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New insights into lymphocyte differentiation and aging from telomere length and telomerase activity measurements[1]

Running title: Aging of lymphocyte subsets

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Conflict of interest

The authors declare that they have no competing financial interests.

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ABSTRACT

αβ $CD8^+$, γδ and NK lymphocytes are fundamental effector cells against viruses and tumors. These cells can be divided into multiple subsets based on their phenotype. Based on progressive telomere attrition from naïve to late effector memory cells, $CD8^+$ T-cell subsets have been positioned along a pathway of differentiation, which is also considered as a process of lymphocyte aging or senescence. A similar categorization has not been clearly established for γδ and NK cell populations. Moreover, the distinction between the aging of these populations due to cellular differentiation or to chronological age of the donor has not been formally considered. Here, we performed systematic measurements of telomere length and telomerase activity in αβ $CD8^+$, γδ and NK lymphocytes based on subset division and across age in order to address these points and better understand the dichotomy between differentiation and temporal aging. This approach enables us to position phenotypically distinct γδ or NK subsets along a putative pathway of differentiation, like for $CD8^+$ T-cells. Moreover, our data show that both cellular differentiation and donor aging have profound but independent effects on telomere length and telomerase activity of lymphocyte subpopulations, implying distinct mechanisms and consequences on the immune system.

INTRODUCTION

αβ $CD8^+$, γδ and NK lymphocytes have in common direct effector functions, including the capacity to eliminate infected or malignant cells, which make them key players of the fight against viruses and tumors (1-3). These effector lymphocytes originate from HSC and HPC in the bone marrow, and go through a process of maturation, which, for αβ and γδ lymphocytes, takes place in the thymus. Mature αβ $CD8^+$, γδ and NK lymphocytes are then disposed to initiate immune responses when encountering antigens, and upon activation to differentiate into fully armed "effector/memory" (EM) cells. Differentiation of mature αβ T-lymphocytes from naïve to highly differentiated effector/memory cells involves functional changes along with modifications in the expression of cell surface receptors (e.g. CD45RA, CCR7, CD27, CD57), which defines their phenotype (4-6). This process of differentiation has also been associated with a progressive aging of these lymphocytes, with an increasing telomere attrition, so that highly differentiated EM cells, characterized by the surface expression of CD57, present the shortest telomere length, and are often considered as senescent T-cells (7, 8). This analogy between effector/memory αβ T-lymphocyte subset definition and senescence nonetheless blurs our perception of what is lymphocyte aging, and begs the question if the latter is a matter of differentiation or of time (i.e. age of the subject).

Moreover, while the differentiation process is relatively well established and accepted for αβ $CD8^+$ T-lymphocytes, it is much less characterized in the case of γδ and NK cell populations. Following their maturation in the thymus, γδ T-cells are usually divided according to the expression of their TCR ($V\delta2^+$ cells that are predominant at birth, and $V\delta2^-$ cells that become more prominent later) (9, 10), as well as their phenotype (based on the expression of classical αβ T-cell receptors like CD45RA, CCR7, CD27, CD57) (11-13). Likewise, based on the expression of cell surface markers (e.g. CD16, NKG2A, NKG2C,

CD57), NK cells can be categorized into different subsets, which have been positioned on putative models of differentiation in previous studies (14-16). While the frequency of these phenotypically distinct γδ or NK subsets in the blood is altered with aging (17-20), questions pertaining the positioning of these phenotypically distinct γδ or NK subsets along a putative pathway of differentiation, and how these populations age with time, remain to be addressed.

Here, we used the power and relevance of the telomere status analysis to better understand differentiation and aging of αβ $CD8^+$, γδ and NK lymphocytes based on subset division and across age. Telomeres are special structures of tandem DNA repeats at the end of chromosomes. In somatic human cells, they are shortened with each cell division due to the inability of DNA polymerase to replicate the extreme 5' end of the lagging strand of DNA, and despite the telomerase enzymatic complex aims at minimizing telomere erosion. Telomere length represents therefore a robust indicator of the proliferative history of a cell, and how close this cell is to reach senescence, somehow reflecting its "age" (21). In the present work, we purified multiple phenotypically distinct subsets of αβ $CD8^+$, γδ and NK lymphocytes from the blood of healthy young, middle aged or elderly adults, and performed systematic measurements of telomere length, as well as telomerase activity in these cells. Our results provide new insights into the positioning of phenotypically distinct subsets of γδ and NK lymphocytes on their respective differentiation pathways, and how αβ $CD8^+$, γδ and NK lymphocytes age overtime in humans.

MATERIALS AND METHODS

Study patients

Three groups of Caucasian volunteers were enrolled in this study: (i) young (18 < age < 25), (ii) middle-aged (30 < age < 55) and (iii) elderly (>75 years old) adults. All individuals were healthy. Individuals with malignancies, acute diseases, or advanced stages of severe chronic diseases, such as chronic inflammatory disease, atherosclerotic disease, congestive heart failure, poorly controlled diabetes mellitus, renal or hepatic disease, or chronic obstructive pulmonary disease, as well as individuals under immunosuppressive therapy were excluded from the study. Venous blood samples were drawn into anti-coagulant tubes and PBMCs were isolated by density gradient centrifugation according to standard protocols.

Flow cytometry

Panels of directly conjugated antibodies to identify subsets of HPC, αβ $CD8^+$ T-cells, γδ T-cells and NK cells are detailed in Table 1. Cell surface staining was performed using standard methodologies. Cells were sorted using an Aria II (Becton Dickinson) and data analyzed using FlowJo v X 10.6 (Tree Star, Inc).

