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Linkage between Dendritic and T Cell Commitments in Human Circulating Hematopoietic Progenitors

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

The relationships between commitments of dendritic cells (DCs) and T cells in human hematopoietic stem cells are not well understood. In this study, we enumerate and characterize conventional DC and plasmacytoid DC precursors in association with T cell and thymus-derived types of NK cell precursors among CD34+ hematopoietic progenitor cells (HPCs) circulating in human peripheral blood. By limiting-dilution analyses using coculture with stroma cells expressing Notch1 ligand, the precursor frequencies (PFs) of DCs in HPCs were found to significantly correlate with T cell PFs, but not with NK cell PFs, among healthy donors. Clonal analyses showed that the majority of T/NK dual- and T single-lineage precursors—but only a minority of NK single-lineage precursors—were associated with the generation of DC progenies. All clones producing both DC and T cell progeny subsets possessed similar expression profiles of proteins and genes related to DC development and functions in both mice and humans (6–8). A recent report using a barcoding technique for single lymphoid–primed multipotent progenitors (LMPPs) suggested that DCs are considered a distinct lineage from myeloid and B cell lineages (9), although the relationships between DC and T cell lineages could not be examined using this technique.

Because DCs contribute to the deletion of autoreactive T cell precursors in the process of negative selection in the thymus, the developmental origin and pathway of murine thymic DCs have been extensively studied in relation to T cell commitment. The CD11b+ cDCs arise from blood precursors that continuously enter the thymus (10, 11). That DC subset derives from bone marrow (BM) DC progenitors that are composed of common macrophage–DC progenitors, common DC progenitors (CDPs), and pre-cDCs (3, 12, 13). In contrast, the CD8+ cDCs develop intrathymically and originate from early T cell progenitors (11, 14, 15). However, contradictory findings have suggested that the thymic CD8+ cDCs are also derived from myeloid precursors (4, 16), or from precursors unrelated to T cell lineage (17). Thymic pDCs were thought to differentiate from lymphoid progenitors (15), but it has recently been reported in a parabiotic study that thymic pDCs originate extrathympically and continually migrate to the thymus (11).

In humans, developmental origin and pathways of thymic DCs were mainly studied in culture (18–20) or in immunodeficient mouse–human chimeras (21) using cord blood (CB) and fetal or newborn thymus for a progenitor source. Results of all these human experiments suggested the presence of common progenitors for T cells and DCs in the thymus, although clonal analyses to confirm a common origin were not conducted. However, due to the lack of human in vivo experimental systems in a physiological setting, a definitive conclusion is thought to be currently unobtainable.

Regardless of whether thymic DCs are derived intrathympically from common progenitors for T cells and DCs or extrathympically from discrete DC lineage progenitors, we assume that possible regulatory mechanisms maintain appropriate numbers of pre-T cells...
and DCs for normal progression of the negative selection in the thymus. In fact, murine thymic DCs displayed kinetics of both generation and decay similar to thymocytes, suggesting a coordinated development of DCs and T cells (22–24). Our hypothesis is that the proportion of DC to T cell precursors entering into the thymus from blood is maintained at a constant level by linkage of commitments between the two lineages at some stage prior to the DC/T split. To test this hypothesis, we sought to establish in vitro functional and quantitative assays of human cDC and pDC progenitors in association with T and NK cell progenitors for the current study. Human peripheral blood (PB) was used as a source of progenitors because these progenitors are assumed to migrate from BM to the thymus through the bloodstream (25).

In our previous study, we developed a cell sorting–based limiting-dilution assay (LDA) and clonal analyses using a 384-well plate for quantification and characterization of T/NK progenitors among CD34-positive/lineage marker–negative (CD34+ Lin−) hematopoietic progenitor cell (HPC) populations circulating in PB of healthy adult humans (26). The surface phenotype of NK cell progenies that developed in the culture represented CD56highCD161−CD16− thymus–derived type (thymic) NK cells. Using single-cell analyses, we classified HPCs into T/NK dual- and T or NK single-lineage precursors. The vast majority of these T and/or NK cell precursor clones were found to be derived from LMPPs or more upstream progenitors that coproduce myeloid cells. The assays used cocultures with OP9-DL1 stroma cells expressing the Notch1 ligand, Delta-like 1 in the presence of Kit ligand (KL), IL-7, and Flt-3 ligand (FL). Some reports described the involvement of Notch 1 signaling in DC development (27, 28), and FL is known to be an essential cytokine for DC development (29). Therefore, it was expected that both DC and T/NK cell commitments would be simultaneously observed in identical culture conditions.

Using the assays, we measured cDC and pDC precursor frequencies (PFs) of PB HPCs from healthy adult human donors in conjunction with T and NK PFs. We also examined CB HPCs for their DC/T potentials as a positive control that is thought to extensively supply progenitors to the thymus. We found that DC PFs correlated with T cell but not with NK cell PFs, suggesting a linkage between DC and T cell potentials in circulating HPCs. Single HPC analyses also indicated a strong linkage between commitments of the two lineages, which may be primed in HPCs prior to bifurcation into T or NK cell lineages.

**Materials and Methods**

**Cytokines and Abs**

Recombinant human KL, FL, IL-3, IL-7, and GM-CSF were purchased from PeproTech. Anti-CD3 (SK7), cytoplasmic CD3 (UCHT1), CD4 (SK-3), CD5 (UCHT2), CD7 (M-T701), CD8 (SK1), CD14 (3G8), CD19 (HIB1B), CD20 (2H7), CD117 (104D2), and HLA-DR (L243) Abs were purchased from BD Biosciences. Anti-CD16a (3G8), CD34 (581), and CD56 (N901) Abs were purchased from Beckman Coulter. Anti-CD14 (TU4K), TCRαβ (BMA031), and TCRγδ (5A6.E9) Abs were purchased from Invitrogen. CD1c (L161), CD11c (Bu15), CD15 (W6/32), CD34 (581), CD141 (M80), CD303 (201A), and CD304 (12C2) Abs were purchased from BioLegend. CD10 (CB-CALLA), CD14 (61D3), and CD123 (6D6) were obtained from eBio. CD127 (R34-34) Ab was purchased from Tombo.

