INTRODUCTION

Homeostasis plays an essential role in the maintenance of normal immune function. For most of adult life, a dedicated balance is conserved between the production of new immune cells and the removal of old or dysfunctional cells and stable cell numbers and function are sustained. However, this balance is believed to be altered in the later stages of life when reduced immune function declines in the elderly. Cross-sectional studies of human age-associated changes have generated a wealth of information and provide a foundation for our current understanding of immune system aging (Douek et al., 1998). Whether the uneven expansion of some T cell clones is due to antigen-driven selection and/or "dysfunction" of homeostatic proliferation with age requires further study.

Age-associated decline of immune function is believed to be mainly due to alterations of immune cells. However, longitudinal changes of human immune cells with age have not yet been adequately addressed. To test the hypothesis that regeneration of lymphocytes and monocytes is robust throughout most of adult life until advanced age, we examined six leukapheresis donors (3 young and 3 middle-aged/old) who donated approximately 10% of their peripheral blood mononuclear cells (PBMC) every other month over 3–5 years. We found the number of both lymphocytes and monocytes were quite stable in the blood of all six donors. As expected, young donors had more T cell receptor excision circles (TRECs), CD31+ cells (CD4 only) and longer telomeres in T cells than did the middle-aged/old donors. Interestingly, more variation in TREC number, Vβ usages, and telomere lengths were observed in young donors during the 3–5 years course of donation whereas the middle-aged/old donors showed a rather striking stability in these measurements. This may reflect a more prominent role of thymic output in T cell regeneration in young than in middle-aged/old donors. Together, these findings provide an in vivo glimpse into the homeostasis of lymphocytes and monocytes in the blood at different ages, and support the notion that regeneration of lymphocytes and monocytes is robust throughout adult life up to the early 70s.

Keywords: T cell, telomere, telomerase aging, leukapheresis, TREC, CD31
Telomeres, the termini of chromosomes, are essential for chromosomal integrity (Blackburn, 2005; de Lange, 2005). Attrition of telomere length occurs due to the inability of conventional DNA polymerases to completely replicate telomeres during chromosomal replication. Our previous studies have shown naive CD4+ T cells possess longer telomeres than do memory CD4+ T cells (Weng et al., 1995), and telomere shortening occurs in both the CD4 and CD8+ T cell compartments with age (Son et al., 2000). Thus, telomere length has been viewed as an indicator of previous cell division history and a predictor for the residual replicative life span of a cell. Telomerase is an enzyme that synthesizes telomeres and compensates for telomere loss during cell division. Telomere length in lymphocytes is a consequence of the interplay of telomerase-mediated telomere synthesis and DNA replication-associated telomere loss. Lymphocytes express telomerase in a tightly regulated fashion during their development and activation (Weng et al., 1996). Resting T cells express low to undetectable levels of telomerase whereas engagement of TCR or stimulation by homeostatic cytokines induces telomerase activity. When telomerase activity is highly induced, telomere length appears to be maintained in these actively dividing T cells (Weng et al., 1997; Li et al., 2005).

Previous studies of lymphocyte regeneration in vivo have been mainly focused on abnormal conditions (Sachenberg et al., 1998; McCune et al., 2000; Neese et al., 2002; Douek et al., 2003; Krupica et al., 2006; Williams et al., 2007; Daguiran et al., 2008). In vivo lymphocyte homeostasis and the influence of age on this process have not been extensively analyzed in normal adults. Here, we report an analysis of six frequent leukapheresis donors (3 young donors with average starting age of 25 and 3 middle-aged/old donors with an average starting age of 61) who donated approximately 10% of peripheral blood mononuclear cells (PBMC) every other month continuously over 3–5 years. The older donors span a large age range (50–73), they are referred to herein as “middle-aged/old” group. We found that the numbers of both lymphocytes and monocytes were quite stable in blood for all six donors over the course of donation, and that the young donors had more TREC+ and longer telomeres in both CD4+ and CD8+ T cells than the middle-aged/old donors. In addition, young donors also had more CD8+ naive CD4+ T cells than did the middle-aged/old donors.