Telomere length measurement

Genomic DNA (gDNA) was extracted from Sorted cells with the QIAmp DNA Mini kit (QIAGEN®, France), following the manufacturer's instruction. The yield of gDNA in sorted cells was between 2ng and 20ng. Quantification of gDNA samples was performed using absorbance measurement method (NanoDrop 2000) and only good quality DNA was included in the assays (i.e. with A260/A280 ratio of 1.7-2.0). Relative telomere length (RTL) was measured as the ratio of standard DNA quantities for

telomere template (T) over single copy gene 36B4 (S) determined using quantitative real-time polymerase chain reactions (PCR), as described previously by Cawthon et al. with some modifications (22). Measurement was performed in triplicates. Telomere and 36B4 PCRs were done on separate plates. The intra-assay coefficient of variation is < 5%. PCR primers were, (Tel-F, 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'; Tel-R, 5'-GCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3') for Telomere PCR and (36B4-F, 5'-CAGCAAGTGGGAAGGTGTAATCC-3'; 36B4-R, 5'-CCCATTCTATCATCAACGGGTACAA-3') for the 36B4 PCR. The final primer concentration was 400nM for all primers. The PCR mix contained Power SYBER Green Master Mix (Life technology) and 2ng of gDNA. qPCR were performed on a 7300 Bio-Rad Thermal cycler. The thermal cycling profile for telomere amplification consisted of a preparation step of 2 min at 50°C, 10 min at 95°C, followed by 35 cycles at 95°C for 15 sec, 56°C for 2 min and 72°C for 1 min. The 36B4 amplification profile was 2 min at 50°C, 10 min at 95°C followed by 35 cycles of 95°C for 15 sec and 58°C for 1 min. To convert the ratio of starting quantity into telomere length in base pairs, a serial dilution of DNA samples from 293T cell line (obtained from the ATCC - ref. CRL-3216, LGC Promochem, Molsheim, France) was run simultaneously in classical southern blot and qPCR.

To examine the intra-assay reproducibility of T/S measurements, we first used a same standard gDNA to set up standard curve reaction in every plate of the study (gDNA from 293T cell line) and second, we prepare a bulk PBMC sample as an internal control to run it in all plates that validate absence of interpolate variability. For this, we determined the coefficient of variation (standard deviation divided by the mean) for T/S for each of the gDNA control samples. The intra-assay geometric mean of the coefficient of variation was <5%. To examine inter-assay reproducibility, we repeated the measurements of T/S in the same 10 DNA samples, in triplicate, on another day, and by another user, paying attention that the specific reaction well positions occupied by each DNA sample were different in these two

independent runs. The slope of the linear regression line through the data was close to one, and the y-intercept near zero. The coefficient of variation for each of the 10 pairs of average T/S values from the two independent runs was determined. The inter-assay geometric mean of the coefficient of variation was 3%.

Telomerase activity measurement

Relative telomerase activity (RTA) was measured using a modified Telomerase Repeat Amplification Protocol (TRAP) assay as described previously (23). Sorted cells were lysed in 200µl of ice cold CHAPS lysis buffer and incubated 30min with shaking at 4°C. The lysate was centrifuged at max speed for 20min at 4°C, and the supernatant was collected. Telomerase extracts were assessed two steps using a SYBR Green real-time quantitate TRAP assay: First, telomerase-mediated extension of an oligonucleotide trimer (TS) (5'-AATCCGTCGAGCAGAGTT-3') at a final concentration of 900nM which serves as a substrate for telomerase, and second, hot start PCR amplification of the resultant product with the oligonucleotide primer pair TS (Forward) and CX (reverse) (5'-GTGTAACCCTAACCCTAACCC-3') at a final concentration of 500nM, in 25 µl final volume with SYBER Green PCR Master Mix (Life technology®). Using the 7300 Bio-Rad Thermal cycler, samples were incubated for 20min at 25°C for Telomerase reaction followed by PCR initial activation step at 95°C for 10 min and amplified in 35 PCR cycles with 30 sec at 95°C (denaturation), 90 sec at 60°C (Annealing) and 30 sec at 72°C (Extension). Standards and negative controls with heat-inactivated samples and lysis buffer only were assayed on each plate. Each sample was analyzed at least in 3 independent assays. Telomerase activity was calculated relative to 293T cell line and expressed as RTA ($Ct_{(293T)}$/ $Ct_{(sample)}$).

Statistical analyses

Univariate statistical analysis was performed using GraphPad prism software. Groups were compared using the non-parametric Kruskal Wallis or Mann Whitney tests. Spearman's rank test was used to determine correlations. *P* values above 0.05 were considered not significant.

Study approval

The study was approved by the local institutional ethics committee (i.e. Comité de Protection des Personnes of the Pitié Salpétrière Hospital, Paris) and all participants provided written informed consent.

RESULTS

Positioning of αβ CD8$^+$, γδ and NK lymphocyte subsets on differentiation pathways

In order to validate our approach and set up standards in our assays, we first performed telomere length and telomerase activity measurements in hematopoietic progenitors (HPC) and αβ CD8$^+$ T-lymphocytes from young adults. We purified total HPC (tHPC), identified in the blood as CD34$^+$ CD45$^+$ lin$^-$ cells, which are derived from hematopoietic stem cells and represent an upstream compartment of lymphocyte development, with strong renewal and differentiation capacity. Within this compartment, common lymphoid progenitors (CLP), which are already lineage committed and thus more differentiated, were further identified, based in a CD45RA$^+$ CD117$^-$ phenotype, and purified (24-27) (Figure 1A). From the same donors, naïve and EM CD8$^+$ T-lymphocytes, either CD57$^-$ or CD57$^+$ were also FACS sorted. As expected, telomere length decreased gradually from tHPC, CLP, naïve, EM CD57$^-$ to EM CD57$^+$ cells, which presented the shortest telomere length, below 5kb (Figure 1B). The evolution of telomerase activity across these subsets showed an equivalent pattern as to telomere length (Figure 1C). tHPC and CLP harbored much stronger telomerase activity, compared to mature CD8$^+$ T-cells, among which naïve cells showed highest and EM CD57$^+$ cells lowest telomerase activity. This is in line with the established CD8$^+$ T-cell differentiation pathway, and confirms that EM CD8$^+$ T-lymphocytes expressing CD57 present the lowest proliferative potential and approach senescence. These data mostly provide means of comparison for γδ and NK lymphocyte subsets in order to place them along a maturation pathway.

