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Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development

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The classical model of hematopoiesis posits the segregation of lymphoid and myeloid lineages as the earliest fate decision. The validity of this model in the mouse has been questioned; however, little is known about the lineage potential of human progenitors. Here we provide a comprehensive analysis of the human hematopoietic hierarchy by clonally mapping the developmental potential of seven progenitor classes from neonatal cord blood and adult bone marrow. Human multilymphoid progenitors, identified as a distinct population of Thy-1$^{neg–lo}$CD45RA$^+$ cells in the CD34$^+$CD38$^-$ stem cell compartment, gave rise to all lymphoid cell types, as well as monocytes, macrophages and dendritic cells, which indicated that these myeloid lineages arise in early lymphoid lineage specification. Thus, as in the mouse, human hematopoiesis does not follow a rigid model of myeloid-lymphoid segregation.

Blood and other highly regenerative tissues are organized as cellular hierarchies derived from multipotent stem cells. Mouse hematopoietic stem cells (HSCs) are defined as lineage-negative (Lin$^-$), Sca-1$^+$, c-Kit$^+$ and CD150$^+$ cells that lack expression of CD135 (Flt3) and CD34, whereas in humans, the Lin$^-$CD34$^+$CD38$^-$ compartment is enriched for HSCs[1,2]. As HSCs differentiate, they give rise to progenitor cells that undergo lineage commitment to one of ten distinct blood lineages. The popular classical model of hematopoiesis postulates that the earliest fate decision downstream of HSCs is the divergence of lymphoid and myeloid lineages that gives rise to common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs)[3,4]. However, clonal analysis has shown that most Lin$^-$Sca-1$^+$c-Kit$^+$Flt3$^+$ lymphoid-primed multipotent progenitors lack erythroid and megakaryocytic potential, which indicates that these lineages branch off before the lymphoid-myeloid split[5–7]. The classical model also predicts that CLPs are the source of all lymphoid cells and that their progeny lack myeloid lineage potential. In contrast, several lymphoid progenitors have been isolated that are able to give rise to B cells, T cells and natural killer (NK) cells. These include Lin$^-$Sca-1$^+$c-Kit$^+$Flt3^{hi}VCAM-1$^-$ lymphoid-primed multipotent progenitors[7], c-Kit^{hi} early lymphoid progenitors expressing recombination-activating gene 1 (ref. 8) and c-Kit$^-$B220$^+$ CLP-2 progenitors expressing the gene encoding the pre-T cell antigen receptor-α[9]. Furthermore, extensive investigation of multilineage outcomes in mouse fetal liver has shown that myeloid output is retained during lymphoid specification[10,11], a finding confirmed by clonal analysis of c-Kit$^+$CD25$^-$ earliest thymic progenitors[12,13]. According to the classical model, during T cell commitment, CLPs first undergo myeloid restriction, followed by the loss of B cell potential. However, the earliest thymic progenitors have been shown to retain myeloid potential but not B cell potential in stromal cocultures and to contribute extensively to thymic granulocyte and macrophage populations[12,13]. Thus, lymphoid development in the mouse seems to be a gradual process marked by several progenitor intermediates that differ in the extent of their lymphoid restriction and retention of myeloid potential[14,15]. There is increasing consensus for revision of the classical model to account for this evidence.

In contrast to mouse hematopoiesis, definitive evidence for a comprehensive model that best describes human hematopoiesis is lacking. Progress has been limited by the paucity of cell surface markers to distinguish pure populations and the absence of assays to detect multilineage outputs from single cells with high cloning efficiency. Human CMPs have been isolated as CD34$^+$CD38$^+$IL-3Rα$^+$CD45RA$^-$ cells from adult bone marrow, but their lineage potential at the clonal level was evaluated only by colony assay[16]. The earliest steps of human lymphoid development remain poorly understood. Human CLPs were first isolated from bone marrow as Lin$^-$CD34$^+$CD10$^+$ cells, only ~3% of which gave rise to B cells and NK cells, but not myeloid or erythroid progeny, in clonal plating on stromal cocultures[17]. Further separation of this population into CD24$^+$ and CD24$^-$ cells has shown that all CLP potential reside in the CD34$^+$CD10$^+$ CD24$^-$ fraction in neonatal cord blood and bone marrow, but the cloning efficiency remains below 5% (ref. 18). Other reports have suggested that at least in cord blood, CLPs

