Telomere Length in Human Natural Killer Cell Subsets

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ABSTRACT: Natural killer (NK) cells are cytotoxic cells that play a critical role in the innate immune response against infections and tumors. In the elderly, the cytotoxic function of NK cells is often compromised. Telomeres progressively shorten with each cell division and with age in most somatic cells eventually leading to chromosomal instability and cellular senescence. We studied the telomere length in NK cell subsets isolated from peripheral blood using "flow FISH," a method in which the hybridization of telomere probe in cells of interest is measured relative to internal controls in the same tube. We found that the average telomere length in human NK cells decreased with age as was previously found for human T lymphocytes. Separation of adult NK cells based on CD56 and CD16 expression revealed that the telomere length was significantly shorter in $CD56^{dim}CD16^{+}$ (mature) NK cells compared to $CD56^{bright}CD16^{-}$ (immature) NK cells from the same donor. Furthermore, sorting of NK cells based on expression of activation markers, such as NKG2D and LFA-1, revealed that NK cells expressing these markers have significantly shorter telomeres. Telomere fluorescence was very heterogeneous in NK cells expressing CD94, killer inhibitory receptor (KIR), NKG2A, or CD161. Our observations indicate that telomeric DNA in NK cells is lost with cell division and with age similar to what has been observed for most other hematopoietic cells. Telomere attrition in NK cells is a plausible cause for diminished NK cell function in the elderly.

KEYWORDS: telomere length; NK cells; flow cytometry; flow FISH

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INTRODUCTION

Telomeres are specialized structures at the end of chromosomes, which consist of repetitive TTAGGG sequences and a number of associated proteins.[1,2] The length of telomere repeats varies between chromosomes and between species. In most human cells, the length of telomere repeats is remarkably heterogeneous[3] ranging from a few to 20 kb depending on the tissue type, the age of the donor, and the replicative history of the cells. The length of telomere repeats was found to gradually decrease with cell divisions (50–200 bp per cell division in normal somatic cells) leading to chromosomal instability and cellular senescence upon replication *in vitro* and with aging *in vivo.*[4,5] Human telomerase is a reverse transcriptase enzyme that has the ability to extend telomeres by adding single-stranded telomeric DNA to the 3′ ends of chromosomes.[6,7] Transfection of the telomerase reverse transcriptase gene into various cells can result in the elongation of telomere length and extension of the *in vitro* replicative life span.[8–13]

A correlation between telomere shortening and life span has been found in cells of the immune system. Effective immune responses in humans rely on a variety of circulating cell types and require rapid expansion of specific T cells or B cells. Previous studies have shown that the average telomere length of memory CD4 and CD8 T lymphocytes from the same donor is significantly shorter than that of naïve T cells.[14,15] In contrast, memory B cells ($CD19^{+}CD27^{+}$) have almost 2 kb longer telomeres compared to naïve B cells ($CD19^{+}CD27^{-}$)[16] and activated B cells appear to prevent telomere shortening by the upregulation of telomerase activity.[17,18]

Over an individual's lifetime, successful host defense relies on the concerted actions of innate and adaptive immunity. Natural killer (NK) cells are an important component of the innate immune system, which can recognize and induce lysis of a variety of target cells including virally infected cells, tumor cells, and allogeneic cells. NK cells can also provide an early source of cytokines and chemokines without prior sensitization.[19,20] NK cells comprise approximately 10–15% of all circulating lymphocytes and are defined phenotypically by their expression of CD56, an isoform of neural adhesion molecule with unknown function, as well as CD16, and lack of expression of CD3.[21] Density of surface expression of CD56 and CD16 can be used to classify functionally and developmentally distinct NK cell subsets.[22,23] $CD56^{bright}CD16^{-}$ NK cells presenting immature NK cells mediate low or no cytotoxicity, proliferate in response to IL-2, and produce high levels of inflammatory cytokines. In contrast, $CD56^{dim}CD16^{+}$ NK cells presenting mature NK cells are potent mediators of cytotoxicity, which have a distinct cytokine and chemokine profile.[22] Furthermore, NK cells express different cell-surface receptors creating a diverse NK cell repertoire, which exhibits specificity in the immune response.[24] A balance between the NK activating and inhibitory receptors on the cell surface controls the cytolytic activity of NK cells, such as human killer cell Ig-like

receptors (KIR), member of the C-type-lectin like receptors, CD94-NKG2A heterodimers, and NKG2D.[24] Peripheral blood NK cells are also equipped with a panel of cell-surface molecules that have been documented to participate in the binding of NK cells to endothelial cells, such as lymphocyte function-associated Ag 1 (LFA-1), CD44, CD2, and CD31.[25,26]