Unexpectedly, young donors exhibited a great deal of variation or fluctuation in TREC levels, Vβ usages, and telomere lengths when compared with more stable measurements observed in the middle-aged/old donors. Together, our findings provide new information on the homeostasis of blood lymphocytes and monocytes in vivo in young and middle-aged/old humans and suggest the regeneration of lymphocytes and monocytes is robust throughout adult life up to the early 70s.

**MATERIALS AND METHODS**

**COLLECTION OF PBMC BY LEUKApheresis OF NORMAL DONORS**

Six normal volunteers 22–70 years old were leukapheresed at the Clinical Research Branch of the National Institute on Aging under an Institutional Review Board-approved protocol. Informed consent was obtained from all subjects. PBMC were further isolated using Ficoll gradient centrifugation and cryopreserved for 1–5 years for subsequent analysis.

**ISOLATION OF T AND B CELLS FROM PBMC**

The procedures for isolating T and B cells from PBMC were previously described (Son et al., 2000). In brief, cryopreserved PBMC were thawed slowly and washed with Hank’s balanced salts solution (HBSS) containing 0.2% bovine serum albumin (BSA), 0.01 M HEPES, and 30 U/ml penicillin and 50 μg/ml streptomycin. CD4+ and CD8+ T cells, and B cells (CD19+) were isolated by positive immunomagnetic separation using Dynabeads (Invitrogen, Life Science) according to the manufacturer’s directions. Purity of isolated T and B cells were typically >95%.

**FACS ANALYSIS OF LYMPHOCYTE MARKERS**

Thawed PBMC were washed twice with HBSS containing 0.2% BSA and 0.1% NaN3 and stained with the following antibodies in different combinations. Fluorescein isothiocyanate (FITC)-labeled antibodies against CD31, phycoerythrin (PE)-labeled CD3+2L, CC+R7, CD28, CD127, PE-Cy5-labeled CD45RA, CD8, PE-Cy5.3-labeled CD4, and allophycocyanin (APC) labeled CD8, CD4, and CD31 were purchased from eBioscience (San Diego, CA, USA). FITC-labeled CD16, CD25, CD36, PE-Cy5-labeled CD31, and APC-labeled CD19 were purchased from Invitrogen. Cells were stained according to the manufacturer’s instructions. stained cells were run on a Calibur flow cytometer or FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA), and the data were further analyzed by CellQuest Pro (BD Biosciences) v5.2.1 or FlowJo v8.8.6 respectively (TreeStar Inc., Ashland, OR, USA).

**TREC ANALYSIS**

TREC analysis was previously described (Douek et al., 2000). In brief, genomic DNA was purified from CD4+ and CD8+ T cells using a Gentra Puregene Cell Kit according to the manufacturer’s instructions (Gentra Systems, Inc., Minneapolis, MN, USA). The procedures for isolating T and B cells from PBMC were further described using a Gentra Puregene Cell Kit according to the manufacturer’s instructions (Gentra Systems, Inc., Minneapolis, MN, USA). The procedures for isolating T and B cells from PBMC were further described using a Gentra Puregene Cell Kit according to the manufacturer’s instructions (Gentra Systems, Inc., Minneapolis, MN, USA).
gene RPL-32. TRECs per μg were converted to TREC per cell based on the DNA content of 1.5 million cells containing 1 μg of DNA (Bazis et al., 2009).