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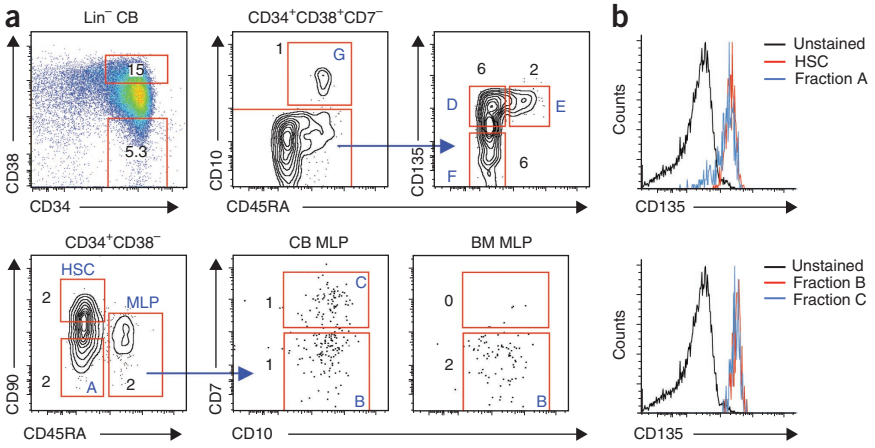

Figure 1 Sorting of human progenitors. (**a**) Sorting of cord blood (CB) mononuclear Lin⁻ cells stained with anti-CD34, anti-CD38, anti-CD90 (anti-Thy-1), anti-CD135 (anti-Flt3), anti-CD45RA, anti-CD7 and anti-CD10; fractions are labeled A–G as in **Table 1**. Top, $CD34^+CD38^{pos–hi}$ population ($CD7^+$ cells gated out; data not shown), gated on CD135, CD45RA and CD10 to separate $CD135^+CD45RA^-$ CMPs (fraction D), $CD135^+CD45RA^+CD10^-$ GMPs (fraction E), $CD135^-CD45RA^-$ MEPs (fraction F) and $CD135^+CD45RA^+CD10^+$ pre-B cells–NK cells (fraction G). Bottom, separation of the $CD34^+CD38^-$ compartment on the basis of expression of CD90 and CD45RA, to distinguish $CD90^+CD45RA^-$ HSCs, $CD90^-CD45RA^-$ MPPs (fraction A), and $CD90^{neg–lo}CD45RA^+$ MLPs; the $CD90^{neg–lo}CD45RA^+$ fraction was further subgated on the basis of expression of CD7 and CD10 (fractions B and C). Bottom right, Lin⁻ bone marrow (BM). Numbers in or adjacent to outlined areas indicate percent Lin⁻ cells in each gate. (**b**) CD135 (Flt3) expression by human HSCs, MPPs (fraction A) and MLPs (fractions B and C). Data are representative of at least 20 independent experiments.

are $CD7^+$ rather than $CD10^+$, and that they reside in the $CD34^+CD38^-$ fraction (cloning efficiency, <5%)[19,20]. These studies failed to detect myeloid potential in the candidate CLP fractions, which led to the assumption that the classical model best describes human hematopoiesis. The existence of at least some cells with multilymphoid progenitor (MLP) potential, defined as any progenitor minimally able to give rise to B cells, T cells and NK cells, in the sorted populations is thus established. However, given the low cloning efficiencies and the absence of single-cell analysis, the lineage potential of rare human MLPs in these fractions cannot be assessed conclusively.

