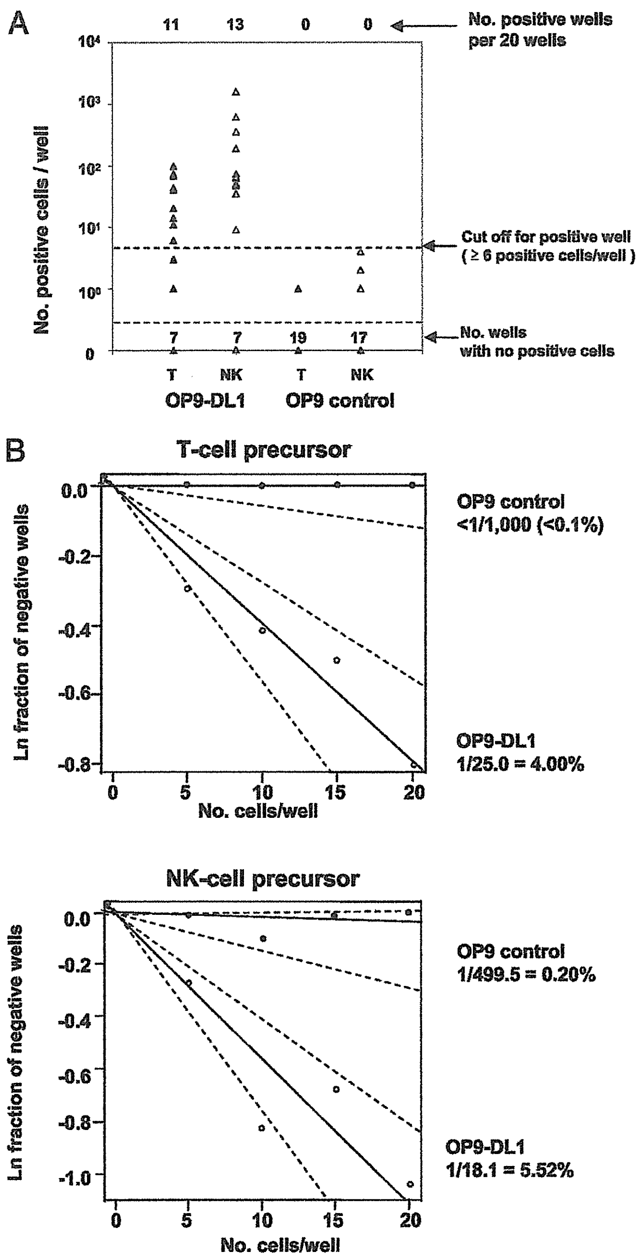

FIGURE 2. LDA for determination of T/NK precursor frequencies in PB CD34⁺Lin⁻ cells by coculture with OP9-DL1 or OP9 control stroma cells. (**A**) CD34⁺Lin⁻ cells in PB from one healthy donor were sorted into wells of a 384-well plate and cocultured with OP9-DL1 or OP9 control cells for 5 wk. Each dot represents the total number of CD7⁺CD5⁺ or CD7⁺CD56⁺ cells generated from twenty CD34⁺Lin⁻ cells in a well, as shown in Fig. 1. (**B**) LDA for determination of T and NK cell precursor frequencies in CD34⁺Lin⁻ cells by coculture with OP9-DL1 or OP9 control cells. One thousand PB CD34⁺Lin⁻ cells from one donor were sorted into wells at the frequency of 20, 15, 10, or 5 cells/well (20 wells for each cell frequency) and cocultured with OP9-DL1 or OP9 control cells for 5 wk. Natural logs of negative-well fractions are plotted against the number of CD34⁺Lin⁻ cells plated per well for T cell (*upper panel*) and NK cell (*lower panel*) precursors using ELDA software. Dotted lines represent 95% confidence intervals. *p* Values of likelihood ratio tests of a single-hit model were 0.50 and 0.47 for T cell and NK cell precursors, respectively. The differences of precursor frequencies between coculture with OP9-DL1 and OP9 control are highly significant for both T and NK cells ($p = 4.7 \times 10^{-9}$ and 2.3×10^{-8}, respectively). The precursor frequencies are converted to the percentage in CD34⁺Lin⁻ cells. Similar results were obtained from two experiments for the other two donors.