TELOMERE LENGTH ANALYSIS BY FLOW FISH
Determination of telomere lengths by flow FISH was previously described (Rufer et al., 1998). Briefly, isolated CD4 and CD8 T cells were permeabilized using cytofix cytoperm (BD Biosciences), and hybridized with telomere-specific FITC-labeled PNA probes (Perkin Elmer, Wallingford, MA, USA) in 70% deionized formamide (Invitrogen) overnight. Cells were stained with 7AAD (BD biosciences). Data were collected on a linear scale on FL1 channel, and the mean fluorescence intensity was determined using Cis-Quest Pro software. Mean fluorescence intensity was normalized using beads (Bangs Laboratories, Inc., Fishers, IN, USA) and then converted to kilobases using an equation derived from a series of samples whose telomere length was measured by both Southern blot and Flow-FISH.

STATISTICAL ANALYSIS
Error bars represent the SD. P-values are the result of a Student’s t-tests and P < 0.05 was considered to be statistically significant.

RESULTS
STABLE LYMPHOCYTE AND MONOCYTE COUNTS IN FREQUENT LEUKAPHERESIS DONORS OVER 3–5 YEARS
To assess the homeostasis of blood leukocytes in healthy human adults, we examined the cell counts of lymphocytes and monocytes of six donors who underwent frequent (every 2–4 months over a period of 3–5 years) leukapheresis (Table 1). In view of the effect of age, we divided these six donors into two age groups: young (mean starting age 25 ± 3) and middle-aged/old (mean starting age 61 ± 3). Lymphocyte and monocyte counts were determined by CBC and are presented in Figure 1. Some fluctuations were found, but the overall cell counts for both lymphocytes and monocytes were stable. We then focused on the subpopulations of lymphocytes, including CD4 and CD8 T cells, B cells (CD19+), and NK cells (CD16+/CD56+) by flow cytometry analysis (Gating strategy in Figure A1 in Appendix).

Table 1 | Subject age, gender, and donation number

| Alias | Gender | Age range | Donations |
|-------|--------|-----------|-----------|
| Y1    | M      | 24–27     | 7 (7)     |
| Y2    | M      | 22–27     | 18 (4)    |
| Y3    | F      | 25–32     | 13 (7)    |
| M1    | M      | 50–54     | 22 (10)   |
| M2    | M      | 62–66     | 17 (10)   |
| M3    | F      | 70–73     | 13 (8)    |

*Normal subjects underwent leukapheresis over 3–5 years once every 2–4 months. Young (Y) subjects with the mean starting age = 25 and middle-aged/old (M) with mean age = 61.

The number of donations in NIA clinic covered in this study. Not all time point over this span were examined. The number in parenthesis indicated times points examined for each donor.

CD4 and CD8 T cells and monocytes appeared stable. In contrast, NK cells exhibited a small increase in middle-aged donors while a decrease of B cells was observed in young donors (Figure 2). Together, these findings suggest that the numbers of lymphocytes and monocytes were well maintained in these frequent blood donors over 3–5 years time regardless of their age.

VARIED THYMIC OUTPUT IN YOUNG AND IN MIDDLE-AGED/OLD ADULTS
To analyze the relative contribution of thymic output in young and middle-aged/old adults, we examined TREC levels in CD4 and CD8 T cells in our donors. In agreement with the previous findings (Dournet et al., 1998; Harris et al., 2005; Naylor et al., 2005; van den Dool and de Boer, 2006), TREC counts in CD4 T cells were higher in young than in middle-aged/old donors (p < 0.05; Figure 3A). Interestingly, we observed three different patterns in young donors: no obvious change, a decrease during the first 2 years but stable after that, and a gradual decline (Figure 3B). In contrast, TREC levels were low but stable in the middle-aged/old donors over similar lengths of time (Figure 3B). This suggests that the thymic output in young is more variable than in middle-aged/old adults.

Next, we analyzed the percentage of CD31+ T cells in the CD4+CD45RA+ T cell compartment. Similar to TREC counts, we observed fewer CD31+ T cells in the CD4+CD45RA+ compartment in middle-aged/old adults than in young donors (p < 0.05; Figure 3C). Furthermore, the percentage of CD31+ CD45RA+CD4 naive T cells was stable in middle-aged/old donors (Figure 3C). Interestingly, the percentage of CD31+ CD45RA+CD4 naive T cells was also stable in young donors, even though some displayed decreasing TREC frequencies (particularly Y3). This suggests TREC levels may be more sensitive than CD31 expression in measuring thymic output. We further analyzed the ratios of naive/memory T cells over the course of leukapheresis and found that there were individual differences in the ratios of naive/memory T cells but did not observe a clear cut age-related difference between young and middle-aged/old donors (Figure A2 in Appendix).