We isolated seven distinct progenitor classes from cord blood and bone marrow samples on the basis of a single panel of seven markers and investigated their developmental potential by clonal analysis under conditions that provided robust support of multiple lineage fates. By assembling such a comprehensive 'road map', we identified human MLPs as a distinct $Thy\text{-}1^{neg–lo}CD45RA^+$ population in the $CD34^+CD38^-$ HSC compartment. We found that MLPs generated all lymphoid cell types, as well as monocytes, macrophages and dendritic cells (DCs), which prompted a revision to the model by which human blood lineages are specified from HSCs.

RESULTS

Clonal assays of human hematopoiesis

To investigate the composition of the human progenitor hierarchy, we used flow cytometry sorting to isolate progenitor ($CD34^+$) fractions on the basis of expression of the markers CD45RA, CD135 (Flt3), CD7, CD10, CD38 and CD90 (Thy-1). Our studies established that this combination of markers provided meaningful separation of human progenitors into functionally distinct subsets. Because age-related developmental changes may affect the composition of the progenitor compartment, we isolated progenitors from neonatal cord blood, which contains a mixture of fetal and adult cells, as well as adult bone marrow. Staining of Lin⁻ or $CD34^+$ cell–enriched samples with this marker panel identified seven distinct progenitor fractions (A–G) in addition to $CD34^+CD38^-Thy\text{-}1^+CD45RA^-$ HSCs (**Fig. 1** and **Table 1**). We were also able to resolve these populations in unfractionated bone marrow and cord blood, which made this panel more suitable for smaller samples or diagnostic applications (**Supplementary Fig. 1**).

The shortcomings of previous approaches were due in part to the lack of assays to efficiently detect the lymphoid and myeloid lineages of single human cells. MS-5 mouse stromal cells support the development of human myeloid, B cell, NK cell and mixed lymphomyeloid colonies in the presence of stem cell factor (SCF), thrombopoietin (TPO), interleukin 7 (IL-7) and IL-2 (ref. 21). Single $CD34^+CD38^-Thy\text{-}1^-CD45RA^-$ cord blood cells proposed to be human multipotent progenitors (MPPs)[22] seeded in these conditions gave rise to all seven possible colony types with a high cloning efficiency (**Fig. 2a**, fraction A; cloning efficiency, 45%). In addition, we used assays with OP9-DL1 stromal cells to detect T cell potential[23] and conventional colony-forming unit (CFU) assays for myeloid and erythroid lineages. Of note, MPPs showed lower efficiency in OP9-DL1 assays, probably because of Notch-mediated inhibition of differentiation[24,25], but they had T cell potential *in vivo* (F.N. *et al.*, unpublished observations). Evidence of the lineage fate potential of any purified population is definitive only when assessment is done at the single-cell level. Thus, we used limiting-dilution analysis or deposition of single cells (single-cell sorting efficiency, **Supplementary Fig. 2**), which resulted in similar estimates of clonogenic potential (**Fig. 2b** and **Supplementary Fig. 3**); this provided the basis for a precise clonal 'readout' of lineage potential.

Table 1 Characterization of progenitor fractions sorted from cord blood and bone marrow

Fraction	Phenotype	Group name	Frequency (% MNC)	Lineage output
–	$CD34^+CD38^-Thy\text{-}1^+CD45RA^-Flt3^+CD7^-CD10^-$	HSC	0.04	All[a]
A	$CD34^+CD38^-Thy\text{-}1^-CD45RA^-Flt3^+CD7^-CD10^-$	MPP	0.04	All[a]
B	$CD34^+CD38^-Thy\text{-}1^{neg–lo}CD45RA^+Flt3^+CD7^-CD10^+$	$MLP7^-$	0.01	B, T, NK, MDC
C	$CD34^+CD38^-Thy\text{-}1^{neg–lo}CD45RA^+Flt3^+CD7^+CD10^+$	$MLP7^+$	0.01	B, T, NK, MDC
D	$CD34^+CD38^+Thy\text{-}1^-CD45RA^-Flt3^+CD7^-CD10^-$	CMP	0.15	EMK, G, MDC
E	$CD34^+CD38^+Thy\text{-}1^-CD45RA^+Flt3^+CD7^-CD10^-$	GMP	0.05	G, MDC
F	$CD34^+CD38^+Thy\text{-}1^-CD45RA^-Flt3^-CD7^-CD10^-$	MEP	0.30	EMK
G	$CD34^+CD38^+Thy\text{-}1^-CD45RA^+Flt3^+CD7^-CD10^+$	B-NK	0.05	B or NK