**Cell preparation**

Human PB samples were collected from 20 healthy in–house volunteer donors (Japanese) with informed consent, following the guidance of the institutional review board (Human Investigation Committee of the Radiation Effects Research Foundation), which approved this study. PBMCs were separated from 10 ml PB samples by Ficoll density gradient centrifugation (Lymphocyte Separation Medium 1077; Wako Pure Chemical Industry). CB mononuclear cells and BM mononuclear cells were purchased from Lonza Walkersville.

**Stroma cells**

Generation of the mouse OP9-DL1 stroma cells engineered to express the GFP and the mouse Delta-like 1 gene has been described previously (30). The OP9-DL1 and the OP9 (31) parental stroma cells were maintained by culturing in α MEM (Life Technologies) supplemented with 20% FBS (HyClone), 4 × 10−5 M β-2-ME, and penicillin-streptomycin at 37 C in an humidified atmosphere flushed with 5% CO2.

**LDA of DC/T/NK precursors**

Procedures and culture conditions for the LDA were described previously (26). Briefly, for progenitor cell culture, OP9-DL1 stroma cells were seeded in wells (50–80% confluence) of a 384-well flat-bottom black plate (BD Biosciences). At least 4 h prior to progenitor cell sorting, the culture medium in each well was replaced by 50 μl phenol red-free α MEM containing 20% knockout serum replacement (Life Technologies), 10−3 M monothioglycerol (Sigma-Aldrich), 50 μg/ml gentamicin (Sigma-Aldrich), 10 ng/ml KL, 10 ng/ml FL, and 10 ng/ml IL-7 (DC/T/NK medium). For progenitor cell sorting, PBMCs and CB mononuclear cells were stained with allopencyocyanin-conjugated anti-CD34 Ab and PE-conjugated anti-lineage markers (anti-CD3, CD14, CD16, CD19, CD20, and CD56 Abs) for 30 min on ice, and dead cells were excluded by 1% propidium iodide staining (BD Biosciences), as shown in Fig. 1A. One thousand CD34Lin− cells were sorted into 80 wells of a 384-well plate at a frequency of 20, 15, 10, or 5 cells/well (20 wells for each factor) by FACS Aria II (BD Biosciences). LDA culture was maintained at 37 C in a humidified atmosphere flushed with 5% CO2 and half of each culture condition’s medium (25 μl) was changed every week. After 5 wk of culture, all cells grown in wells were harvested by vigorous pipetting and washed with PBS containing 5 mM EDTA, 0.01% NaN3, and 1% FBS (washing buffer [WB]). For detection of cDC and pDC lineage progenies by Cy5 (Beckman Coulter), cells were stained with a PE–conjugated mixture of the lineage markers and CD7, PE–Cy7–conjugated HLA-DR, allopencyocyanin-conjugated CD11c, and PerCP/CY5.5-conjugated CD123 Abs in WB. Stained cells were resuspended in 200 μl WB containing 1 μg/ml DAPI (Invitrogen) to exclude dead cells by flow cytometry. OP9-DL1 cells and their cell debris were gated out using GFP fluorescence. After gating on DR−CD7−Lin− cells, CD11cLin−CD123low and CD11cLin−CD123high progeny cells in one well were divided into two equal parts for immuno- fluorescent staining, as described above. The absolute number of each progeny per well was calculated from the number of events on a flow cytogram in a volume (160 μl) of cell suspension. A well exhibiting six or more positive events was designated as positive. PFs of cDC, pDC, T, and NK cells were calculated by online analysis using ELDa software (32), available on the home page of Walter Elisa Hall Institute Bioinformatics Division (http://bioinf.wehi.edu.au/software/elda/index.html).

For LDA of PB HPC subpopulations, PBMCs were stained with allopencyocyanin-conjugated CD34, PE–conjugated lineage markers, PE–Cy7–conjugated cKit (CD117), and FITC–conjugated CD10 or CD7 Abs.

For differentiation kinetics and phenotype analyses, CD34Lin− cells were cultured in some experiments at 500 cells/well using a 24-well plate in the same conditions as those used for the 384-well plates.

**Clonal analysis**

Procedures for single HPC analysis were previously described (26). Briefly, CD34Lin−, CD34Lin−cKit+highCD7−, and CD34Lin−cKit+lowCD10− cells in PB were sorted into a total of ∼280 wells for each experiment, using 384-well plates with a single cell per well. The conditions of OP9-DL1 coculture for single HPC culture were the same as those for LDA, described above. All cells in a well of a 384-well plate exhibiting generation of progenies after a 5-wk culture were transferred to one well of a 96-well plate, in which OP9-DL1 cells were preseeded. After culture for 5–10 d in the presence of KL, IL-7, and FL, cells were split into two wells of a 96-well plate. Cells in each of the two wells were analyzed for cDC/pDC or T/NK/myeloid lineage markers by flow cytometry, as described above. A well exhibiting three or more positive events was designated as positive. This cutoff value for clonal analysis was chosen because for LDA batch progeny cells in one well were divided into two equal parts for immuno- fluorescence staining, as described above.