It has been reported that the number of NK cells increases and that modifications of NK cell cytolytic activity occur with advancing age.[27–30] A recent study observed an age-associated loss of telomere length and reduction of telomerase activity in $CD16^+$ NK cells of octogenarians.[31] However, little is known about the telomere biology in the NK cell subsets in terms of the expression of different surface molecules and activating or inhibitory receptors. To further characterize the telomere length heterogeneity in peripheral blood cells of normal individuals, we analyzed the length of telomere repeat sequences in NK cell subpopulations that were purified on the basis of phenotypic properties using the partially automated flow FISH technique.[32]

MATERIALS AND METHODS

Subjects

Peripheral blood mononuclear cells (PBMCs) were obtained with informed consent from eight healthy male and female individuals by density centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). The mononuclear cells were frozen in tissue culture medium containing 20% fetal bovine serum (FBS) and 10% dimethylsulfoxide (DMSO). Following thawing, cells were washed and resuspended in phosphate-buffered saline (PBS) containing 2% FBS (staining buffer) for staining or sorting.

Flow Cytometry and Cell Sorting

Leukocytes were stained with fluorescent-labeled monoclonal antibodies against human CD3, CD4, CD8, CD19, CD56, CD16 (all purchased from Becton Dickinson, San Jose, CA), Vα24, and CD161 (Immunotech, Marsielle, France). After labeling, $CD3^+$, $CD4^+CD3^+$, $CD8^+CD3^+$, $CD19^+CD3^-$, $CD16^+CD3^-$, $CD56^+CD3^-$, $V\alpha24^+$, and $V\alpha24^+CD161^+$ cells were sorted on a FACSVantage (Becton Dickinson) cell sorter and immediately cryopreserved in 10% DMSO, 20% FBS, and 70% RPMI (freezing medium) for analysis by flow FISH at a later time point.

Human NK Cell Enrichment and NK Cell Subsets Sorting

Human NK cells were isolated from normal donor leukocytes using NK cell enrichment cocktail (StemCell Technologies, Vancouver, BC) according to the

manufacturer's directions. Enriched NK cells were stained with monoclonal antibodies against human CD56 (Becton Dickinson), CD16 (Becton Dickinson), CD94 (Immunotech), KIR (NKB1, Becton Dickinson), NKG2A (Immunotech), NKG2D (R&D Systems, Inc., Minneapolis, MN), LFA-1 (Becton Dickinson), and CD161 (Becton Dickinson). After labeling, $CD56^{dim}CD16^{+}$, $CD56^{bright}CD16^{-}$, positive and negative cells for CD94, KIR, NKG2A, NKG2D, LFA-1, and CD161 antibodies were sorted and immediately cryopreserved in freezing medium for further flow FISH analysis.

Telomere Length Analysis by Fluorescence In Situ *Hybridization and Flow Cytometry (Flow FISH)*

The average length of telomere repeats at chromosome ends in individual peripheral blood leukocytes was measured by automated flow FISH as previously described.[14,32,33] Briefly, sorted T cells, B cells, NK cells, and natural killer T (NKT) cells were hybridized with or without 0.3 μg/mL telomere-specific fluorescein isothiocyanate (FITC)-conjugated $(CCCTAA)_3$ PNA probe, washed, and counterstained with 0.01 μg/mL LDS 751 (Exciton Chemical Co. Inc., Dayton, OH). To convert the fluorescence measured in sample cells hybridized with the FITC-labeled telomere PNA probe into kilobases of telomere repeats, fixed bovine thymocytes with known telomere length (internal control) were processed simultaneously with each sample.[32] FITC-labeled fluorescent beads were used to correct for daily shifts in the linearity of the flow cytometer and due to possible fluctuations in the laser intensity and alignment. Flow cytometric data collection was performed on a Becton Dickinson FACSCalibur and the data analysis was performed using CellQuestPro (Becton Dickinson).