γδ T-lymphocytes were divided and purified according to Vδ2 expression as well as their phenotype: CD45RA$^+$CD27$^+$ (presumably naïve) versus non CD45RA$^+$CD27$^+$ (presumably EM) cells (Figure 1D). We used also the expression of CD57 as an additional marker to dissect further the non CD45RA$^+$CD27$^+$

γδ T-cell population. Vδ2^{+} and Vδ2^{-} CD45RA^{+}CD27^{+} presented long telomere length, compared to non CD45RA^{+}CD27^{+} cells, and equivalent to the one found in naïve αβ CD8^{+} T-lymphocytes (Figure 1E). Among non CD45RA^{+}CD27^{+} γδ T-cells, CD57 expressing subsets showed the shortest telomeres, usually below 5kb. Telomerase activity variations paralleled telomere length changes across these subsets (Figure 1F). In accordance with CD8^{+} T-lymphocytes, these data portray CD45RA^{+}CD27^{+} γδ T-cells as naïve, and CD57^{+} non CD45RA^{+}CD27^{+} γδ T-cells as EM cells approaching senescence.

NK cells were separated and FACS sorted into five distinct subsets based on the expression of CD56, NKG2A, NKG2C and CD57 (Figure 1G). Our telomere data confirmed differences in telomere length between CD56^{bright} and CD56^{dim} NK cells as previously reported (15, 28), and extended such differences to other NK cell subsets, showing that they were not equivalent to one another and could be also positioned according to a hierarchy. Together, these changes in telomere length and telomerase activity across subsets are in line with a differentiation of NK cells from early differentiated CD56^{bright} to CD56^{dim} NKG2A^{+}NKG2C^{-}, NKG2A^{-}NKG2C^{-} and then NKG2A^{-}NKG2C^{+} (Figures 1H and 1I). They strongly support earlier observations, based on transcriptional and functional characterization of equivalent subsets, and *in vivo* reconstitution experiments, of a linear NK cell differentiation process (14, 15). CD57 expression on NKG2A^{-} NKG2C^{+} NK cells highlighted again the population closest to terminal differentiation and senescence. Overall, telomere length and telomerase activity measurements in lymphocyte populations from young adults enable us to position γδ T-cell and NK cell subsets along differentiation pathways similarly to αβ CD8^{+} T-lymphocytes. Of note, a strong correspondence was found between telomere length and telomerase activity with all populations studied (Figure 2). The decrease in telomerase activity seems to be closely related to the stage of differentiation. This suggests that telomerase activity is regulated at the level of the cellular differentiation process, decreasing as cells

differentiate further. It implies that as αβ $CD8^+$, γδ and NK lymphocytes proliferate and differentiate, they will suffer obvious telomere attrition, and ultimately approach cellular senescence.

Impact of aging on mature αβ $CD8^+$, γδ and NK lymphocytes

After providing new insights into the relationship between telomere erosion and lymphocyte differentiation, we next aimed at studying the evolution of lymphocyte telomere length according to individual aging. For this purpose, we performed telomere length and telomerase activity measurements in each of the same lymphocyte subpopulations studied earlier, but this time, in older subjects (i.e. middle aged and elderly adults aged more than 75 years). Importantly, all αβ $CD8^+$, γδ and NK cell populations and their respective subsets presented reduction in telomere length with increasing subject age (Figures 3A, 3B and 3C). This applied also to CLP, in line with our recent findings on tHPC (29). In the elderly, the telomeres of all cells, including naïve T-cells, early differentiated NK cells and upstream CLP, reached lengths equivalent to 5kb or below, i.e. to levels found for highly differentiated $CD57^+$ lymphocytes, considered as approaching senescence in young adults. We also observed a steady reduction in telomerase activity in all lymphocyte subpopulations (i.e. including CLP, naïve and early differentiated cells) with increasing age of the subjects (Figures 3D, 3E and 3F). Telomere length and telomerase activity were therefore tightly linked across age, similar to their correspondence across lymphocyte differentiation. Overall, age related telomere attrition and telomerase activity reduction highlight an overall aging of the all lymphocyte populations, including early compartments of lymphocyte differentiation, which may approach telomere based cellular senescence in the elderly.

Of note, similar to observations with naïve $CD8^+$ T-lymphocytes (Figure 4A) (29), there were strong correlations between telomere lengths in tHPC and in naïve ($CD45RA^+CD27^+$) γδ T-cells or early

differentiated ($CD56^{bright}$) NK cells (Figures 4B and 4C). This relationship suggests a direct link between the upstream source of lymphocyte development and downstream mature lymphocyte pools with regards to telomere status, so that short telomeres and reduced telomerase activity of progenitors may be passed on to progeny cells like γδ and NK lymphocytes. This may explain, at least partially, the strongly reduced telomere length observed in naïve T-cells and early differentiated NK cells from old individuals, while these cells have in theory not encountered any antigen or stimulator, and not differentiated yet.

Influence of cytomegalovirus infection

Altogether, our data implies that both cellular differentiation and donor age influence telomere status of lymphocyte populations. Additionally, chronic infection with cytomegalovirus (CMV), which is a large double-stranded DNA herpesvirus with a high prevalence in humans (50–90% of individuals are seropositive), has a strong influence on the immune aging profile, and might be another parameter to considerer in this context. Through its stimulation of αβ $CD8^+$, γδ and NK cell compartments, this persistent virus is known to influence greatly the frequency and distribution of their respective subsets (30), as well as telomere length of the total $CD8^+$ T-cell pool (31). CMV infection is indeed associated with decreased proportions of naïve T-lymphocytes, but mostly an overrepresentation of EM $CD57^+$ $CD8^+$ T-cells (32, 33), EM $CD57^+$ $V\delta2^-$ T-cells (18, 34, 35), and $NKG2A^-$ $NKG2C^+$ $CD57^+$ NK cells (20, 36-38), which are known to include many CMV specific or reactive cells. This disequilibrium between naïve and EM $CD57^+$ T-cells, which harbor very different telomere lengths, can by itself explain the reduced telomere length reported in the total $CD8^+$ T-lymphocyte compartment of CMV seropositive individuals. Nonetheless, we aimed at investigating if CMV infection could impact directly the telomere status of specific lymphocyte subsets. Our data were therefore reanalyzed to take in account the CMV serostatus of individuals. For this purpose, we concentrated only on young and middle aged subjects,