**Surface phenotyping of DC/T/NK progenies**

For analyses of DC subsets, progeny cells from PB HPCs in coculture with OP9-DL1 cells were stained with PerCP/CY5.5-conjugated CD141, CD303,
or CD304, and allophycocyanin-Cy7–conjugated CD1c, in combination with a mixture of lineage markers and CD7, HLA-DR, CD11c, and CD123 Abs, as described above. To determine the stage of T cell maturation, progeny cells were stained with PE-Cy7–conjugated CD7, PerCPCy5.5–conjugated CD4, and allophycocyanin-Cy7–conjugated CD8 Abs, in combination with CD5 and CD7 Abs. For TCR expression, cells were stained with allophycocyanin-conjugated CD7, PE-Cy7–conjugated CD5, allophycocyanin-Cy7–conjugated CD3, PE-conjugated TCRαβ, or TCRγδ Abs. To allow us to detect CD127 expressions in progeny cells, progenies were precultured with DC/T/NK medium in the absence of IL-7 for 3 d. For CD127 expressions in CD34+CD7+ cells, progenies were stained with PE-conjugated CD7, allophycocyanin-Cy7–conjugated CD3, and allophycocyanin–conjugated CD127 Abs. For characterization of NK lineage progenies and PB NK cell subsets, cells were stained with PerCPCy5.5–conjugated CD16 and allophycocyanin–conjugated CD127 Abs, in combination with CD7 and CD56 Abs.

**FIGURE 1.** Growth and differentiation kinetics of DC lineage progenies generated from PB HPCs. (A) Representative flow cytograms of CD34+Lin− cells (HPCs) in PB (left panel) and CB (right panel) mononuclear cells. (B) Representative flow cytograms of HLA-DR+Lin−CD11c−CD123high-pDC lineage and HLA-DR+Lin−CD11c−CD123low-cDC lineage and progenies generated from PB CD34+Lin− cells in 5-wk OP9-DL1 coculture in the presence of KL, IL-7, and FL. (C and D) Time courses of cDC and pDC lineage differentiation from PB CD34+Lin− cells. PB CD34+Lin− cells from four donors were cultured at a concentration of 500 cells/well in a 24-well plate in which OP9-DL1 stroma cells had been preseeded in the presence of KL, IL-7, and FL. Percentage (C) and absolute number (D) of cDC and pDC lineage progenies generated from CD34+Lin− cells are plotted against time (weeks). Solid lines connect the average values. (E) Time course of cDC and pDC lineage differentiation from CD34+Lin− cells of one donor. Each dot represents the total number of cDCs (left panel) or pDCs (right panel) generated from 20 CD34+Lin− cells in a well of a 384-well plate. Twenty wells were analyzed for each of 3, 4, 5, and 6 wk. A well exhibiting six or greater positive events was designated a positive well for LDA. Similar results were obtained from two experiments for two other donors.
**Methylcellulose colony assay**

CFU-granulocyte/macrophage (GM) and burst-forming unit erythroid (BFU-E) in CD34+CD7+ CD5^2 CD56^2 cell populations generated from CD34+Lin^- cells in 4-wk OP9-DL1 cocultures were assayed in methylcellulose cultures using 96-well plates. Briefly, CD34^+CD7^+ CD5^2 CD56^2 were sorted into wells at one cell per well in 50 μl culture containing 1.2% methylcellulose (Stem Cell Technology) with erythropoietin (6 U/ml), KL (20 ng/ml), G-CSF (20 ng/ml), and IL-3 (20 ng/ml). The methylcellulose cultures were microscopically observed after 14 d to look for the presence of a colony in each well.

**Cytokine measurement**

At the fourth week of OP9-DL1 coculture using a 24-well plate, cDC and pDC fractions were sorted and cultured with 50 μl DC/T/NK medium in the presence of GM-CSF (10 ng/ml) and IL-3 (10 ng/ml) for 5 d, respectively, using a 384-well plate (5000 cells/well). Poly I:C (0.2 μg/ml) and R848 (2 μM) for TLR stimuli were added to cDC and pDC culture, respectively, at day 3 after the initiation of the culture. After 5-d culture, supernatants were collected and assayed for IL-6 and IFN-α production. IL-6 and IFN-α assays were conducted using Quantikine HS ELISA (R&D Systems) and VeriKine ELISA (PBL IFN Source) kits, respectively, according to the manufacturers’ instructions.

**Statistics**

Spearman’s rank correlation analysis, Wilcoxon’s signed rank sum test, Mann–Whitney U test, and χ² test were conducted using SPSS 16.01 software (SPSS).

**Results**

**LDA of cDC/pDC precursors in HPCs**

PB and CB HPCs (Fig. 1A) were cultured with OP9-DL1 stroma cells in the presence of KL, FL, and IL-7. Generation of cDC and pDC lineage progenies in OP9-DL1 cultures was determined by flow cytometric detection of CD123^low^CD11c^+^ and CD123^high^
CD11c+ cells in HLA-DR+ CD7− Lin− cell populations, respectively (Fig. 1B). Although variations among individual donors were numerous, the absolute numbers of both progenies per well on average were found to reach plateau levels after 4–5 wk of culture in both 24- and 384-well plates (Fig. 1C–E). When we seeded 20 CD34+Lin− cells into wells of 384-well plates, we...
observed the development of cDCs or pDCs in approximately half of the wells, but the numbers of DC progenies could differ by >100-fold from well to well (Fig. 1E). Based on our previous report describing LDA for T/NK cell precursors (26), we decided on six or more positive events per well as the cutoff value for a positive well in the LDA for cDC and pDC precursors. Although this cutoff value was somewhat arbitrary, regression curves of negative fractions for both cDC and pDC precursors fit well with this model (Fig. 2A). Furthermore, this PF assay showed high reproducibility in nonparametric correlation analysis between the first and second measurements among seven adults (cDC: \( p = 0.89, p = 0.007 \); pDC: \( p = 0.82, p = 0.023 \)). The PFs in HPCs were converted to the percentages of precursors in the CD34+Lin- population to more easily assess the population size of progenitors (Fig. 2A). We simultaneously enumerated CD7+CD5- T cell and CD7+CD56- NK cell PFs for each donor (Supplemental Fig. 1), as described previously (26).