>95% of wells in the OP9 control culture were negative (240 wells examined for three volunteer donors in total). Although this cutoff value was somewhat arbitrary, regression curves of negative fractions for both T and NK cell precursors fit well with this model (Fig. 2B). Furthermore, this precursor frequency assay showed high reproducibility by a nonparametric correlation analysis between the first and second measurements (Supplemental Fig. 1). As a corollary, NK/T ratio was highly repeatable. The precursor frequencies in CD34⁺Lin⁻ cells were converted to the percentages of precursors in CD34⁺Lin⁻ population to assess the population size of progenitors with ease (Fig. 2B).

Characterization of T/NK-lineage progenies

CD7⁺CD5⁺ cells did not express CD3, CD4, and CD8 in ~85% of positive wells (125 wells were analyzed in total from six volunteer donors) in LDAs from PB CD34⁺Lin⁻ cells (Supplemental Fig. 2). Only 6% of the positive wells expressed both CD4 and CD8 but remained surface CD3 negative. Nevertheless, because nearly all CD7⁺CD5⁺ cells produced cytCD3, these cells were certainly committed to the T cell lineage.

The majority of CD7⁺CD56⁺ cells generated in LDAs of PB CD34⁺Lin⁻ cells expressed CD161 (NK receptor) but not CD16, CD159a (NKG2A), granzyme B, or cytCD3 (Fig. 3A, 3B). Further culture with IL-15 in the absence of stroma cells induced expressions of CD159a, granzyme B, and cytCD3 in CD7⁺CD56⁺ NK cell progenies (Fig. 3B). Those findings indicate that CD7⁺CD56⁺ progenies generated in OP9-DL1 coculture were immature NK cells. Moreover, the presence of IL-15 from the beginning of OP9-DL1 coculture induced expressions of CD159a, granzyme B, IFN-γ, and cytCD3 but partially inhibited T cell development (Supplemental Fig. 3). We also found by LDA that, in the absence of Notch 1 signaling, IL-15 could induce CD7⁺CD56⁺ cells significantly but less effectively than in the presence of Notch 1 signaling (Supplemental Fig. 3).

Frequencies of T/NK precursors in PB and BM CD34⁺Lin⁻ cells

We obtained the frequencies of T/NK precursors in CD34⁺Lin⁻ cells from PB ($n = 20$) and BM ($n = 3$) (Table I). Both frequencies of T and NK cell precursors in PB CD34⁺Lin⁻ cells ranged approximately from 1 to 7% among 20 healthy donors. No significant difference between PB and BM was observed for both frequencies of T and NK cell precursors ($p = 0.27$ and $p = 0.16$ in Mann–Whitney *U* test, respectively), although this may be due to the small number of BM samples.

Associations of T and NK cell precursor frequencies in PB with age were analyzed for the 20 donors (Fig. 4). The frequency of T cell precursors was found to significantly decrease with age (Spearman's rank correlation; $p = 0.0005$), but no significant association of NK cell precursor frequency with age was detected ($p = 0.50$). Consequently, the NK/T ratio significantly increased with age ($p = 0.002$).

In addition, the proportion of CFU-GM (total proportion of CFU-G, -M, and -GMmix) in PB CD34⁺Lin⁻ cells in the 20 donors ranged from 3.0 to 19.4% (median, 11.2%), which did not show significant age-association (Spearman's rank correlation; $p = 0.92$). Similarly, no significant association with age ($p = 0.26$) was observed for the proportion of burst-forming unit erythroid in PB CD34⁺Lin⁻ cells (median, 29.6%; range, 11.1–53.6%). The CD34⁺Lin⁻ cell proportion (median, 0.066%; range, 0.015–0.150%) in PBMCs showed no significant decline with age ($p = 0.68$).

Clonal analyses of T/NK differentiation from a single PB CD34⁺Lin⁻ cell

To examine whether the difference in NK/T ratio among donors might be attributed to the difference in relative prevalence of T/NK