Similar to CD4 T cells, TREC frequencies in CD8 T cells were higher in young than in the middle-aged/old donors (p < 0.05; Figure 4A). Individually, we observed some fluctuation in TREC counts in both young and middle-aged/old donors (Figure 4B). We then analyzed naive CD8 T cells defined by CD45RA+ and CD62L+ or CCR7+ and observed the difference between young and middle-aged/old adults (Figure 4C). As the relationship between CD31 expression and recent thymic emigrants of CD8 T cells has not been defined, we did not observe a close association between TREC counts and the number of naive CD8 T cells (Figures 4B,C). In addition, we analyzed CD28+CD8 T cells and found that the percents of CD28+CD8 T cells were relatively stable over time in both young and middle-aged/old donors (Figure A3 in Appendix).

MORE FLUCTUATIONS OF TCR β VARIABLE GENE USAGE IN YOUNG THAN IN MIDDLE-AGED/OLD DONORS
To ascertain if the usage of another key factor of T cell homeostasis and function changes over time, we examined TCR β variable
gene (Vβ) usage in these six donors longitudinally with a panel of antibodies specific for 24 different Vβ genes. We investigated the percent of CD4 and CD8 T cells that stained positive for one of the 24 different Vβ chains contained in our panel of antibodies. The total percentage of CD4 and CD8 T cells covered by these Vβ antibodies was approximately 50% each and similar between young and middle-aged/old donors (Table 2). The magnitude of measurable fluctuations of each Vβ change was determined by measuring the same Vβ’s two to three different times from the same donors and the same sample (time point). The SD of these differences was calculated for each Vβ and used as a measure to determine actual changes in Vβ usage over time. To evaluate the

Table 2 | Vβ staining and change in CD4 and CD8 T cells overtime

| Donor | CD4 | CD8 | Time span (month) |
|-------|-----|-----|------------------|
|       | Percent | Increase | Decrease | Percent | Increase | Decrease |       |
| Y1    | 49.7 | 3, 13.2* | 5.3, 71, 1*, 5.1* | 475 | 14 | 46 |
| Y2    | 475 | 3, 13.2* | 5.3, 71, 1*, 5.1* | 42.4 | 3 | 59 |
| Y3    | 42.4 | 2, 17 | 38.8 | 8, 14, 21.3 | 26 |
| M1    | 38.5 | 1 | 34.5 | 1, 23 | 25 |
| M2    | 470 | 38.4 | 8 | 32 |
| M3    | 61.3 | 11 | 46.9 | 5.2* | 35 |

*CDR Vβ repertoire usage was determined using a panel of 24 fluorochrome-conjugated antibodies against individual Vβ epitopes in PBMC along with antibodies against either CD4 or CD8. The sum of the percent of T cells staining positive for each Vβ gene are shown for each donor.
*The SD of measurement error for each Vβ gene was calculated (see Results). The Vβ gene designation without * indicates the change ≥ 1 SD but < 2 SD, and with * indicates change ≥ 2 SD.
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PBMC regeneration in blood donors

FIGURE 2 | Frequent leukapheresis did not alter frequency of PBMC constituents. Donors underwent leukapheresis every other month and the relative percentages of component cell types were determined from PBMC by FACS analysis. Young donors (left panels) were compared with middle-aged donors (right panels). Starting from the top CD4, CD8, NK cell (CD16/CD56⁺), B cells (CD19⁺), and monocyte (CD14⁺) were examined. Time values on the abscissa correspond to the time values on the abscissa in Figure 1.