since old individuals present overall short telomere lengths and are generally CMV seropositive, which represent biases to compare CMV negative versus positive individuals. EM $CD57^+$ $CD8^+$ T-cells, EM $CD57^+$ $V\delta2^-$ T-cells, and $NKG2A^-$ $NKG2C^+$ $CD57^+$ NK cells from CMV seropositive presented the lowest medians in telomere length among all populations studied, although the differences did not reach statistical significance comparing these subsets between CMV seropositive and CMV seronegative subjects (Figure 5). This indicates that, while the virus likely has a specific impact, these highly differentiated cells present short telomere lengths independently of CMV infection, likely through other stimuli. Of note, similar non-significant trends were also observed for other less differentiated subsets, including naïve αβ or γδ T-cells, as well as CLP, which may suggest a possible influence of CMV on upstream compartments on lymphocyte differentiation.

DISCUSSION

In the present work, we have used the power of telomere length and telomerase activity measurements applied to multiple subsets of effector lymphocyte populations like αβ $CD8^+$, γδ and NK cells to better characterize both their cellular differentiation and aging. Our data represent further evidence that γδ T- and NK cells can be positioned along putative pathways of linear differentiation, as it has been done for αβ $CD8^+$ T-cells. The positioning and classification into naïve ($CD45RA^+CD27^+$), early EM ($CD57^-$ and non $CD45RA^+CD27^+$) and late EM ($CD57^+$ and non $CD45RA^+CD27^+$) γδ T-cells, or early to late differentiated ($CD56^{bright}$ => $CD56^{dim}$ $NKG2A^+NKG2C^-$ => $CD56^{dim}$ $NKG2A^-NKG2C^-$ => $CD56^{dim}$ $NKG2A^-NKG2C^+$ => $CD56^{dim}$ $NKG2A^-NKG2C^+CD57^+$) NK cells, represent important insights to better understand and follow the development of immune responses mediated by these cells. It is nonetheless important to mention that the present work is not all-inclusive, as more T- and NK cell subsets can be identified using additional cell surface markers and thus analyzed, which could complement the present classification. As it is the case for $CD8^+$ T-cells, CD57 expression emerges also as a valid marker to identify most differentiated γδ T- and NK cells. Moreover, the observation that $V\delta2^+$ $CD45RA^+CD27^+$ T-cells have significantly longer telomeres and stronger telomerase activity than $V\delta2^-$ $CD45RA^+CD27^+$ T-cells (P=0.006), suggests differences in the maturation process of these two naïve populations in the thymus. One may speculate that $V\delta2^+$ γδ T-cells might go through a shorter process of maturation in the thymus compared to $V\delta2^-$ γδ T-cells. Although the cause of such differential γδ T-cell development remains to be determined and may be related to thymic TCRγδ ligands (39), the $V\delta2^+$ γδ T-cell overrepresentation in early life may be a consequence. Of note, early differentiated $CD56^{bright}$ NK cells show also a trend towards shorter telomere length compared to naïve αβ and γδ T-cells, which suggests that the formation of this compartment might involve more cellular proliferation. This may be explained by the fact that early differentiated NK cells likely represent a rather homogeneous population, generated

through the expansion of precursors, in contrast to the production of a polyclonal pool of individual naïve T-cells.

The negative regulation of telomerase activity along cellular differentiation is important and implies that, as lymphocytes proliferate and differentiate, their telomeres will inexorably shorten, so that highly differentiated cells from these lineages may reach a state of cellular senescence due to telomere attrition. This process may be perceived as aging of the lymphocytes. However, one needs also to consider aging of the donors itself. With advanced age of donors, we also observed reduced telomerase activity and important shortening of telomeres in αβ $CD8^+$, γδ and NK lymphocytes, affecting primarily naïve, early differentiated, as well as lymphoid progenitor cell subsets. While waning telomerase activity and telomere erosion occur with both cellular differentiation and donor aging, discriminating between these two contexts is important. On the one hand, 'aging' of lymphocytes can occur in young adults through the generation of highly differentiated $CD57^+$ lymphocytes, which are close to senescent cells. On the other hand, *a priori* 'young' cells (i.e. CLP, naïve and early differentiated cells) appear old in elderly people. These two types of 'aged' cells are fundamentally different both with respect to their function in the immune response, and to the mechanisms resulting in their 'aging'. CLP, naïve and early differentiated cells are necessary to renew the pool of lymphocytes and initiate immune responses, while highly differentiated lymphocytes display strong effector functions, but are not destined to expand greatly themselves. One should therefore not make the confusion between young adult old cells and old adult young cells. Likewise, one need to consider that 'young' cells (i.e. naïve and progenitor cells) in old adults are very distinct from their counterparts in young adults, and hold very different proliferative, renewal and differentiation potential. With regards to the causes of 'aging' in these two settings, one may oppose a programmed reduction of telomerase activity and cellular expansion capacity related to increased differentiation of lymphocytes, versus the waning potential of primary immune resource passed

on to progeny lymphocyte populations related to time and increasing individual age. The respective mechanisms will need to be elucidated in details.

Last, we considered the potential impact of CMV, which is known to be a key player of the immune changes occurring with age. While CMV influences greatly the expansion and frequency of highly differentiated αβ, γδ T- and NK cell subsets (30), its impact on telomere length and therefore aging of these specific populations is less evident. Even if highly differentiated cells from CMV seropositive donors presented lowest telomere length median, the specific influence of CMV mediated stimulation on this aspect is harder to determine with certainty, as highly differentiated cells have short telomeres *per se*. The seeming CMV related effect on telomere length of less differentiated subsets may suggest a possible influence of CMV upstream, on lymphocyte differentiation compartment, possibly through a general mobilization of lymphopoiesis to generate EM cells, and through CMV driven increased inflammation, which is known to impact hematopoiesis (40).