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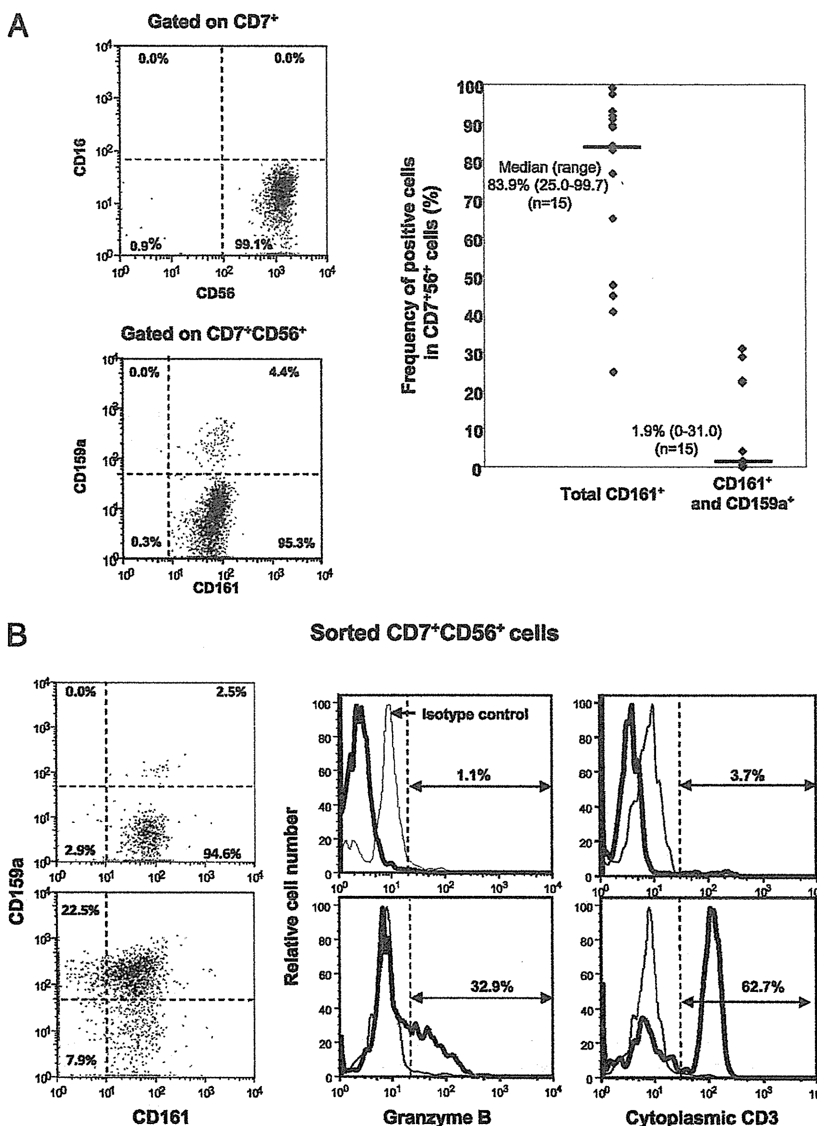

FIGURE 3. Characterization of CD7$^+$CD56$^+$ cells generated from CD34$^+$Lin$^-$ cells. (**A**) Representative flow cytograms of CD16 and CD56 expressions in CD7$^+$-gated cells (*upper panel*) and CD161 (NK receptor) and CD159a (NKG2A) expressions in CD7$^+$CD56$^+$-gated cells (*lower panel*) generated from 20 CD34$^+$Lin$^-$ cells in a well of 384-well plate after 5 wk of culture (*left panel*), and frequencies of total CD161-positive cells and CD161/CD159a double-positive cells (mature phenotype) in CD7$^+$CD56$^+$ cells from 15 wells showing six CD7$^+$CD56$^+$ events and over (*right panel*). Bars in the graph represent medians of the frequencies. (**B**) Induction of CD159a, granzyme B, and cytCD3 in CD7$^+$CD56$^+$ progenies by further culture with IL-15. No significant or very low expressions of these mature NK phenotypes were observed in CD7$^+$CD56$^+$ cells sorted from 5-wk mass culture of CD34$^+$Lin$^-$ cells with OP9-DL1 cells (*upper panels*). Significant expressions of those phenotypes were detected in CD7$^+$CD56$^+$ cells after 3-wk culture with IL-15 (10 ng/ml) in the absence of stroma cells (*lower panels*).

dual- and T or NK single-lineage precursors, we performed clonal analyses of progenies generated from single PB CD34$^+$Lin$^-$ cells for the two donor groups with high and low NK/T ratios (Table II). In this analysis, precursors were classified to T/NK dual-, T single-, and NK single-lineage precursors by the surface phenotype of their progenies (Fig. 5A), including precursors producing small numbers of progenies (six events and more per well) (Supplemental Fig. 4). In the low NK/T ratio donor group, the majority of progenies were generated from T/NK dual-lineage precursors, and the rest of the progenies were derived from T or NK single-lineage precursors (Table II). In contrast, the majority of progenies from the high NK/T ratio group were found to be derived from NK single-lineage precursor clones. The relative prevalence of the T/NK precursor clones significantly differed between the low and high NK/T ratio groups (χ^2 test; $p < 10^{-6}$).