Both cDC and pDC precursor assays using OP9 controls showed that ~70% of precursor-generated DC progenies depended on Notch 1 signaling, but significant parts of the precursor population, especially cDC precursors, were able to produce DC progenies in the absence of Notch 1 signaling (Fig. 2A). Notch1-independent DC precursors may be myeloid-derived DC progenitors: only a few T or NK cell precursors were observed in OP9 controls (Fig. 2A). In fact, ~90% of DC-positive wells in OP9 control cocultures for LDA (31 wells of 35 wells among four donors) exhibited coexistence of CD14+CD15+ monocyte and/or CD14+CD15+ granulocyte lineage cells (Supplemental Fig. 1). This result was confirmed by the presence of PB HPC clones producing both DCs and myeloid cells, but was not associated with lymphoid progenies, as shown below. As expected, generation of T/NK lineage cells was IL-7 dependent, but generation of DCs was IL-7 independent in the OP9-DL1 cocultures (Fig. 2B). It is noteworthy that generation of CD7+CD5- CD56+ cells (Supplemental Fig. 1) was Notch 1 dependent and IL-7 independent (Fig. 2). Because >50% of these CD7+CD5- CD56+ cells were found to express CD34 (Supplemental Fig. 1), this cell population should have comprised multipotential HPCs enriched in lymphoid progenitors at the stage prior to bifurcation into T and NK cell lineages. Single-cell methylcellulose colony assays confirmed that CD34+CD7+CD5- CD56+ cells retained monocyte–granulocyte potential but lacked erythroid potential (% CFU in total sorted cells, median [range], CFU-GM: 14% [7–27]; BFU-E: 0% [0–0] [number of donors = 3]), whereas CD34+Lin- CD7+ cell population retained both myeloid and erythroid potentials (CFU-GM: 20% [14–26]; BFU-E: 13% [9–25] [number of donors = 3]).

Characterization of DC, T cell, and NK cell lineage progenies

The major population of PB HPC-derived cDC progenies comprised CD1c+CD141- cells, and the minority was composed of CD1c-CD141+ or CD1c-CD141+ (Fig. 3A). In contrast, pDC progenies did not express CD1c at all, but did express CD303 and CD304. The surface phenotypes of both cDC and pDC progenies were similar to those of mature cDCs and pDCs in adult PB (33, 34), although expression levels of CD141 and CD304 in cDC and pDC progenies, respectively, were somewhat lower than PB mature DCs (Supplemental Fig. 2). cDCs isolated from OP9-DL1 cocultures were found to produce IL-6 with poly(I:C), TLR-3 ligand stimulation only after additional culture with GM-CSF (Fig. 3A). Similarly, pDCs showed R848 (TLR-7 and TLR-8 ligand)-stimulated IFN-α production that also required additional culture in the presence of IL-3. These results indicate that both DC lineage progenies in the present culture conditions are functionally immature and that their maturation is induced by appropriate cytokine signals.

We previously reported that nearly all CD7+5+ T lineage progenies produced cytoplasmic CD3ε but did not exhibit surface CD3 expression after a 5-wk coculture with OP9-DL1 (26). When the CD7+5+ cells were cultured with fresh OP9-DL1 cells for an additional 5 wk, ~10–50% of the cells expressed CD3 at the stages of CD4/CD8 double positive and CD4 single positive (Fig. 3B). The majority of surface CD3+ cells coexisting in the culture revealed immature CD4 single-positive or CD4/CD8 double-positive phenotypes, and the surface CD3+ T cells were found to express TCRβ or TCRγδ. These observations agreed with a previous report by other researchers describing T cell differentiation of human adult BM HPCs in OP9-DL1 coculture (35).

![PFs in HPC (%)](image)

<table>
<thead>
<tr>
<th>Cell Lineage</th>
<th>Median PF (Range) in HPCs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDCs</td>
<td>5.14 (1.57–9.12)</td>
</tr>
<tr>
<td>pDCs</td>
<td>1.85 (0.02–4.59)</td>
</tr>
<tr>
<td>T cell</td>
<td>3.13 (0.97–4.52)</td>
</tr>
<tr>
<td>NK cell</td>
<td>3.62 (2.44–6.45)</td>
</tr>
</tbody>
</table>

Table I. Frequencies of cDC and pDC precursors in PB and CB HPCs

PFs of each cell lineage were obtained for PB and CB samples from 20 adults (age range: 28–64) and 3 neonates, respectively, by LDA. Most T and NK cell PFs from adults were obtained in our previous study (26).

![FIGURE 4.](image)

Correlation of PF between DC and T or NK cell lineages in PB HPCs from healthy donors. Correlation of PF between cDC and T or NK cell lineages (upper graphs) and between pDC and T or NK cell lineages (lower graph) in PB from 20 donors (10 females, open circles; 10 males, closed circles; age range: 28–64). Spearman’s rank correlation coefficients (\( p \)) are shown in each panel with \( p \) values.
As also shown in our previous report (26), CD7+56+ NK lineage progenies expressed CD161 but not CD16, suggesting that these cells are similar to the thymic NK cells observed in PB (Supplemental Fig. 2). We confirmed this consideration by finding the expression of CD127 (Fig. 3B), which is known to be expressed in thymic NK cells but lacking in BM-derived CD16+ NK cells (36, 37).

**Correlation of PFs between cDC/pDC and T/NK cell lineages in PB HPCs**

We obtained the frequencies of cDC and pDC precursors in HPCs from PB (n = 20) and CB (n = 3) (Table I). Frequencies of cDC and pDC precursors in PB HPCs ranged from 1 to 10% and from 0.1 to 5% among 20 healthy donors, respectively. Differences between cDC and pDC PFs were highly significant (p = 8.8 × 10^{-5} in a paired Wilcoxon’s signed rank sum test). PFs of cDCs and pDCs in CB HPCs were much higher than those in PB HPCs (p = 0.006 and p = 0.006, respectively, in Mann–Whitney U tests). Similarly, T and NK PFs were 5- to 10-fold higher for CB HPCs than for PB HPCs (Table I). Because we previously showed that PB HPCs had levels of T and NK precursor activity similar to BM HPCs (26), the differences of both DC PFs between PB and BM HPCs were not significant (data not shown).

cDC PFs in adult PB HPCs were found to weakly but significantly correlate with T cell PFs (p = 0.53) (Fig. 4). Interestingly, pDC PFs showed strong correlation with T cell PFs (p = 0.75) for the same donors. In contrast, neither cDC nor pDC PFs showed any significant correlation with NK cell PF (Fig. 4). As expected, the correlation between cDC and pDC PFs was found to be significant (p = 0.63) (Fig. 4).