Statistical Analysis

All statistical procedures were performed with SPSS™ for Windows™ v.6.1 (SPSS inc., Chicago, IL). The Mann–Whitney *U* test was used to determine statistically significant differences of the telomere length comparisons between B cells and T cells or NK cells, $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$, $NKG2D^{+}$ and $NKG2D^{-}$, and $LFA\text{-}1^{+}$ and $LFA\text{-}1^{-}$ NK cells in this study. *P* values less than 0.05 were considered significant.

RESULTS

Telomere Length in Sorted T, B, and NK Cells

The median telomere length in sorted T cells ($CD3^{+}$), B cells ($CD19^{+}CD3^{-}$), and NK cells ($CD16^{+}CD3^{-}$ and $CD56^{+}CD3^{-}$) of seven

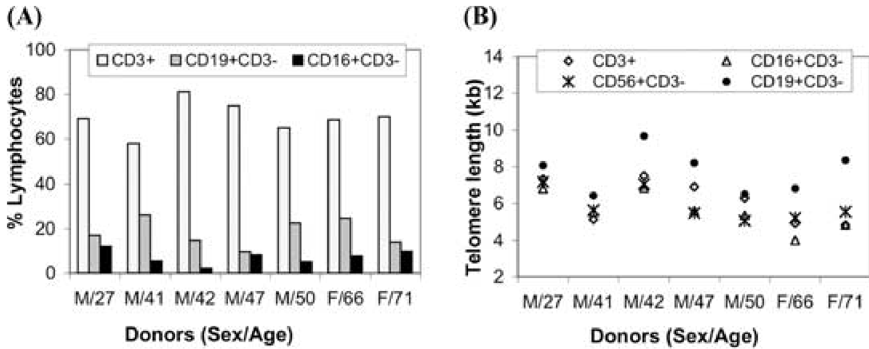

FIGURE 1. Telomere length in sorted T ($CD3^+$), B ($CD19^+CD3^-$), and NK ($CD16^+CD3^-$) cells from healthy individuals measured by flow FISH. (**A**) Proportion of T cells, B cells, and NK cells among lymphocytes. (**B**) Telomere length (kb) in sorted T, B, and NK ($CD16^+CD3^-$ and $CD56^+CD3^-$) cells from seven individual donors. The difference of telomere length between B cells and T cells ($P < 0.05$) and NK cells ($P < 0.05$) is statistically significant.

individuals was measured by automated flow FISH. The proportion of T cells, B cells, and NK cells in density separated mononuclear blood cells from those individuals is shown in FIGURE 1 A. The median telomere length in T cells, B cells, and the NK cell populations is shown in FIGURE 1 B. In all individuals B cells had longer telomeres compared to T cells and NK cells. The mean telomere length in B cells was 7.7 ± 0.6 kb, in T cells 6.1 ± 0.6 kb, and in NK cells 5.9 ± 0.4 kb. The most pronounced difference in telomere length between B cells and T cells or NK cells was observed in donor F/71 (3.5 kb and 2.8 kb) and resulted from the relatively longer telomeres in B cells in this donor. The difference in median telomere length between B cells and T cells (or NK cells) was statistically significant ($P < 0.05$). B cells showed the least decline in telomere length with age and B cells from old donors F/66 and F/71 had longer telomeres (resp. 6.8 and 8.3 kb) than B cells from some of the younger donors as M/41 and M/50 (resp. 6.4 and 6.5 kb, FIG. 1B).