Surprisingly, ~98% of the T/NK precursor clones examined (n = 109) simultaneously generated CD7$^-$CD14$^+$CD16$^-$ and/or CD7$^-$CD14$^+$CD16$^+$ myeloid cells in the cultures (Fig. 5B, Table III). The tested clones included T/NK dual-, T single-, and NK single-lineage precursors (n = 33, 14, and 62, respectively). May–Giemsa staining confirmed that CD7$^-$CD14$^+$CD16$^-$ or CD7$^-$CD14$^+$CD16$^+$ cells were monocytic or granulocytic, respectively (Fig. 5C). Therefore, we conclude that the majority of T/NK precursors in adult PB can retain myeloid differentiation potential.

Discussion

The presence of HSCs in human PB was demonstrated previously by autologous transplantation of PB WBCs (32). However, characterization of circulating HSCs in steady state has been hampered mainly because of their rarity. Although G-CSF can mobilize PB HSCs, it is not known whether the functional properties of such mobilized PB HSCs are the same as those of nonmobilized PB HSCs. In this study, we have developed novel assays for the functional enumeration and characterization of rare PB T/NK precursors using cell sorting–based limiting-dilution and clonal

Table I. Frequencies of T and NK cell precursors in PB and BM CD34$^+$ Lin$^-$ cells

Cell Sources (No. of Donors)	Median Precursor Frequencies (Range) in %	
	T	NK
PB (n = 20)	3.44 (0.97–5.29)	3.63 (2.44–6.45)
BM (n = 3)	4.47 (1.71–8.42)	2.54 (0.41–4.10)

LDA measurements were performed with PB and BM samples from 20 volunteers (age range, 28–64 y) and 3 donors (commercially available, age/gender: 35/female, 43/female, and 44/male), respectively.

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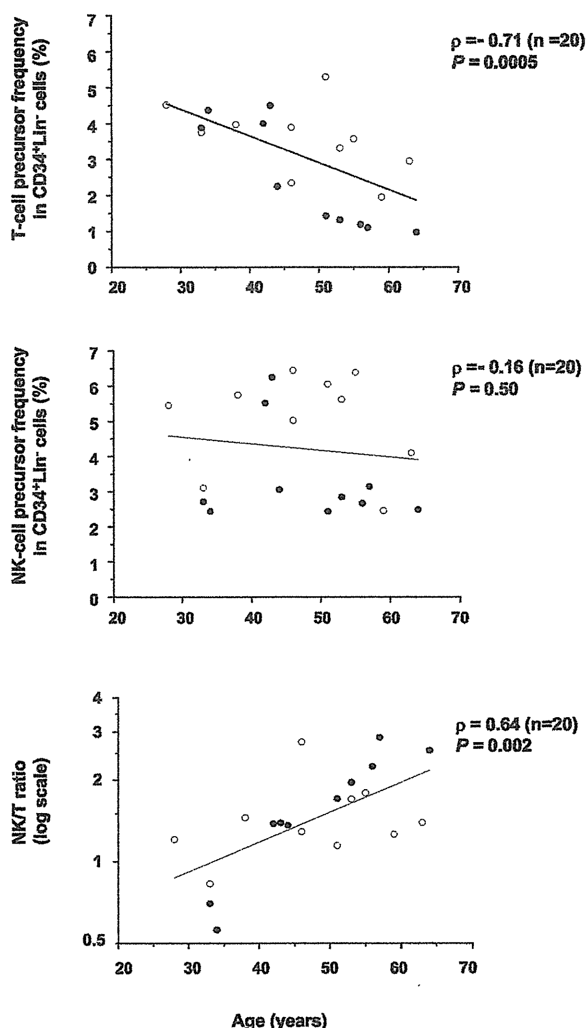

FIGURE 4. Association of T and NK cell precursor frequencies in PB CD34$^+$Lin$^-$ cells with donor age. Frequencies of T (*upper panel*) and NK cell (*middle panel*) precursors in PB from 20 donors (10 females, O; 10 males, ●) as shown are plotted against age. Ratio of NK to T precursor frequency (NK/T ratio) is also plotted on a log scale (*lower panel*). Spearman's rank correlation coefficients (ρ) are shown with *p* values.

analyses. Microculture using a 384-well plate with a small observation field and small volumes of culture medium made it easy to observe cell growth under the microscope and to maintain a small number of progenitors ex vivo. This assay can be used to measure the T/NK precursor frequencies in PB CD34$^+$Lin$^-$ cells

Table II. Different distributions of precursor clones producing T- and/or NK-lineage progenies between the donor groups with low and high NK/T ratio