Frequency (percent of cord blood mononuclear cells (% MNC)) and lineage output of progenitor fractions (A–G) sorted from cord blood and bone marrow, based on seven-parameter flow cytometry with various combinations of cell surface markers (column 2; **Fig. 1**). EMK, erythroid and megakaryocyte.
[a]Multipotency of HSC and MPP fractions demonstrated *in vivo* (F.N. *et al.*, unpublished observations).

Human myeloid progenitors

In our analysis of lineage potential on MS-5 stroma, progenitor fractions D and E (**Table 1**) gave rise exclusively to myeloid colonies but

not B cell or NK cell colonies (**Fig. 2b**,**c** and **Supplementary Table 1**) with cloning efficiency ranging from 54% (fraction D, bone marrow) to 44% (fraction E, cord blood) or 29% (fraction D, cord blood, and fraction E, bone marrow). With the exception of fraction E from cord blood, these cells had no T cell potential (**Fig. 2d**). Both fraction D and fraction E gave rise to myeloid colonies in colony-forming cell assays, and fraction D also generated erythroid and myelo-erythroid colonies, consistent with common progenitor of myeloid lineages (CMP; **Fig. 2e**). In contrast, we never observed erythroid colonies from cells of fraction E, consistent with a more restricted progenitor of granulocyte and monocyte lineages (granulocyte and monocyte progenitor (GMP); **Fig. 2e**). It is unclear why GMPs in cord blood had substantial T cell potential; however, a similar finding has been reported for mice[26]. CMPs from cord blood, but not those from bone marrow, had serial replating potential, albeit with a lower capacity than that of multipotent cells (**Supplementary Fig. 4**). In contrast to the Flt3$^+$ fractions, cells in fraction F produced no colonies in the MS-5 or OP9-DL1 assay (**Fig. 2b–d** and **Supplementary Table 1**) but gave