Telomere Length in Sorted NK Cell Subpopulations

The proportion of $CD56^{dim}CD16^+$ mature NK cells and $CD56^{bright}CD16^-$ immature NK cells in an enriched NK cell population was analyzed by flow cytometry (FIG. 2 A). The majority of the enriched NK cells from eight individuals were $CD56^{dim}CD16^+$ mature NK cells (mean: 69.6%, range: 62.1–80.9%), and only a small proportion (mean: 3.7%, range: 1.3–7.1%) were $CD56^{bright}CD16^-$ immature NK cells (FIG. 2 C, upper panel). An example of the fluorescence histograms obtained by flow FISH analysis of $CD56^+CD16^+$ and $CD56^+CD16^-$

populations is shown in FIGURE 2B. The telomere fluorescence of mature $CD56^{+}CD16^{+}$ NK cells was more heterogeneous compared to the more immature $CD56^{bright}CD16^{-}$ immature NK cells. This difference in fluorescence histograms most likely represents a more diverse replication history of the mature NK cells, similar to the more heterogeneous telomere fluorescence in memory compared to naïve T cells observed previously.[14] All the eight donors had longer telomeres in $CD56^{+}CD16^{-}$ immature (6.3 ± 0.6 kb) than in $CD56^{+}CD16^{+}$ mature NK cells (5.5 ± 0.6 kb) and this difference was statistically significant ($P < 0.05$). The maximum telomere length difference for mature and immature NK cells was 1.5 kb (donor M/47, FIG. 2 C, lower panel). To further analyze the telomere length in more specialized NK cell subpopulations, the enriched NK cells were sorted on the basis of CD94, KIR, NKG2A, NKG2D, CD161, and LFA-1 expression. CD94 was expressed on most of the cells (mean: 71.3%, range: 65.6–76.4%) as was NKG2D (mean: 76.6%, rang: 60–94.1%) and LFA-1 (mean: 86.4%, range: 78.1–95%). NKG2A was expressed on a more variable proportion of the cells (mean: 43.2%, range: 27.4–53.1%) and KIR and CD161 were expressed on only a minority (resp. mean: 24.4%, range: 0–47.1% and mean: 17.2%, range: 8.4–23%) of the enriched NK cells (FIG. 2 D).

The telomere length was significantly longer in $NKG2D^{+}$ (5.5 ± 0.6 kb) compared to $NKG2D^{-}$ (5.9 ± 0.7 kb) NK cells from the same donor ($n = 8, P < 0.05$). This was also true for LFA-1^{+} (5.6 ± 0.5 kb) compared to LFA-1^{-} (6.5 ± 0.6 kb) NK cells ($n = 8, P < 0.05$). However, the telomere length was more heterogeneous in $CD94^{+}$ (mean: 5.4 kb, range: 3.0–7.9 kb), $CD94^{-}$ (mean: 5.9 kb, range: 4.4–7.5 kb), $NKG2A^{+}$ (mean: 5.9 kb, range: 4.7–7.2 kb), $NKG2A^{-}$ (mean: 5.3 kb, range: 3.4–7.2 kb), KIR^{+} (mean: 5.1 kb, range: 2.6–7.2 kb), KIR^{-} (mean: 5.9 kb, range: 5.0–7.3kb), $CD161^{+}$ (mean: 6.0 kb, range: 4.7–7.7 kb), and $CD161^{-}$ (mean: 6.2 kb, range: 4.0–8.3 kb) NK cell populations (FIG. 2D) and the expression of these markers was not significantly different. The difference between cells that do or do not express these markers was not significant.

Telomere Length in Sorted Human NKT Cells

We also compared the telomere length in sorted NKT ($V\alpha24^{+}CD161^{+}$) cells and $V\alpha24^{+}$ T cells. The proportion of $V\alpha24^{+}$ T cells (mean: 0.62%, range: 0.14–0.89%) within lymphocytes was higher than NKT cells (mean: 0.29%, range: 0.11–0.71%) for all seven individuals (FIG. 3, *upper panel*). The $V\alpha24^{+}$ T cells (6.2 ± 0.6 kb) had significantly longer telomeres than NKT cells (4.9 ± 0.8 kb) ($n = 7, P < 0.05$) (FIG. 3, *lower panel*).