	No. of Clones Producing T and/or NK Progenies (% Total)			
NK/T Ratio	T and NK	T	NK	Total
Low	32 (71.1)	5 (11.1)	8 (17.8)	45 (100)
High	13 (26.5)	2 (4.1)	34 (69.4)	49 (100)

A total of 45 clones from three donors in the low NK/T group was analyzed for T/NK dual-lineage, T single-lineage, and NK single-lineage progenies, as shown in Fig. 4A (15, 10, and 20 clones from donors with NK/T ratios of 1.45, 1.39, and 1.38, respectively.) A total of 49 clones from three donors in the high NK/T group also was analyzed and compared with the low NK/T group (23, 17, and 9 clones from donors with NK/T ratios of 2.76, 2.86, and 2.57, respectively). The difference in relative prevalence of the T/NK precursor clones between the low and high NK/T groups was highly significant (χ^2 test; $p < 10^{-6}$).

expanded on OP9-DL1 layers in the presence of KL, IL-7, and FL. Our results showed that the proportions of T and NK cell progenitors in PB CD34$^+$Lin$^-$ cells were not significantly different from those in BM CD34$^+$Lin$^-$ cells. The results suggest that the proportional compositions of T/NK progenitor populations in PB may be comparable with those in BM, although the mechanism for export of progenitors to PB from BM in steady state is unknown.

Our findings indicate that Notch signaling is required for both T- and NK-lineage commitment of human PB progenitors in culture. Because the majority of CD7$^+$CD5$^+$ progenies generated in coculture with OP9-DL1 cells were found to express cytCD3 but have a triple-negative phenotype, the T cell maturation from adult PB progenitors cannot proceed further under the present culture condition. This result may not be concordant with previous reports describing full T cell maturation of human CB CD34$^+$ cells in OP9-DL1 coculture (24). Full maturation from adult PB progenitors may require longer periods of coculture or thymic environments (33, 34).

Our observation that in vitro NK cell differentiation from PB progenitors requires Notch 1 signaling is concordant with previous studies describing development of mouse BM and human CB NK progenitor cells (25, 27, 35, 36). However, a recent study using knockout mice described that Notch 1 deficiency completely abrogated early thymic progenitors and subsequent T cell development but did not affect thymic NK cell development at all (37). Thus, the role of Notch1 signaling in mouse NK cell development is dispensable in vivo, but its role for in vivo human NK cell commitment remains unknown. Furthermore, in the current study, IL-15 induced the expression of molecules associated with NK cells such as cytCD3, granzyme B, and CD159a in the CD7$^+$ CD56$^+$ cells generated from the OP9-DL1 coculture. The essential role of IL-15 in NK cell development is well accepted in both mice and humans (38, 39). Our observation is consistent with previous reports describing IL-15 augmentation of Notch activation–induced NK cell differentiation from CB CD34$^+$ cells (26, 27). It also was found that IL-15 could partially but significantly induce CD7$^+$CD56$^+$ cells in the absence of Notch 1 signaling. Taken together, our data suggest that both Notch 1 and IL-15 signaling are involved in the induction of NK cell commitment of human PB progenitors in vitro and that IL-15 but not Notch 1 is essential for further maturation of the NK-committed cells (CD7$^+$ 56$^+$ cells). If that is the case, the role of Notch 1 in NK cell development may be dispensable in humans in vivo.

In the current study, the frequency of T cell precursors derived in vitro from PB CD34$^+$Lin$^-$ cells significantly decreased with age, but the NK cell precursor frequency did not significantly change. Age-related increases in mature NK cells in the periphery are associated with an increase in the NK subset dimly expressing CD56 (CD56^{dim}), whereas CD56^{high} NK cells remain stable with age (8, 9, 40). The majority of NK-lineage progenies from PB progenitor express high levels of CD56 but not CD16, as shown in the current study. The CD56^{high}CD16$^-$ NK subset found in the periphery is thought to originate in the thymus and is functionally different from CD56^{dim}CD16$^+$ NK subset (41); therefore, the majority of NK-lineage progenies generated from PB progenitors may be thymic-derived NK cells. If that is the case, the results showing no age-associated changes in NK precursor frequency is in accordance with the insignificant changes of the CD56^{high} NK subset with aging, as mentioned above.