In agreement with the TREC changes, we observed more Vβ usage changes in young donors than in middle-aged/old donors in both CD4 and CD8 T cells (Table 2).

DIFFERENT CHANGES OF TELOMERE LENGTHS IN YOUNG AND MIDDLE-AGED/OLD LEUKAPHERESIS DONORS

Telomere attrition has been well documented during cell division and aging. To determine if telomere length changed in CD4 and CD8 T cells of young and middle-aged/old leukapheresis donors, we used a flow-FISH method and measured the telomere lengths of CD4 and CD8 T cells at multiple time points during the 3–5 year donation. Telomeres were measured by mean fluorescence intensity (MFI) using flow cytometry and then converted from mean fluorescence intensity to kilobase pairs. Consistent with the above findings, telomere length was more stable in middle-aged/old than in young donors over 3–5 years.
FIGURE 4 | CD8 TREC levels were variable in both young and middle-aged donors. (A) TREC counts (left) and the percentages of naïve CD8 T cells (right) are presented ($P < 0.05$). (B) TREC counts in CD8 T cells isolated from young (left panels) and middle-aged (right panels) donors are shown. (C) Naïve (CD45RA+ and CD62L+ or CCR7+) CD8 T cells were examined. PBMC were counterstained with CD8 and CD45RA and CD62L or CCR7 and examined by FACS analysis.

FIGURE 5 | Telomere attrition was higher in young than in middle-aged adults. (A) Telomere length of CD4 and CD8 T cells from young and middle-aged donors are presented as mean and SD. (B) The CD4 T cell telomere lengths of each donor are shown, young (left), and middle-aged (right). (C) CD8 T cell telomere lengths of T cells from each donor are shown, young (left), and middle-aged (right).

(Figure 5B). The telomere attrition rates are 245 bp/year and 5 bp/year for young and middle-aged/old donors CD4 cells, respectively (Figure 5B). The mean telomere attrition rates are 119 bp/year and 22 bp/year for young and middle-aged/old donors CD8 cells, respectively (Figure 5C). Compared to the previously reported telomere attrition rates in CD4 and CD8 T cells from cross-sectional analysis, the telomere attrition rates were much higher in young donors and lower in the middle-aged donors. It remains to be determined whether this is a general characteristic of telomere length change in young and middle-aged adults.
As expected, we found that young donors had more TREC systems examining human T cell homeostasis (Sachsenberg et al., 1998; McCune et al., 2000; Neese et al., 2002; Douek et al., 2003; Kruppica et al., 2006; Williams et al., 2007; Dagurindau et al., 2008). The design of this study enabled us to examine T cell regeneration in blood donors. PBMC regeneration in blood donors (Bains, I., Antia, R., Callard, R., and Yates, A. J. (2009). Quantifying the CD31 expression in human naïve CD4+ T cell subsets and proportionally expands the CD31+ subset in a PLK-dependent manner. Blood 113, 2995–3007. Banu, L., Atay, K., Gallard, B., and Yelis, A. J. (2009). Quantifying the development of the peripheral naive CD4+ T-cell pool in humans. Blood 113, 5480–5487.

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APPENDIX

FIGURE A1 | Gating strategy for flow cytometry analysis of blood lymphocytes. PBMC were isolated and stained with various antibodies and analyzed by FACS.
FIGURE A2 | Relative stable ratio of naïve/memory CD4+ T cells of six frequent leukapheresis donors throughout 3–5 years. PBMC were isolated and stained with CD4, CD45RA, and CCR7. Naïve CD4+ T cells were defined by CD4+ CD45RA+ CCR7+ and memory cells were defined by CD4+ CD45RA− CCR7+−/−.

FIGURE A3 | CD8+ CD28− T cells of six frequent leukapheresis donors throughout 3–5 years. PBMC were isolated and stained with CD8 and CD28. The percents of CD8+ CD28− in total CD8 T cells are presented.