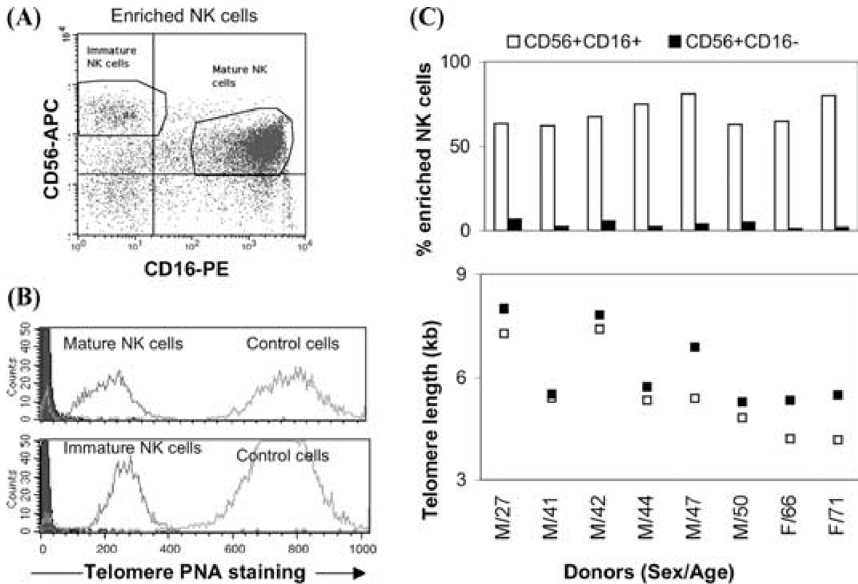

FIGURE 2. Percentages and telomere length of NK cell subsets within enriched NK cell populations. (**A**) The enriched NK cells were stained with anti-CD56-APC and CD16-PE and the CD56$^+$CD16$^+$ (mature NK) and CD56$^+$CD16$^-$ (immature NK) cells in the indicated windows were sorted. (**B**) Representative staining obtained by flow FISH analysis of CD56$^+$CD16$^+$ and CD56$^+$CD16$^-$ populations relative to the bovine thymocytes with known telomere length used as control cells. (**C**) Proportion of CD56$^+$CD16$^+$ (mature NK, *open boxes*) and CD56$^+$CD16$^-$ (immature NK, *black boxes*) NK cells and corresponding telomere length. Cells were sorted from enriched NK cell populations from eight healthy donors ($P < 0.05$). (**D**) Telomere length in sorted NK cell subpopulations measured by flow FISH. Proportions and telomere length of NK subpopulations (CD94$^+$, CD94$^-$, KIR$^+$, KIR$^-$, NKG2A$^+$, NKG2A$^-$, CD161$^+$, CD161$^-$, NKG2D$^+$, NKG2D$^-$, LFA-1$^+$, and LFA-1$^-$) within enriched NK cells are shown in upper and middle lower panels as well as upper middle and lowest panels, respectively. Telomere length from the same donor was significantly longer in NKG2D$^+$ than NKG2D$^-$ NK population ($P < 0.05$), so was in LFA-1$^+$ than LFA-1$^-$ NK cells ($P < 0.05$).

DISCUSSION

In this study, we measured the telomere length in NK cell subpopulations using the automated flow FISH method, in which hybridized fluorescently labeled telomere probe in cells of interest is measured relative to internal control cells (bovine thymocytes) in the same tube.[32,33] We observed a marked heterogeneity in the telomere length in adult peripheral NK cell subpopulations. The pattern of telomere length in NK cells is similar to what has been described previously in T cells.[34,35] In each of eight donor samples studied, the CD56^{dim}CD16$^+$ mature NK cells were found to have shorter telomeres than the CD56^{bright}CD16$^-$ immature NK cells. Furthermore, we showed that NK cells carrying NKG2D,