Clonal analyses of PB CD34$^+$Lin$^-$ cells showed that many progenitor clones were able to produce both T and NK progenies. Because the LDA did not discriminate single- and dual-lineage precursors, precursor frequencies obtained from the LDA are considered to simply reflect the total number of events of lineage

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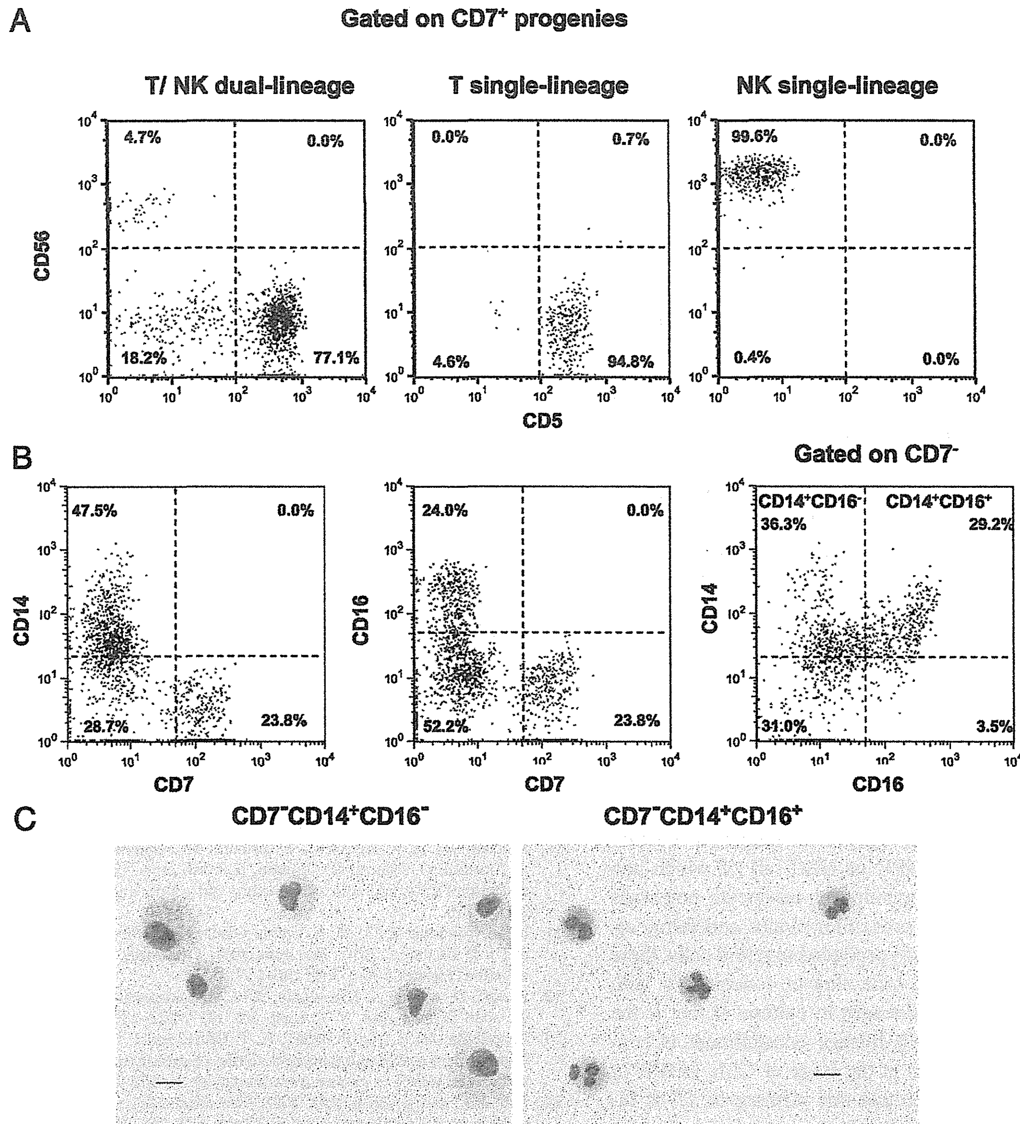