Taken together, our results show that both cellular differentiation and donor aging, but not CMV infection, have profound effects on telomere length and telomerase activity of lymphocyte populations, and therefore their proliferative potential. Although these effects may be considered as cellular 'aging', they should not be confused with one another, as they have distinct meanings, mechanisms and consequences on the immune responses.

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TABLE AND FIGURE LEGENDS

Table I. Panels of directly conjugated antibodies to identify subsets of HPC, αβ $CD8^+$ T-cells, γδ T-cells and NK cells

Figure 1. Telomere length and telomerase activity in αβ $CD8^+$, γδ and NK lymphocyte subsets

Representative flow cytometry staining and sorting gates for $CD34^+$ progenitor, αβ $CD8^+$**(A)**, γδ **(D)** and NK **(G)** lymphocyte subsets. Telomere length in FACS sorted $CD34^+$ progenitor, αβ $CD8^+$ **(B)**, γδ **(E)** and NK **(H)** lymphocyte subsets from young adults (n=9) Telomerase activity in FACS sorted $CD34^+$ progenitor, αβ $CD8^+$ **(C)**, γδ **(F)** and NK **(I)** lymphocyte subsets from young adults (n=9). The Mann-Whitney or Kruskall Wallis tests were used for comparing 2 groups or 3 groups, respectively. Each dot represents an individual. Bars indicate the median.

Figure 2. Relationship between telomere length and telomerase activity in lymphocyte populations

Correlations between telomere length and telomerase activity in $CD34^+$ progenitors and αβ $CD8^+$ T-cells **(A),** γδ T-cells **(B),** and NK cells **(C)**. The Spearman's rank test was used to determine correlations.

Figure 3. Telomere length and telomerase activity in lymphocyte subsets across age

Telomere length in FACS sorted $CD34^+$ progenitor, αβ $CD8^+$ **(A)**, γδ **(B)** and NK **(C)** lymphocyte subsets from young (n=9), middle aged (n=10) and elderly (n=10) adults. Telomerase activity in FACS sorted $CD34^+$ progenitor, αβ $CD8^+$ **(D)**, γδ **(E)** and NK **(F)** lymphocyte subsets from young (n=9), middle aged

(n=10) and elderly (n=10) adults. The Kruskall Wallis test was used for comparing groups. Bars indicate the median.

Figure 4. Relationship between telomere length in CD34$^+$ progenitors and mature lymphocytes

Correlations between telomere length in CD34$^+$ progenitors and αβ CD8$^+$ T-cells **(A),** γδ T-cells **(B),** and NK cells **(C)**. The Spearman's rank test was used to determine correlations.

Figure 5. Telomere length in αβ CD8$^+$, γδ and NK lymphocyte subsets according to CMV status

Telomere length in FACS sorted CD34$^+$ progenitor, αβ CD8$^+$ **(A)**, γδ **(B)** and NK **(C)** lymphocyte subsets from CMV negative (n=10) or positive (n=9) young or middle aged adults. The Mann-Whitney test was used for comparing groups. Bars indicate the median.

Table I

PANEL	ANTIBODY	FLUOROCHROME	SUPPLIER AND REFERENCE
HPC	CD34	PE	BD BIOSCIENCES - 345802
	Lineage cocktail	FITC	BD BIOSCIENCES - 340546
	CD45RA	V450	BD BIOSCIENCES - 560362
	CD117	PE-Cy7	BECKMAN COULTER - B49221
αβ $CD8^+$ T cell	CD3	FITC	BD BIOSCIENCES - 555916
	CD4	HV500	BD BIOSCIENCES - 560768
	CD8	BV650	BD BIOSCIENCES - 563822
	CD45RA	ECD	BECKMAN COULTER - IM2711U
	CD27	AF700	BIOLEGEND - 302814
	CD57	Pacific Blue	BIOLEGEND - 322316
γδ T cell	CD3	BV650	BIOLEGEND - 317323
	TCR PANγδ	PE	BECKMAN COULTER - IM1418U
	TCR Vδ2	FITC	BECKMAN COULTER - IM1464
	CD45RA	ECD	BECKMAN COULTER - IM2711U
	CD27	AF700	BIOLEGEND - 302814
	CD57	Pacific Blue	BIOLEGEND - 322316
NK cell	CD3	BV650	BIOLEGEND - 317323
	CD16	APC H7	BD BIOSCIENCES - 560195
	CD56	PE-Cy7	BD BIOSCIENCES - 557747
	NKG2A	APC	BECKMAN COULTER - A60797
	NKG2C	PE	R&D - MAB1381
	CD57	Pacific Blue	BIOLEGEND - 322316