**Clonal analyses of DC/T/NK differentiation from a single PB HPC**

To examine whether the significant correlations between T cell and cDC or pDC PFs might historically be due to the high prevalence of common DC/T precursors, we performed clonal analyses of T, NK, cDC, pDC, and myeloid lineage progenies generated from a single HPC in PB and CB by flow cytometry (Supplemental Fig. 3). The number of progenies per well (clone) was distributed largely in both PB and CB (Supplemental Fig. 4), but cloning efficiency did not differ greatly among the four different progenitors (~60–70%). In that analysis, lymphoid precursors were classified as T/NK dual-, T single-, and NK single-lineage precursors based on the surface phenotype of their progenies (Table II), as described previously (26). DC precursors also were classified as cDC/pDC dual-, cDC single-, and pDC single-lineage precursors (Fig. 5A) in combination with T/NK classification (Fig. 5B, Table II). In addition, three other categories of the HPC clones that generated CD7+CD5+CD56− cells without the presence of CD7+CD5+ T or CD7+CD5+ CD56+ NK cell progenies (Supplemental Fig. 1), myeloid cells in association with no CD7+ lymphoid progenies, and DC progeny alone were included in the classification (Table II). Findings obtained from the clonal analyses are summarized below.

Nearly all pDC-producing clones, except for one CB clone, were found to be associated with cDC progenies, whereas ~60 and 40% of cDC producer clones were not associated with pDCs in PB or CB HPCs, respectively (Table II). Among PB HSC clones, the majority (~91%: 75 + 16) of pDC-producing clones (or cDC/pDC dual producers) were associated with lymphoid lineage, including CD7+CD5−CD56− cells. In contrast, only 64% (48 + 16) of cDC single-lineage clones revealed lymphoid association, and 29% of those were myeloid-associated DCs (Table II). Viewing DC potential data (Table II) from the lymphoid side, >80% of PB T/NK dual- and T single-precursor clones were associated with generation of DC progenies (Fig. 5C), whereas only ~20% of PB NK single-lineage producers were associated with DC progenies. Furthermore, distribution of relative prevalence of cDC and pDC association in CD7+CD5−CD56− clones did not differ from that in total T and/or NK cell clones (Fig. 5C), suggesting that DC commitment may have mainly been determined at the stage prior to T/NK bifurcation. Among CB, almost no NK single-lineage clones were observed (Fig. 5B, Table II), and nearly all T/NK dual- and T single-lineage clones produced DC progenies (Fig. 5C).

**Table II. Classification of T/NK cell and DC potential of PB and CB HPC clones**

<table>
<thead>
<tr>
<th>PB HPC Precursor Clones</th>
<th>Number of Clones Producing DCs</th>
</tr>
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<tbody>
<tr>
<td>T and NK</td>
<td>29^a (49%) 22^b (24%) 0 11 (10%) 62 (61%)</td>
</tr>
<tr>
<td>T</td>
<td>6^b (10%) 13^b (14%) 0 4 (3%) 23 (22%)</td>
</tr>
<tr>
<td>NK</td>
<td>9^c (15%) 10^c (11%) 0 67 (63%) 86 (82%)</td>
</tr>
<tr>
<td>T/NK subtotal</td>
<td>44^d (75%) 45^d (48%) 0 82 (76%) 171 (165%)</td>
</tr>
<tr>
<td>CD7+CD5+CD56−</td>
<td>9^e (16%) 15^e (16%) 0 11 (8%) 35 (32%)</td>
</tr>
<tr>
<td>Myeloid without CD7+ cells^e</td>
<td>5 (9%) 27 (29%) 0 184 216</td>
</tr>
<tr>
<td>DCs alone</td>
<td>0 8 (9%) 0 — 8</td>
</tr>
<tr>
<td>Total</td>
<td>58 (100%) 95 (100%) 0 277 430</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CB HPC Precursor Clones</th>
<th>Number of Clones Producing DCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>T and NK</td>
<td>134^f (92%) 63^f (71%) 0 3 (3%) 200 (200%)</td>
</tr>
<tr>
<td>T</td>
<td>7^g (5%) 16^g (18%) 1^g 13 (12%) 37 (36%)</td>
</tr>
<tr>
<td>NK</td>
<td>2^h (1%) 0 0 2 (2%) 2 (2%)</td>
</tr>
<tr>
<td>T/NK subtotal</td>
<td>143^i (99%) 79^i (89%) 1^i 16 (15%) 239 (238%)</td>
</tr>
<tr>
<td>CD7+CD5+CD56−</td>
<td>2^j (1%) 8^j (9%) 0 4 (4%) 14 (14%)</td>
</tr>
<tr>
<td>Myeloid without CD7+ cells^e</td>
<td>0 2 (2%) 0 26 28</td>
</tr>
<tr>
<td>DCs alone</td>
<td>0 0 0 — 0</td>
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<tr>
<td>Total</td>
<td>145 (100%) 89 (100%) 1 46 281</td>
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Progenies from 430 and 281 clones in PB and CB HPCs were obtained by single-cell sorting of CD34^Lin− cells from nine and three donors, respectively. The DC potential was classified as cDCs and/or pDCs in each T and/or NK precursor clone.

^aThe presence of myeloid cells including CD14+CD15+ monocytic and/or CD14+CD15+ granulocytic cells but not CD7+ lymphoid cells (Supplemental Fig. 3).

All clones producing both DCs and lymphoid cells gave rise to myeloid cells.