FIGURE 5. T/NK- and myeloid-lineage progenies derived from a single PB CD34$^+$Lin$^-$ cell. PB CD34$^+$Lin$^-$ cells from each donor were sorted into more than 560 wells of 384-well plates at the frequency of one cell per well. After 5-wk culture, wells exhibiting cell growth were analyzed for the surface phenotypes of T/NK and myeloid lineages. (**A**) Representative flow cytograms of CD5 and CD56 expressions in CD7$^+$-gated progenies from a single CD34$^+$Lin$^-$ cell. Each precursor was classified to T/NK dual-lineage (*left panel*), T single-lineage (*middle panel*), and NK single-lineage (*right panel*). (**B**) Myeloid cell progenies generated from a single CD34$^+$Lin$^-$ cell which simultaneously produced T- and/or NK-lineage cells. Representative flow cytograms of CD7$^-$CD14$^+$ (*left panel*) and CD7$^-$CD16$^+$ (*middle panel*) myeloid cells generated from a precursor that produces both of T and NK dual-lineage progenies as shown in (A). CD16$^+$ cells expressed a low level of CD14 (*right panel*). Similar results were obtained for T single- and NK single-lineage precursor clones. (**C**) May-Giemsa staining confirmed that CD7$^-$CD14$^+$CD16$^-$ cells (*left panel*) and CD7$^-$CD14$^+$CD16$^+$ cells (*right panel*) were monocytic and granulocytic, respectively. Scale bars, 10 μm. Images were acquired by BIOREVO model BZ-9000 (Keyence) with Plan Apo ×40/0.95 numerical aperture objective lens (Nikon) using BZ-II Viewer and BZ-II Analyzer software.

commitment in the CD34$^+$Lin$^-$ progenitor population. Clonal analyses also demonstrated that the distributions of single- and dual-lineage T/NK precursor clones significantly differed between the donors whose NK/T ratios were high and low. Therefore, the

Table III. Myeloid potential of precursor clones producing T- and/or NK-lineage progenies

	No. of Clones Producing Myeloid Cells				
T/NK Precursor Clone	Mo	Gr	Mo and Gr	No Mo or Gr	Total
T and NK	4	1	28	0	33
T	4	0	10	0	14
NK	36	0	24	2	62
Total (% total)	44 (40.4)	1 (0.9)	62 (56.9)	2 (1.8)	109 (100)

One hundred nine T and/or NK precursor clones in total were obtained by single-cell sorting of CD34$^+$Lin$^-$ cells from five donors. The presence of CD14$^+$CD16$^-$ monocytic (Mo) and/or CD14$^+$CD16$^+$ granulocytic (Gr) cells in each clonal cell population were detected by flow cytometry, as shown in Fig. 5B. The presence of myeloid potential was classified to Mo and/ or Gr in each T and/or NK precursor clone.

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difference of NK/T ratio among donors was attributable to the difference in relative prevalence of single-and dual-lineage T/NK precursor clones. Particularly, high proportions of NK single-lineage precursors were observed in donors with high NK/T ratios, suggesting that high NK/T ratios in aged donors are the result of an increase in NK single-lineage progenitors. This indicates that the age-associated shift of T/NK differentiation can be explained by changes of lineage commitment at T/NK bifurcation point in each progenitor cell. It is noteworthy that the relative prevalence of NK single-lineage precursor clones in CB $CD34^{+}Lin^{-}$ cell populations was very low (0.8% of total single-and dual-lineage T/NK-precursor clones), which is in strong contrast to the adult PB (S. Kyoizumi, Y. Kubo, J. Kajimura, K. Yoshida, T. Hayashi, K. Nakachi, L. F. Young, M.A. Moore, M.R.M. van den Brink, and Y. Kusunoki, manuscript in preparation). Therefore, our results support our hypothesis that bifurcation of T/NK coprogenitors shifts from T to NK cell lineage with aging.

We observed that generation of T- and/or NK-lineage progenies from progenitor clones was associated with myeloid differentiation. That finding indicates that the pool of T/NK cell precursors in human PB is constituted by T/NK/myeloid common progenitors. Recent work with human BM and CB cells also established the existence of lymphoid progenitors with myeloid potential (23). Our results are concordant with previous observations in adult mice that the progenitors in blood with efficient T-lineage potential were CLP (20) and LMPP (17) and that thymic immigrants retain myeloid potential (14–16), although the myeloid potential may not manifest under physiological conditions, as reported by other researchers (20, 21, 42, 43). However, because we did not examine the differentiation potentials of the progenitor clones to other lineages such as B cell, erythrocyte, and megakaryocyte lineages, it is unknown whether these clones were CLP, LMPP, MPP, or HSC, all of which can display combined lymphoid and granulocyte–monocyte potentials in vitro. Because nearly all of the progenitor clones generating either T or NK single-lineage progenies produced myeloid cells, these clones may bifurcate into T or NK cells after or concurrently with dichotomy of T/NK and myeloid lineages in culture. If that is the case, an age-associated shift to NK-lineage commitment is considered to be primed in progenitors at the stage of CLP, LMPP, or a more upstream stage. We assume that this priming of T/NK bifurcation occurs in HSCs, because such age-associated changes should be accumulated in long-lived and self-renewing stem cells but not in CLP, LMPP, or MPP with transient life spans. It can be presumed that aging might affect the expression levels of genes regulating T/NK-lineage commitment (11). Age-associated epigenetic changes in such genes of HSCs may imprint the biased lineage commitment at a tail branch of the differentiation tree (44).

