

The length of telomeres is by some authors regarded as a major determinant of cellular ageing. The age-related shortening of telomeres of NK cells found in our investigation was also described by Mariani and coworkers (9). In their study only octogenarians revealed significantly shorter telomeres relatively to the septuagenarians and young, however, no significant differences were found in the telomere length of the young and septuagenarians (9). This observation with our finding of shorter telomeres (than in the young) in the old subjects (mean age 78 years) may suggest that there is an “age threshold” of telomere shortening in the NK cells. Further studies on a larger population could prove this suggestion.

The length of telomeres is to a great part dependent on the activity of telomerase. The oldest seniors in our study showed significantly lower NK cell telomerase activity than the old ones, whose NK cells had lower telomerase activity than the young subjects. Similar results related to septuagenarians and octogenarians obtained Mariani and coworkers (9). However, in their study, only octogenarians but not septuagenarians presented significantly lower telomerase activity than the young individuals.

Since IL-2 is a very potent immunomodulator acting on various lymphocyte populations we compared telomerase activity in NK cells isolated from the young, old and the oldest subjects. Interestingly, IL-2 increased telomerase activity to a similar range in the all investigated age groups. This is a novel observation as no similar investigations on NK cells isolated from healthy people in the context of ageing were performed. Kawauchi and coworkers described increase of telomerase activity in IL-2 stimulated cells of NK-92 cell line established from a patient with progressive NK cell lymphoma (38). It is known that telomerase activity is induced during activation of lymphoid cells (39) and its activity is probably important in determining the capacity of lymphoid cells to proliferation and clonal expansion (40).

In conclusion, we demonstrated that the NK component of the innate immunity is well preserved during ageing. The NK cells of very old and old subjects effectively respond to activation by increasing intracellular concentration of interferon  $\gamma$  and an efficient increase of telomerase activity. The sensitivity of NK cells of nonagenarians to antigenic stimulation appears to contribute to healthy ageing and longevity.

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Author's address: Lucyna Kaszubowska, Department of Histology, Medical University of Gdansk, Debinki 1, 80-211 Gdansk, Poland. Tel.: (+48) 58-349-14-37. Fax: (+48) 58-349-14-36; e-mail: lkras@amg.gda.pl