^Number of clones accompanying monocyte and/or granulocyte progenies in parentheses.
FIGURE 5. Analyses of cDC/pDC and T/NK lineage progenies derived from a single PB and CB HPCs. PB and CB HPCs from each donor were sorted into >280 wells with 384-well plates in OP9-DL1 cocultures at a frequency of one cell per well. After 5-wk culture, wells exhibiting cell growth were analyzed for surface phenotypes of cDC/pDC and T/NK lineages. (A) Representative flow cytograms of CD11c and CD123 expressions in HLA-DR+Lin- gated progenies from a single CD34+Lin- cell. Each precursor was classified as cDC/pDC dual lineage (upper panel) or cDC single lineage (lower panel). (B) Different distributions of precursor clones producing T and/or NK lineage progenies between PB and CB CD34+Lin- cells. Classifications of T/NK dual-lineage, T single-lineage, and NK single-lineage progenies were noted in the previous report (26). Distribution of each lineage is expressed as the relative prevalence calculated from the data listed in Table II. The difference in distribution of the T/NK precursor clones between PB and CB was highly significant by χ2 test based on the number of clones (p < 1 × 10−5). (C) Classification of precursor clones producing cDC/pDC dual- or pDC single-lineage in combination with T/NK dual-lineage, T single-lineage, NK single-lineage, and CD7+CD5+CD56− phenotypes in PB and CB HPCs. Distribution of each lineage is expressed as a relative prevalence obtained from the data listed in Table II. The difference in distribution of the cDC/pDC precursor clones between T/NK dual- and NK single-lineage is highly significant by χ2 test (p < 1 × 10−6). The difference in distribution of the cDC/pDC precursor clones in association with T/NK lineage between PB and CB was highly significant by χ2 test (p < 1 × 10−6). Different distributions of the cDC/pDC precursor clones in association with T/NK lineage and NK single-lineage did not differ from that with T and/or NK phenotype in PB HPCs (p = 0.11).

More than 95% of T and/or NK clones from both PB and CB were associated with monocytes and/or granulocytes (PB: 165/171; CB: 238/239) (Table II), suggesting that the majority of T/NK lineage clones are derived from LMPPs or more upstream HPCs, as described previously (26). It is noteworthy that all the clones producing both T/NK and DC progenies gave rise to myeloid cells (PB: 89/89; CB: 223/223) (Table II).

DC/T/NK potentials in different PB HPC subpopulations

To more precisely assess which subset of PB HPC is an origin of cDC, pDC, T, and NK cell precursors detected by OP9-DL1 co-culture, we conducted LDA for cKit+CD7+, cKit+CD10+, and cKit−CD10− subsets in addition to the total cKit+ and cKit− subsets among a PB HPC population (Fig. 6A). The proportions of total cKit+ and cKit− cells in HPCs from three donors were ~60% (median: 55.9; range: 55.5–63.2) and 40% (median: 37.7; range: 27.4–38.3), respectively. Because PFs of all the four different lineages in the total cKit+ population were ~5-fold higher than the PFs of cKit−, nearly 90% of each lineage precursor is thought to exist in the cKit+ population. CD7+ HPCs in both cKit+ and cKit− subsets decreased considerably but remained at significant levels. In both subsets, pDC potential was very low or absent. cKit−CD10+ cells, which may be a human counterpart of the murine CLP, showed relatively high lymphoid potentials among cKit− HPC populations, but very low DC potentials. It is noteworthy that both cKit+CD7+ and cKit−CD10− subsets retained myeloid potentials by single-cell analyses, as shown below. Furthermore, few CD127+ cells were detected in any PB HPC subsets (Fig. 6A).

Interestingly, single-cell analyses of CD34+CD7+ and CD34+CD10− progenitors showed that >20% T/NK dual- and T single-lineage precursors were associated with generation of DC progenies, whereas only 4% NK single-lineage precursors were associated with DC progenies (Table III). Moreover, only ~30% of T- and/or NK-producing clones were accompanied by myeloid cells, whereas all the clones producing both T cells and cDCs gave rise to myeloid cells (Table III).

We also examined CD127 expression in progenitor subpopulations generated in vitro from PB HPCs by coculture with OP9-DL1 cells. During 3–4 wk of coculture, PB HPCs were found to generate and retain CD34+CD7+, CD34+CD7−, and CD34−CD10− cell populations (Fig. 6B). As noted with in vivo HPCs, in vitro generated CD34+CD7+ and CD34+CD7− cells both lacked CD127 expression. The CD34+CD7− cell population mainly contains CD5+ T and CD56− NK lineage-committed progenies expressing CD127.
Discussion

In the current study, we developed assays for the functional enumeration and characterization of rare PB cDC/pDC precursors, using cell sorting–based LDA and clonal analyses. Because the conditions of coculture with OP9-DL1 stroma cells in the presence of KL, FL, and IL-7 are identical to the precursor assay for T/NK cells (26), we were able to analyze the relationship between cDC/pDC and T/NK cell commitment at the single-cell level. Using these assays, we tested our hypothesis that the proportion of DC to T cell precursors circulating in PB is maintained through linkage of lineage commitments. To determine the lineage commitment, we used surface phenotyping of the progenies generated in culture. Although the progenies in each lineage, as defined by their surface phenotypes, remained in an immature state, we demonstrated that these progenies could mature in appropriate conditions. cDC and pDC progenies did not produce TLR agonist-stimulated cytokines, but did mature into IL-6– or IFN-α–producing cells by culturing with GM-CSF or IL-3, respectively. Similarly, NK lineage progenies were immature but became NKG2A–, granzyme B–, and IFN-γ–expressing cells after short-term culture with IL-15, as seen in our previous study (26). T-lineage progenies also exhibited differentiation up to the surface TCR+ cell stage after prolonged culture with OP9-DL1 cells. Thus, lineage classification by flow analyses of surface phenotypes is apparently valid for testing the hypothesis.