T/NK–differentiation potential altered because of the age of PB donors may contribute to an age-associated shift of the human immune system from acquired to innate immunity. Extensive evidence indicated that the HSC number in BM increased substantially with age in mice (45, 46). However, HSCs from old mice showed functional deficiencies, including diminished repopulating ability and a biased lineage potential from lymphopoiesis toward myelopoiesis (44, 47–50). In humans, a recent study also found that aged immunophenotypic HSCs increased in frequency and exhibited myeloid-biased differentiation potential compared with young HSCs (6). Our findings of PB myeloid–committed progenitors also support that myeloid potential does not decrease with age in contrast to T cell potential, although it is unknown whether human LMPP contributes to myelopoiesis under physiological conditions.

In conclusion, we have developed a novel assay to assess T/NK potential of PB HSCs and demonstrated an age-associated increase of NK over T cell development for PB HSCs.

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Disclosures

The authors have no financial conflicts of interest.

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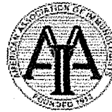

The Journal of Immunology

Age-Associated Changes in the Differentiation Potentials of Human Circulating Hematopoietic Progenitors to T- or NK-Lineage Cells

Seishi Kyoizumi,* Yoshiko Kubo,* Junko Kajimura,* Kengo Yoshida,* Kazue Imai,* Tomonori Hayashi,* Kei Nakachi,* Lauren F. Young,† Malcolm A. Moore,‡ Marcel R. M. van den Brink,† and Yoichiro Kusunoki*

Age-associated changes of T and NK cell (T/NK) potential of human hematopoietic stem cells are unknown. In this study, we enumerate and characterize T/NK precursors among $CD34^{+}Lin^{-}$ cell populations circulating in normal human adult peripheral blood (PB) by a limiting-dilution assay using coculture with OP9-DL1 stroma cells expressing Notch 1 ligand, Delta-like 1. The frequency of T cell precursors in $CD34^{+}Lin^{-}$ cells was found to decrease with donor age, whereas the ratio of NK to T cell precursor frequency (NK/T ratio) increased with age, suggesting that lymphoid differentiation potential of PB progenitors shifts from T to NK cell lineage with aging. Clonal analyses of $CD34^{+}Lin^{-}$ cells showed that differences in the NK/T ratio were attributable to different distributions of single- and dual-lineage T/NK precursor clones. Because nearly all of the clones retained monocyte and/or granulocyte differentiation potentials in coculture with OP9-DL1 cells, T/NK precursors in PB are considered to be contained in the pool of T/NK/myeloid multipotent progenitors. The age-associated increase in NK over T cell commitment might occur in precursor cells with T/NK/myeloid potential. ***The Journal of Immunology*, 2013, 190: 6164–6172.**

One of the most characteristic features of immunological aging is the decline of T cell production associated with thymic involution. Consequently, the peripheral naive T cell pool gradually reduces in size during aging. The main causes of age-associated thymic dysfunction are thought to involve impairments in both hematopoietic stem cells (HSCs) and thymic microenvironment (1–3). Studies in mice have shown the age-related loss of T cell potential in both prethymic and intrathymic progenitors (4, 5), and a recent report suggested that human HSCs exhibited myeloid-biased differentiation potentials with aging (6). Furthermore, a recent paper reported that T cell potential was lower in human adult bone marrow (BM) than cord blood (CB), suggesting high T cell potential in human neonate HSCs (7). However, precise age-associated changes in the T cell potential of human adult HSCs and downstream progenitors have not been well characterized.

In contrast with age-associated decline in T cell development, both the proportion and absolute number of NK cells in the periphery have been reported to increase with age (8–10). These findings suggest that aging has either no effect or a positive effect on NK-lineage differentiation, which could lead to the observed increase in peripheral NK cells. The T/NK-lineage differentiation pathway is well defined, and it is widely accepted that both T and NK cells are generated from bipotent T/NK progenitors (11). We hypothesize that the bifurcation of T/NK coprogenitors shifts from T to NK cell lineage with aging, leading to the differences observed in T and NK cell numbers and proportions with aging. In addition to this, we hypothesize that the microenvironment supporting NK production may be unchanged or enhanced during aging in stark contrast to the decline of microenvironment for T cell development.

To test the above hypotheses, we sought to establish in vitro functional and quantitative analyses of T and NK cell progeni-

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