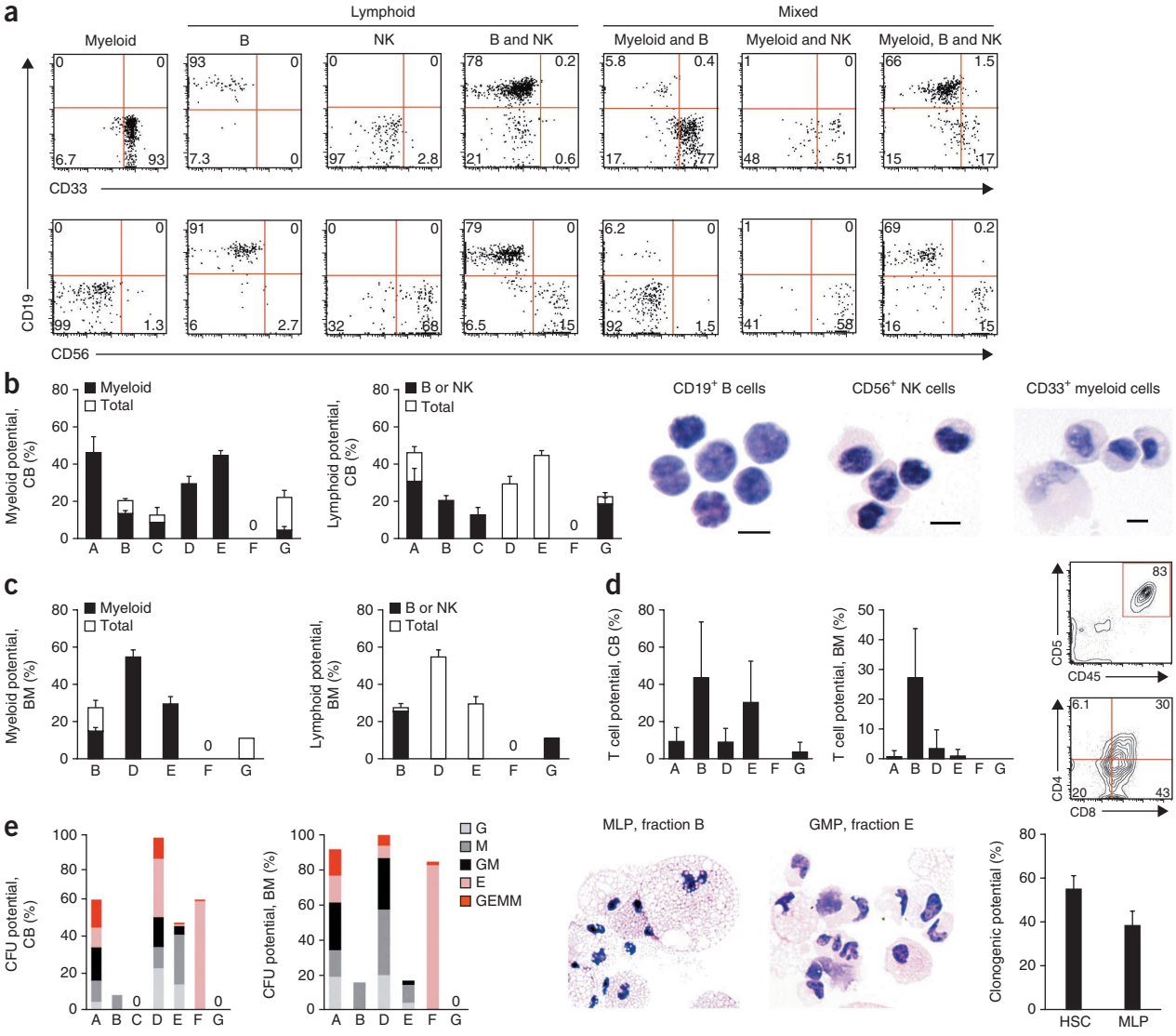

Figure 2 Clonal analysis of candidate cord blood and bone marrow progenitor fractions. (**a**) Flow cytometry analysis of the multilineage output of individual wells seeded with single cord blood MPPs (fraction A) and cultured for 4 weeks on MS-5 stroma with SCF, TPO, IL-7 and IL-2; only CD45$^+$ events are presented. Numbers in quadrants indicate percent cells in each. (**b**,**c**) Cloning efficiency of myeloid lineages (left) and lymphoid lineages (right) of single cord blood progenitors (**b**) or bone marrow progenitors (**c**) deposited onto the MS-5 stroma by flow cytometry sorting (fractions A–G, horizontal axes). Bars indicate total cloning efficiency; filled portion indicates the proportion of myeloid potential (myeloid plus mixed colonies) or lymphoid potential (lymphoid plus mixed colonies). Far right (**b**), morphology of cells (fraction B) isolated from single wells confirms lineage assignment. Scale bars, 10 μm. (**d**) T cell potential of cord blood progenitors (left; 8 weeks) or bone marrow progenitors (middle; 4 weeks) seeded at limiting dilution on OP9-DL1 stroma. Results are presented as limiting-dilution frequency (error bars, upper limits of 95% confidence interval). Right, marker profiles of T cells cultured on OP9-DL1 stroma; numbers in outlined areas (top) indicate percent CD5$^+$CD45$^+$ cells, and numbers in quadrants (bottom) indicate percent cells in each. (**e**) CFU assay (left) of the colony-forming efficiency of myeloid and erythroid lineages of single cord blood and bone marrow progenitors deposited by flow sorting. G, granulocytic; M, macrophage; GM, mixed myeloid; E, erythroid; GEMM, myelo-erythroid. Middle, Giemsa stain of MLP and GMP colonies. Original magnification, ×100. Right, colony-forming efficiency of cord blood MLPs and HSCs cultured for 4 d on OP9 stroma. Data are representative of three independent experiments (**a**) or four (**b**) or two (**c**–**e**) experiments with independent cord blood samples and over 12 wells for each fraction per experiment (mean and s.e.m., **b**,**c**,**e**).