LDA showed that the frequencies of DC precursors in PB HPCs are significantly correlated with the frequencies of T cell precursors, but not with those of NK cell precursors. This finding was corroborated by results obtained from single-cell analyses of HPCs, as follows: >80% PB T/NK dual- and T single-lineage clones coproduced cDCs and/or pDCs, whereas only ~20% PB NK single-lineage producers were associated with DC differentiation. Furthermore, the difference of correlation coefficients with T cell PF between cDC and pDC precursors (r = 0.53 and 0.75, respectively) can also be explained by the results of clonal analyses, as follows: ~60% precursor clones producing pDC were associated with T cell progenies, whereas less than half of cDC-producing clones were associated with T cell commitment. In addition, a larger prevalence of myeloid cell–associated cDC single-lineage clone is thought to contribute to the lower correlation coefficient between T and cDC PF.

Interestingly, clonal analyses of PB HPCs showed that almost all pDC-producing clones were associated with cDC progenies; these findings strongly suggest that HPC-derived pDC are generated via CDPs. This result agrees with that of mouse CDPs, which at clonal levels give rise to cDCs and pDCs in FL-supplemented cultures (38, 39). Therefore, although we did not directly examine the PF of CDPs for the LDA in the current study, it can be inferred that the PF of human putative CDPs also correlates with that of T cells. LDA found that CD34+CD127+ cells had only low pDC potential, irrespective of their cKit expression levels, indicating that, in

**FIGURE 6.** Frequencies of cDC, pDC, T, and NK cell precursors in different subsets of PB HPCs and CD127 expression in subsets of in vitro generated HPCs. (A) Representative flow cytograms and gating strategy for sorting cKithigh, cKitlow, cKitlowCD7+, cKitlowCD10+ subsets of PB HPCs and for CD127 expression in CD7+ PB HPCs (upper panels). PFs of cDCs, pDCs, T cells, and NK cells were obtained by LDA of each HPC subset using OP9-DL1 coculture (lower panel). (B) Flow cytometric analyses of CD127 expression (upper) in CD34+CD7+, CD34-CD7+, and CD34+CD7- cells generated in 4-wk bulk culture of PB HPCs. CD5 and CD56 expressions were analyzed for the three HPC subsets (lower).
Table III. Classification of T/NK cell and DC potential in CD34+CD7+ and CD34+CD10+ clones

<table>
<thead>
<tr>
<th>In Vivo CD34+CD7+ and CD34+CD10+ Clones</th>
<th>Number of Clones Producing DCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cDCs and pDCs</td>
</tr>
<tr>
<td>T and NK</td>
<td>0</td>
</tr>
<tr>
<td>T</td>
<td>0</td>
</tr>
<tr>
<td>NK</td>
<td>2 (1)</td>
</tr>
<tr>
<td>T/NK subtotal</td>
<td>2 (1)</td>
</tr>
<tr>
<td>CD7+CD5−CD56−</td>
<td>0</td>
</tr>
<tr>
<td>Myeloid without CD7+ cells</td>
<td>0</td>
</tr>
<tr>
<td>DCs alone</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
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</table>

CD34+Lin− CD7− or CD34+Lin− CD10− were sorted from five donors at single cell per well and cultured with OP9-DL1 cells for 4–5 wk. The presence of T/NK/DC progenies was analyzed and classified, as noted in Table II. The difference in distribution of total DC producer clones was significant by χ² test (Yates’ correction) (p = 0.002), based on the number of clones between the total T lineage (T/NK dual-lineage plus T single-lineage phenotypes) and NK single-lineage phenotype (T: 8 producer clones versus 25 nonproducer clones; NK: 3 producer clones versus 79 nonproducer clones).

*Number of clones accompanying monocyte and/or granulocyte progenies in parentheses.

conjunction with the expression of CD7, HPCs lose CDP potentials. Thus, the lineage split between the majority of putative CDPs and T/NK precursors occurs prior to CD7 expression. Similar findings on divarication of DC/T-NK lineages have been reported by the analysis of progenies differentiated from human fetal liver CD34+CD38− cells in mouse fetal thymus organ culture (19), although cDCs and pDCs were not discriminated in these studies.

It is important to note that clonal analyses of PB HPCs showed that all clones producing both DC and T cell progenies were accompanied by granulocyte and/or monocyte progenies. Similar results have been obtained from clonal analyses of CD7+ or CD10+ HPCs. These findings lead to our inference that DC progenies of T/DC clones may be generated via common granulocyte–monocyte progenitors (GMPs). If this is the case, GMPs give rise to the above-mentioned human putative CDPs via macrophage–DC progenitors according to the well-accepted model of the myeloid DC differentiation pathway (3, 12, 13), as discussed below.

Although data are not shown in the current study, human PB HPCs were found to retain B cell potential at very low levels in comparison with T/NK cell potentials (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). Furthermore, because PB-derived CD34+CD7+ and cKit+CD10− cells have T and/or NK cell potentials, but only low levels of B cell potential, these HPC subpopulations are thought to mainly consist of T/NK-oriented lymphoid progenitors. These progenitor populations may be the human PB counterpart of murine PB CLPs (40), based on their expression of CD7 or CD10, but human PB CD7+ or CD10+ progenitors in the current study did not express CD127, which is detected in murine CLPs. Nevertheless, both human PB CD7+ or CD10+ progenitors and murine PB CLPs (40) retained DC and myeloid potential in vitro at low but significant levels.

CB HPCs have a high potential for both T/NK and cDCs/pDCs in comparison with adult PB HPCs. This may be related to extensive development of the thymus required for the establishment of a total immune system in the early stages of human life. Nearly all T/NK cell progenitor clones were T/NK dual- or T single-lineage producers, whereas only 1% of T/NK lineage clones were NK single lineage, which is in contrast to the proportions of NK single-lineage producers in adult PB-derived clones. More than 90% of CB T and/or NK cell progenitor clones gave rise to cDCs and/or pDCs. Therefore, these findings are in accord with the linkage between DC and T cell commitments. As observed in PB HPCs, all CB HPC clones producing both T cells and DCs also gave rise to myeloid cells.