Fig. 1

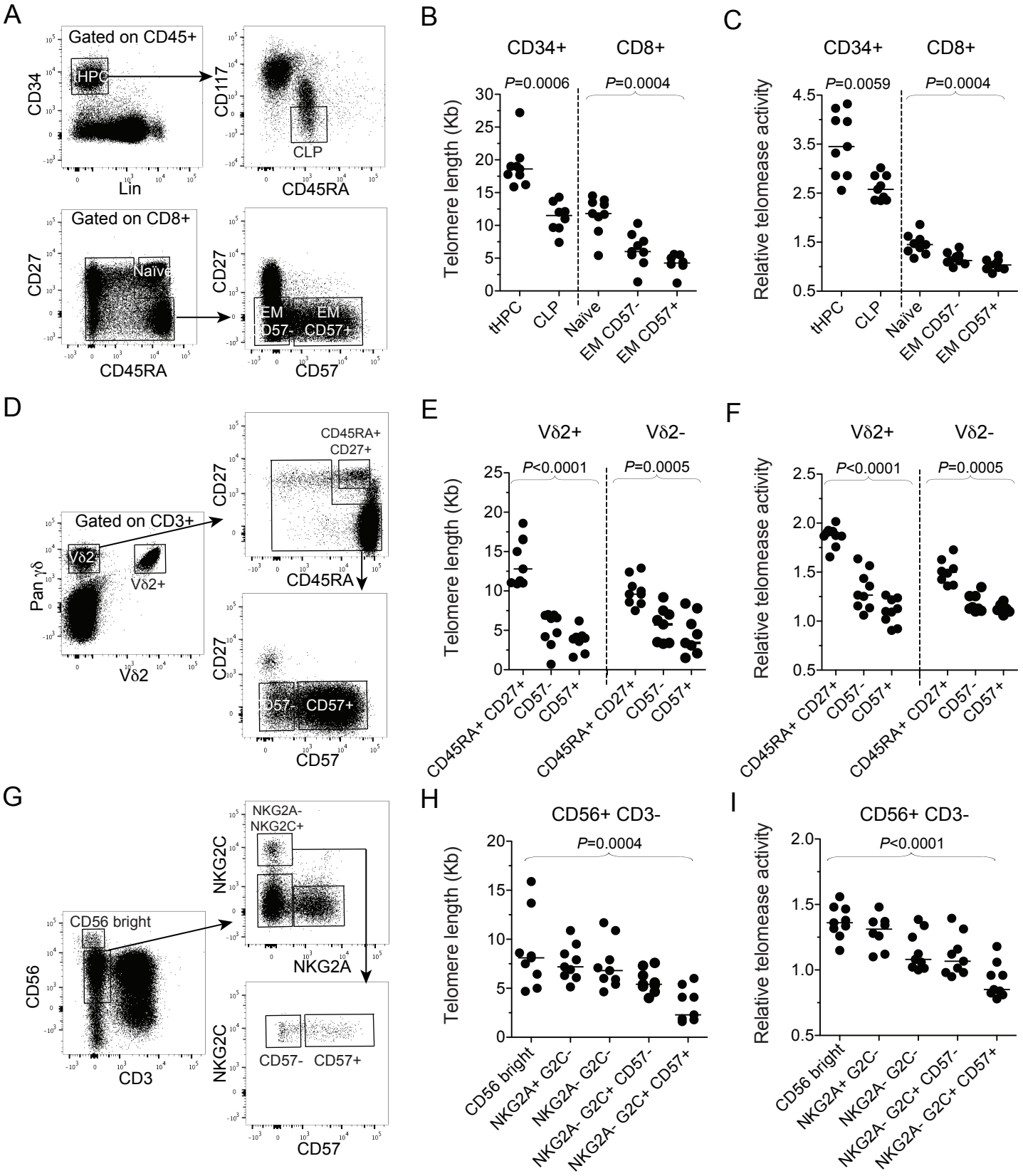

A
Gated on CD45+
HPC
CD34
Lin
CD117
CLP
CD45RA
Gated on CD8+
CD27
Naive
CD45RA
CD27
EM CD57-
EM CD57+
CD57
B
CD34+
CD8+
P=0.0006
P=0.0004
Telomere length (Kb)
tHPC
CLP
Naïve
EM CD57-
EM CD57+
C
CD34+
CD8+
P=0.0059
P=0.0004
Relative telomease activity
tHPC
CLP
Naïve
EM CD57-
EM CD57+
D
Gated on CD3+
Vδ2
Pan γδ
Vδ2+
Vδ2
CD45RA+
CD27+
CD27
CD45RA
CD27
CD57-
CD57+
CD57
E
Vδ2+
Vδ2-
P<0.0001
P=0.0005
Telomere length (Kb)
CD45RA+ CD27+
CD57-
CD57+
CD45RA+ CD27+
CD57-
CD57+
F
Vδ2+
Vδ2-
P<0.0001
P=0.0005
Relative telomease activity
CD45RA+ CD27+
CD57-
CD57+
CD45RA+ CD27+
CD57-
CD57+
G
CD56 bright
CD56
CD3
NKG2A-
NKG2C+
NKG2C
NKG2A
NKG2C
CD57-
CD57+
CD57
H
CD56+ CD3-
P=0.0004
Telomere length (Kb)
CD56 bright
NKG2A+ G2C-
NKG2A- G2C-
NKG2A- G2C+ CD57-
NKG2A- G2C+ CD57+
I
CD56+ CD3-
P<0.0001
Relative telomease activity
CD56 bright
NKG2A+ G2C-
NKG2A- G2C-
NKG2A- G2C+ CD57-
NKG2A- G2C+ CD57+

Fig. 2

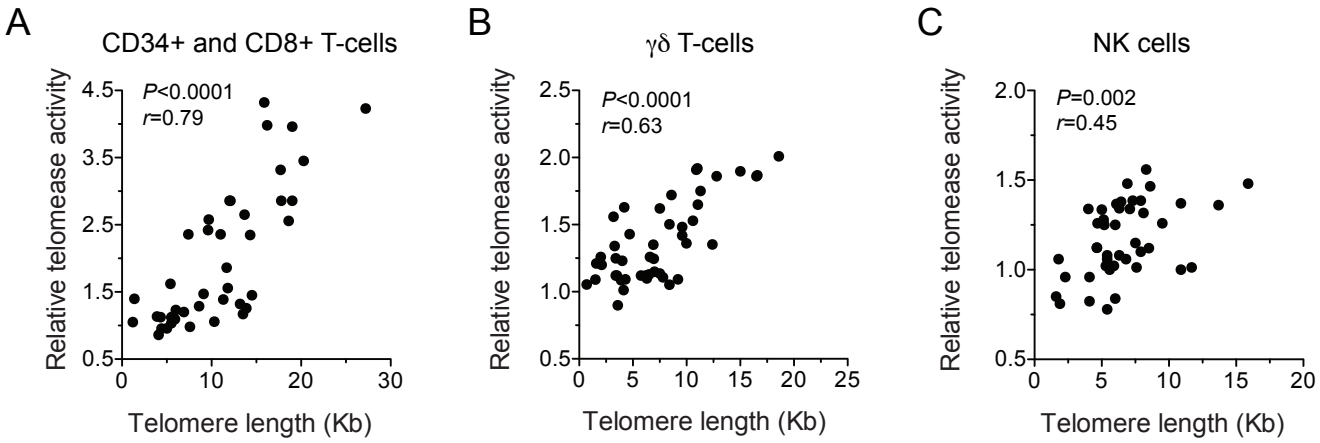

A
CD34+ and CD8+ T-cells
P<0.0001
r=0.79
Relative telomease activity
Telomere length (Kb)
B
γδ T-cells
P<0.0001
r=0.63
Relative telomease activity
Telomere length (Kb)
C
NK cells
P=0.002
r=0.45
Relative telomease activity
Telomere length (Kb)