Table 2 Limiting-dilution analysis of human MLP fractions

Cells per well	Wells (*n*)	Positive wells	Phenotype: Myeloid	Phenotype: Lymphoid (B, N, BN)	Phenotype: Myelo-lymphoid (MB, MN, MBN)
Fraction B ($CD34^+CD38^-Thy\text{-}1^-CD45RA^+CD10^+CD7^-$), cord blood					
4	12	9 (75%)	1	4 (0, 1, 3)	4 (0, 1, 3)
2	36	14 (39%)	0	7 (1, 4, 2)	7 (4, 1, 2)
1	96	18 (19%)	0	6 (2, 2, 2)	12 (2, 3, 7)
Total	144	41	1	**17 (43%)**	**23 (57%)**
			1.0 myeloid: 1.1 B cell: 1.3 NK cell		
Fraction C ($CD34^+CD38^-Thy\text{-}1^-CD45RA^+CD10^+CD7^+$), cord blood					
5	24	10 (42%)	0	4 (0, 1, 3)	6 (1, 0, 5)
2	24	4 (17%)	0	4 (2, 1, 1)	0 (0, 0, 0)
1	36	4 (11%)	0	1 (0,0,1)	3 (0,1,2)
Total	84	18	0	**9 (50%)**	**9 (50%)**
			1.0 myeloid: 1.7 B cell: 1.7 NK cell		
Fraction B ($CD34^+CD38^-Thy\text{-}1^-CD45RA^+CD10^+CD7^-$), bone marrow					
4	24	15 (58%)	1	8 (2,3,3)	6 (3,0,3)
1	48	13 (27%)	1	6 (2,4,0)	6 (0,4,2)
Total	72	28	2	**14 (50%)**	**12 (43%)**
			1.0 myeloid: 1.1 B cell: 1.4 NK cell		

Limiting-dilution analysis of candidate human MLP fractions B and C isolated from cord blood and bone marrow (fraction C is not found in bone marrow) and deposited by flow sorting into individual wells with MS-5 stroma, then cultured for 4 weeks with SCF, TPO, IL-7 and IL-2; myeloid, lymphoid or myelo-lymphoid colonies of seven different subtypes (**Fig. 2a**) were identified with a panel of lineage markers. Bolding indicates colony types representing >90% of total output for each fraction (probable lineage output); 'Positive wells' indicates wells containing human cells; phenotype indicates wells containing cells of myeloid, lymphoid or myelo-lymphoid lineage (colony types in parentheses). MB, myeloid and B cell; MN, myeloid and NK cell; MBN, myeloid, B cell and NK cell. Ratios of lineage output (bottom row of each fraction): myeloid = M + MB + MN + MBN colonies; B cell = B + BN + MB + MBN colonies; NK cell = N + BN + MN + MBN colonies. Data are pooled from two or more independent experiments with 12 or more wells per cell dose per fraction each.

rise to erythroid colonies in CFU assays, with no detectable myeloid potential, consistent with a restricted megakaryocytic and erythroid progenitor (MEP; **Fig. 2e**). These results establish the identity of key myeloid progenitor types from both neonatal and adult sources and indicate that myeloid commitment in human hematopoiesis proceeds along a developmental path consistent with the classical model.