Taking account all the findings obtained in the current study, we propose a model for differentiation pathways and linkage of cDC/pDC/T/NK cell lineages from human PB HPCs (Fig. 7). PB CD34+Lin−CD7− cells expressing high levels of cKit give rise to both cDCs and pDCs, most probably via GMPs/CDPs, as mentioned above. Because this population also produces CD34+CD7+CD5−CD56− cells, a GMP-CDP/CD34+CD7+ split occurs mainly at the CD34+CD7− stage. CD34+CD7− cells retain T and/or NK cell potentials as well as myeloid/DC potential at a low but significant level, indicating that CD34+CD7− cells branch off into T/NK lineage cells and GMPs/CDPs. Because CD34+Lin−CD7− cells retain both lymphoid and myeloid potential, we define these cells as CD7−LMPPs in the model. Our model agrees with recent findings that all subtypes of murine thymic DCs, including CD11b+cDCs, CD8+cDCs, and pDCs, are derived from myeloid
precursors (4, 16) or from precursors with no history of CD127 expression (17). All PB HPC clones producing both T cell and DC progenies gave rise to myeloid cells, so DC precursors linked with T cell commitment may differentiate to cDCs/pDCs through a pathway common to myeloid DC differentiation, as shown in Fig. 7. Our model does not necessarily assume that T lineage–linked DC commitment and differentiation occur in the thymus: DCs generated from T-linked myeloid progenitors in BM may migrate to thymus. Furthermore, no PB HPCs, including CD7<sup>-</sup> LMPPs, expressed CD127 at a significant level. Neither cDC nor pDC generation required IL-7 in OP9-DL1 coculture.

In the present culture conditions, Notch 1 is required for differentiation of T and NK lineage cells from PB HPCs (Fig. 7). Both processes, from CD34<sup>+</sup>CD7<sup>-</sup> to CD34<sup>+</sup>CD7<sup>+</sup> and from CD34<sup>+</sup>CD7<sup>-</sup> to T or NK cell lineage, were found to be Notch dependent. In the absence of Notch 1, nearly all CD34<sup>+</sup> cells are committed to myeloid lineages, including monocytes, granulocytes, and DCs. It is well known that Notch 1 signaling is essential for generation of mature naïve T cells in the mouse thymus (41), although the precise role of Notch 1 in thymic DC commitment in vivo is somewhat controversial (42). An essential role for Notch signaling in DC development has been demonstrated only for splenic and small intestine CD11b<sup>+</sup> cDCs using mice lacking RBP-J gene transcript (43) and Notch 2 receptor (44), respectively. Conversely, deletion of the Notch 1 gene converted pro-T cells to cDCs and pDCs in the thymus (45). For humans, it has been reported that Delta-1 stimulated pDC differentiation from CB and BM CD34<sup>+</sup> cells (27). We also demonstrated that OP9-DL1 cells promoted differentiation of cDCs, in addition to pDCs, from PB and CB HPCs, but the role of Notch 1 signaling in human DC development in vivo is still unknown. We assume that T cell commitment of DC-linked T cell progenitors may essentially require Notch signaling, but DC commitment of these progenitors might be indirectly induced by crosstalk between myeloid DC-inducing signal and Notch 1 downstream signal. If this is the case, DC-linked T cell progenitors might differentiate to DC lineage by myeloid DC-inducing signal in the absence of Notch 1 stimulation. In our previous study, Notch 1 signaling induced thymic NK cell commitment much more effectively than IL-15 did in culture, although IL-15 can induce NK cell commitment in the absence of Notch 1 and it is essential for functional maturation of thymic NK cells (26).

As discussed above, the strong correlation of PFs between T cell and pDC or putative CDP suggests a linkage between T cell and DC commitments (Fig. 7). The high prevalence of HPC clones retaining both T and DC potentials also supports this assumption. If our model of the differentiation pathway (Fig. 7) is correct, this linkage is primed in progenitors at the stage of CD34<sup>+</sup>Lin<sup>-</sup> cKit<sup>high</sup> CD7<sup>-</sup> population. In other words, the dichotomy fate of downstream T/NK bipotent progenitor is determined by the upstream event of DC/T-NK split. If a T/NK bipotent progenitor experiences the DC commitment at the stage of DC/T-NK split, the bipotent progenitor will be directed to T cell lineage with high probability. It is important to note that analyses of HPC clones generated from PB CD34<sup>+</sup>Lin<sup>-</sup>CD7<sup>-</sup> or CD10<sup>+</sup> showed that DC progenies generated from these progenitors are more frequently associated with T/NK dual or T single-lineage precursor clones than with NK single-lineage clones. These findings support the concept that linkage between T and DC commitment is retained at the downstream CD34<sup>+</sup>CD7<sup>-</sup> or CD10<sup>+</sup> stage (Fig. 7). In contrast, commitment of human PB HPCs to thymic NK cell lineage is thought to be independent of the T/DC linkage (Fig. 7). In this context, our previous study showed that the ratios of T to NK cell PFs varied among individuals but were fairly constant in each individual for as long as 6 mo (26). This observation suggests that the bifurcation of coprogenitors to T and/or NK cell lineages is not stochastic, but is presumed to be also primed in HPCs at some upstream stage.

Molecular mechanisms involved in the linkage between T and DC lineage commitments remain to be determined. Transcription networks for commitment of each lineage might be cross-linked by common transcription factors between the two lineages, and we think that the linkage signal might be intrinsically imprinted in long-lived HSCs, but not in CLPs, LMPPs, or multipotent progenitors with transient life spans, because such linkages would be stably integrated in the long-term throughout human life. Extrinsic signals from niche including cytokines may be instructive for DC development (29), but they also seem to be transient. Recent mouse studies using a barcoding technique for a single LMPP suggested that the fate of the progenitors is imprinted in an early stage of hematopoiesis (9). Thus, intrinsic signals linking the two lineages may be generated in HSCs and propagated through intermediate stages to a tail branch on the differentiation tree.

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Disclosures
The authors have no financial conflicts of interest.

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