Fig. 3

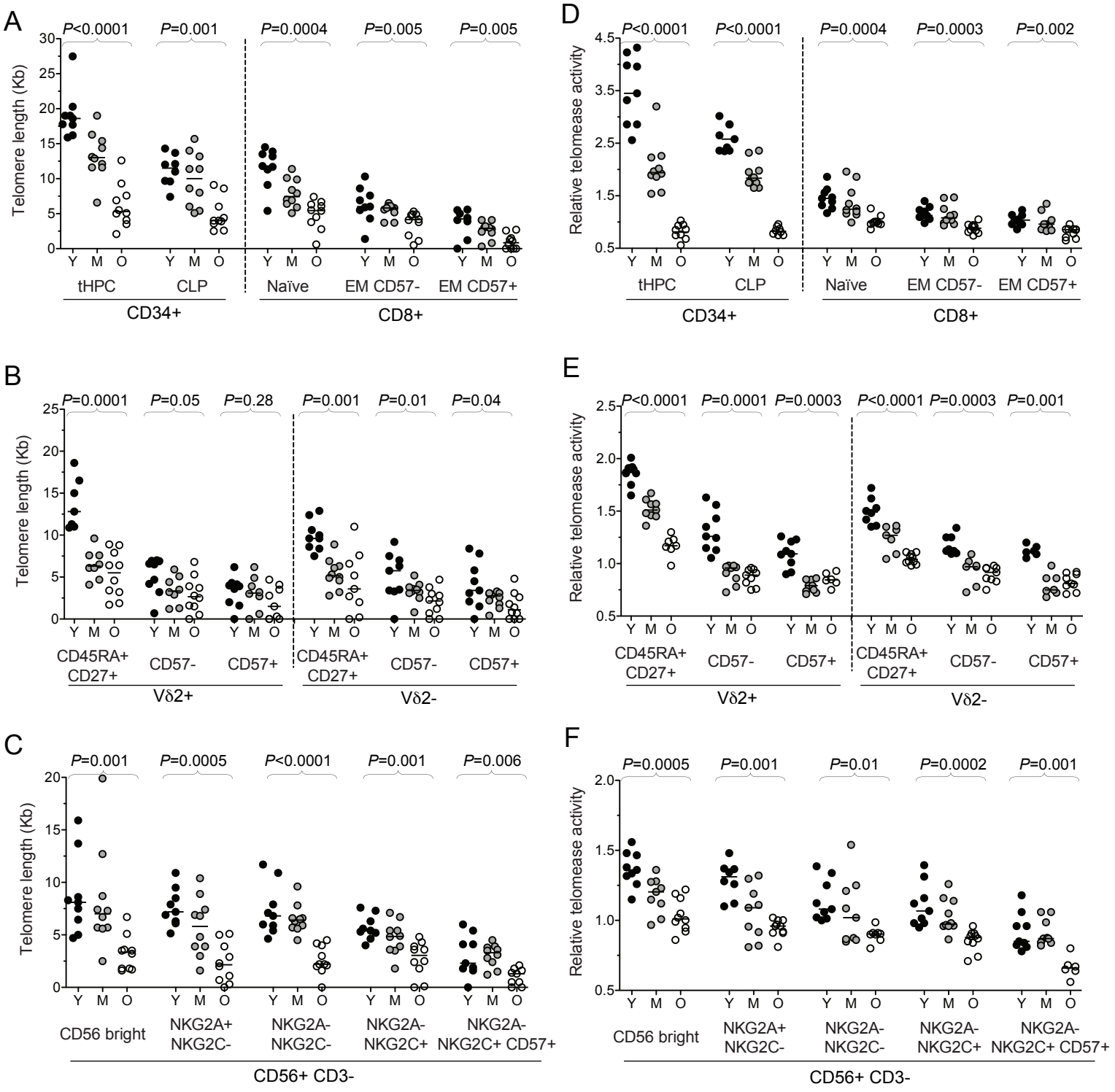

A
P<0.0001
P=0.001
P=0.0004
P=0.005
P=0.005
Telomere length (Kb)
Y M O
tHPC
CLP
Naïve
EM CD57-
EM CD57+
CD34+
CD8+
D
P<0.0001
P<0.0001
P=0.0004
P=0.0003
P=0.002
Relative telomease activity
B
P=0.0001
P=0.05
P=0.28
P=0.001
P=0.01
P=0.04
CD45RA+ CD27+
CD57-
CD57+
Vδ2+
Vδ2-
E
P<0.0001
P=0.0001
P=0.0003
P<0.0001
P=0.0003
P=0.001
C
P=0.001
P=0.0005
P<0.0001
P=0.001
P=0.006
CD56 bright
NKG2A+ NKG2C-
NKG2A- NKG2C-
NKG2A- NKG2C+
NKG2A- NKG2C+ CD57+
CD56+ CD3-
F
P=0.0005
P=0.001
P=0.01
P=0.0002
P=0.001