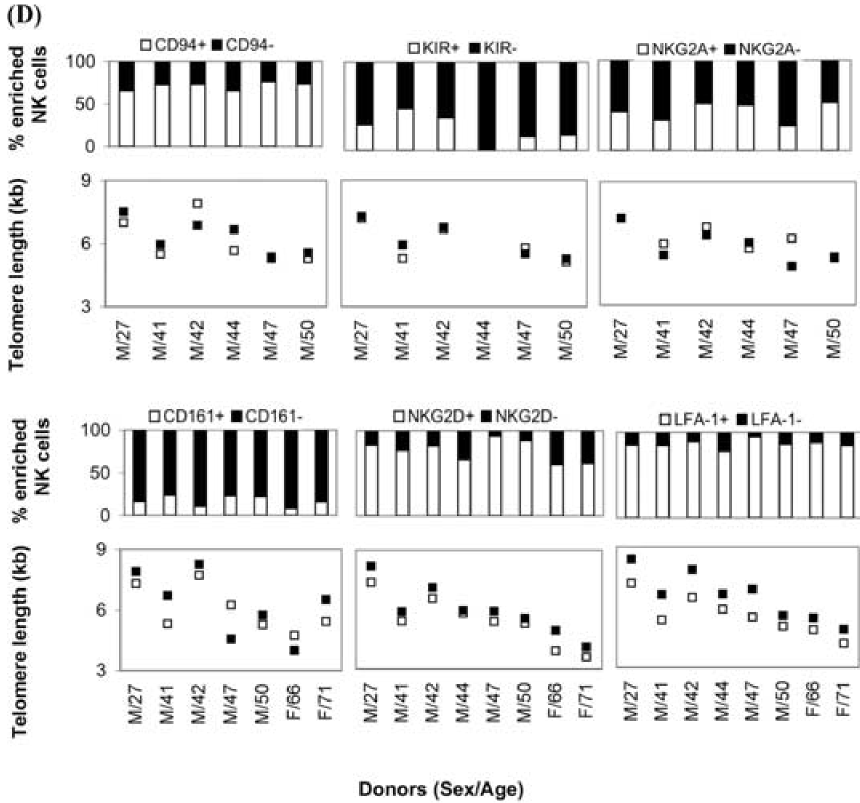

FIGURE 2. Continued.

a NK cell-activating receptor, have significantly shorter telomeres compared to NK cells that do not express this receptor. Moreover, NK cells expressing the adhesion molecule LFA-1 (which may potentiate the cytolytic NK-effector function) also had shorter telomeres than the LFA-1-negative NK cells.

Our data on telomere length analysis of subpopulations of NK cells supports the hypothesis that cells with the greatest proliferative potential have the longest telomeres. $CD56^{dim}CD16^{+}$ mature NK cells appear to be more terminally differentiated than the $CD56^{bright}CD16^{-}$ immature NK cells in terms of their higher cytotoxicity against NK-sensitive targets and distinct cytokine and chemokine profile.[22] During normal aging NK cells show a phenotypic and functional shift toward cells with a more mature NK cell phenotype.[36] $CD56^{bright}$ NK cells are considered to be immature NK precursor cells with a potential to differentiate into $CD56^{dim}$ mature NK cells.[37] The observed differences in telomere length between $CD56^{dim}CD16^{+}$ mature and $CD56^{bright}CD16^{-}$ immature NK cells are compatible with this notion.