Fig. 4

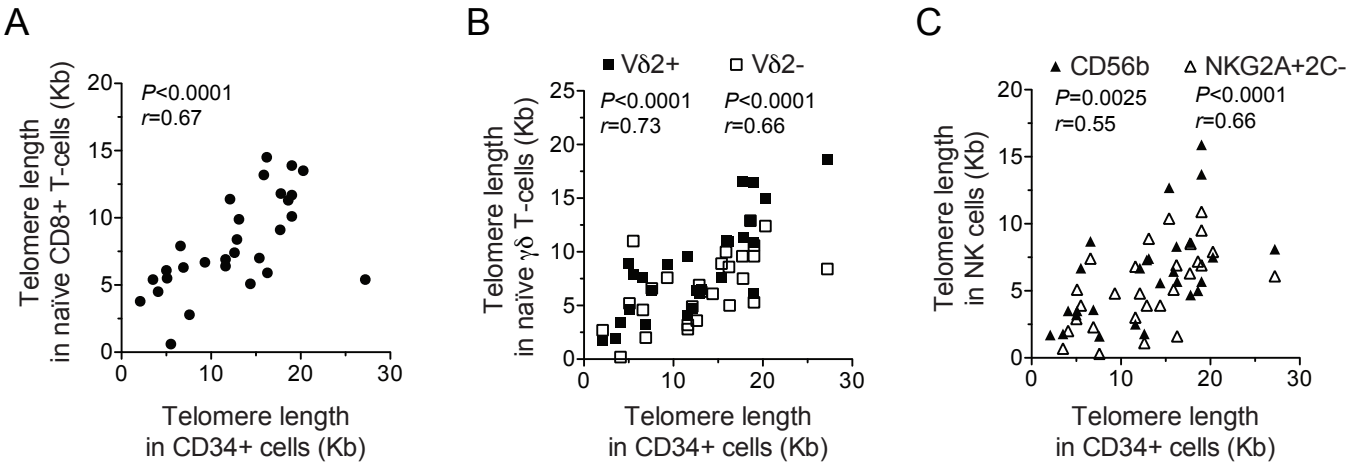
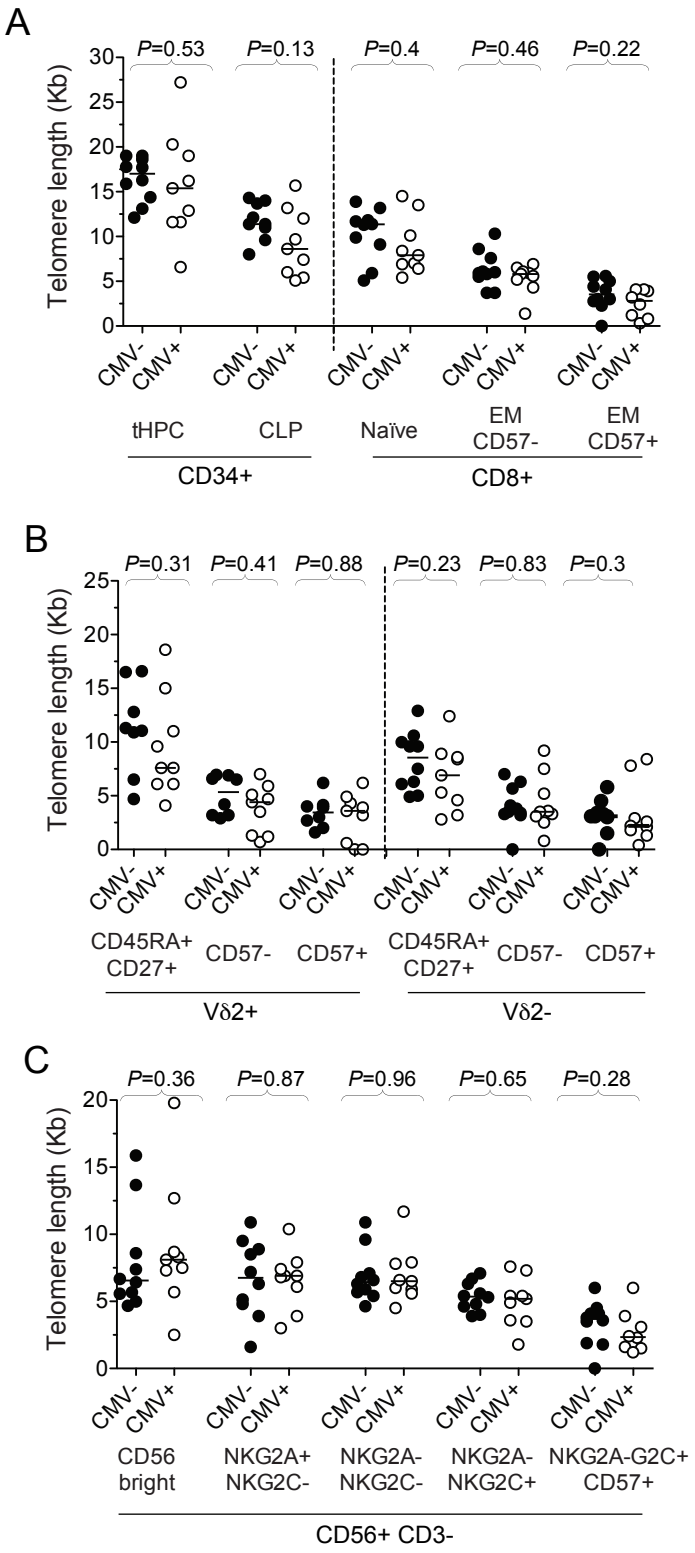

A
P<0.0001
r=0.67
Telomere length in naïve CD8+ T-cells (Kb)
Telomere length in CD34+ cells (Kb)
B
Vδ2+
Vδ2-
P<0.0001
r=0.73
P<0.0001
r=0.66
Telomere length in naïve γδ T-cells (Kb)
Telomere length in CD34+ cells (Kb)
C
CD56b
NKG2A+2C-
P=0.0025
r=0.55
P<0.0001
r=0.66
Telomere length in NK cells (Kb)
Telomere length in CD34+ cells (Kb)

Fig. 5


A
P=0.53
P=0.13
P=0.4
P=0.46
P=0.22
Telomere length (Kb)
CMV-
CMV+
tHPC
CLP
Naïve
EM CD57-
EM CD57+
CD34+
CD8+
B
P=0.31
P=0.41
P=0.88
P=0.23
P=0.83
P=0.3
CD45RA+ CD27+
CD57-
CD57+
Vδ2+
Vδ2-
C
P=0.36
P=0.87
P=0.96
P=0.65
P=0.28
CD56 bright
NKG2A+ NKG2C-
NKG2A- NKG2C-
NKG2A- NKG2C+
NKG2A-G2C+ CD57+
CD56+ CD3-