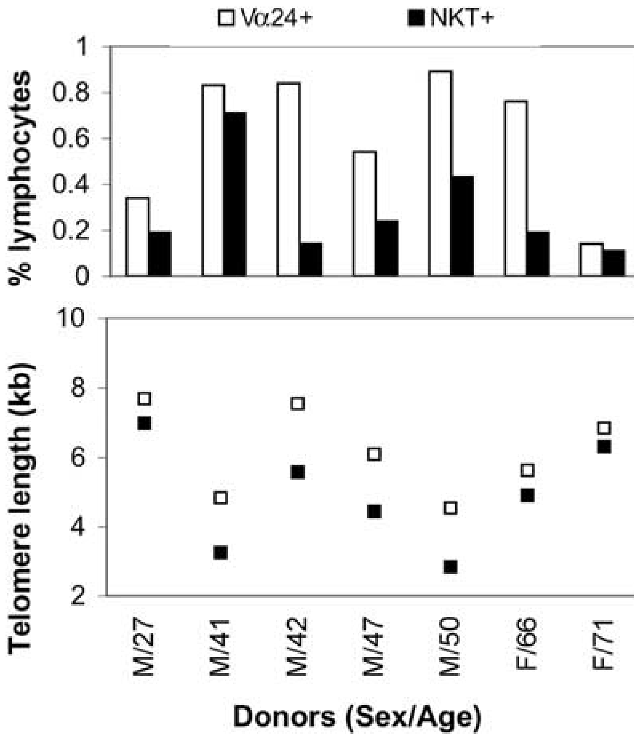

FIGURE 3. Telomere length in sorted NKT cells measured by flow FISH. (*upper panel*) Proportion of NKT cells and Vα24$^+$ T cells within lymphocytes. (*lower panel*) Telomere length in kilobases of sorted NKT and Vα24$^+$ T cells. The Vα24$^+$ T cells have a significantly longer telomere length than NKT cells ($P < 0.05$).

It has been reported that the high density of adhesion molecule LFA-1 may increase the cytolytic-effector function of NK cells,[21] which can be regulated by L-selectin.[38] We observed that LFA-1$^+$ NK cells have shorter telomeres. This observation could reflect differences in replicative history or function of the NK cells expressing high levels of LFA-1 during the early immune response to foreign pathogens.[38]

NK cells are known to be activated by many tumor cells and virus-infected cells.[39,40] NK cells express a large number of different cell-surface receptors that deliver either activating or inhibitory signals, which are involved in the proper regulation of NK cell functions.[41–43] However, the interactions between these two receptor types, which dictate the balance between rest and activation in NK cells, have not yet been clearly defined. Engagement of NKG2D on NK cells can trigger cytolytic activity and can also elicit cytokine and chemokine production.[39,44,45] NK cells expressing NKG2D have shorter telomeres than those that do not, suggesting that activated NK cells typically have replicated

more frequently *in vivo* than unactivated NK cells. Unactivated NK cells from mice have been shown to often respond poorly to target cells.[46] However, the telomere length profile is strikingly heterogeneous in NK cells carrying inhibitory and activating NK receptors, such as CD94, NKG2A, KIR, or expressing the surface molecule CD161. Most likely, these receptors are expressed variably among different NK cells (e.g., KIR is not expressed on NK cells of individual M/44 at all).

In seven individuals studied, the sorted B cells have longer telomeres than T cells and NK cells from the same donor. These results are consistent with previous findings from baboons[47] and humans showing that germinal center B cells and certain memory B cells may have longer telomeres, which is most likely due to the high level of telomerase in the germinal center.[16,18]

Vα24 NKT cells are innate immune cells constituting a lymphocyte lineage sharing characteristics of both T cells including a semi-invariant TCR with uniform usage of α-chain variable gene segment 24 (Vα24), preferentially paired with β-chain variable gene segment 11(Vβ11), and NK cells including the expression of the NK cell marker CD16,[48,49] and play immunoregulatory roles in autoimmunity and tumor immunity.[50] By using flow FISH, we found that NKT cells have shorter telomeres than Vα24$^+$ T cells in all seven individuals and shorter than CD3$^+$ T cells and CD161$^+$ NK cells in six of seven individuals (except the oldest individual F/71). The number of Vα24 NKT cells has been reported to be reduced in elderly humans[51] and in patients with systemic lupus erythematosus,[52] and rheumatoid arthritis.[53] The murine Vα14 NKT cells are lost during acute infection with lymphocytic choriomeningitis virus.[54] Compared to T and NK cells, NKT cells are unique in acquiring a memory-activated phenotype before birth,[55] which may account in part for the shorter telomeres in NKT cells.

Taken together, we conclude that NK cell subpopulations with a mature phenotype have shorter telomeres compared to those with an immature phenotype. Furthermore, NK cells with an effector phenotype or expressing activating receptors also have relatively short telomeres. Our observations support the concept that telomere length dynamics in NK cells are similar to that observed in T cells and granulocytes. At advanced age such telomere attrition could result in the disappearance of long-lived, mature cytotoxic NK cells and compromise immunity against foreign cells, virally infected cells, and tumor cells.

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