Human MLPs

Published reports of human MLPs with B cell, T cell and NK cell potential have placed them in the $CD10^+CD24^-$ or $CD38^-CD7^+$ fraction[17,19]. To refine that analysis, we determined the lineage potential of progenitor fractions expressing the lymphoid markers CD7 or CD10. CD10 was expressed by a subset of $CD34^+CD38^+$ cells (fraction G) and a distinct fraction of Thy-$1^{neg–lo}CD45RA^+$ cells in the $CD34^+CD38^-$ stem cell compartment (**Fig. 1** and **Table 1**). Cells in fraction G gave rise to B cell and NK cell colonies on MS-5 stroma, with a bias for the NK cell lineage, and lacked appreciable myeloid potential in CFU assays (**Fig. 2b**,**c**,**e** and **Supplementary Table 2**; cloning efficiency, 24% (cord blood) or 13% (bone marrow)). This fraction had no detectable T cell potential in OP9-DL1 assays (**Fig. 2d**), which indicated that these cells were precursors of B cells and NK cells but not MLPs.

We next tested the developmental potential of Thy-$1^{neg–lo}CD45RA^+$ cells in the $CD34^+CD38^-$ compartment. In cord blood, these cells expressed CD10 and we were able to subcategorize them into $CD7^-$ populations (fraction B) and $CD7^+$ populations (fraction C); in contrast, bone marrow cells were uniformly $CD7^-$ (**Fig. 1**). These cells composed 1–2% of Lin^- cord blood and their frequency was unchanged in adult bone marrow (**Supplementary Fig. 5**). In limiting-dilution analysis and single-cell plating on MS-5 stroma, every colony generated by cells in fraction B from cord blood contained lymphoid (B cells, NK cells or B cells–NK cells), and 57% of colonies also contained $CD33^+CD11b^+$ myeloid cells (**Fig. 2b** and **Table 2**; cloning efficiency, 19%). However, these progenitors never produced myeloid colonies without lymphoid progeny. We obtained similar results with cells in fraction B isolated from bone marrow (**Fig. 2c** and **Table 2**; cloning efficiency, 27%), with no differences between neonatal and adult samples in their output of myeloid, B cell or NK cell lineage (**Table 2**). Cells in fraction B showed robust T cell potential on OP9-DL1 stroma (**Fig. 2d**), with higher cloning efficiency and proliferative potential from cord blood than that of adult cells, consistent with the diminished output of T lymphocytes with aging[27] (**Fig. 2d**; cloning efficiency, 45% (cord blood) or 27% (bone marrow)). Thus, these progenitors could be identified as MLPs that were not restricted to the lymphoid lineages and hence they could not be defined as CLPs, which are expected to be lymphoid restricted.

To assess the myeloid potential of human MLPs, we used CFU assays. Cord blood and bone marrow MLPs gave rise to macrophage CFU colonies, independently established on the basis of their $CD14^+CD11b^+$ phenotype and cell morphology (**Fig. 2e**). No granulocytic CFU colonies arose from MLPs. As GMPs always gave rise to a mixture of granulocytic and macrophage CFU colonies under the same conditions (**Fig. 2e**), we can conclude that MLPs retain only macrophage potential. Although only 10% of freshly sorted cord blood MLPs formed colonies, CFU efficiency was increased considerably by preculture of these cells on OP9 stroma. After 4 d of preculture on OP9 stroma, 50% of MLPs generated macrophage CFU colonies, similar to Thy-1^+ HSCs (**Fig. 2e**, right). MLP-derived colonies could not be replated (**Supplementary Fig. 4**), which indicated that MLPs do not have self-renewal ability. Thus, single MLPs could give rise to B cells, T cells, NK cells and macrophages but lacked granulocytic or erythroid lineage potential.

We next tested the developmental potential of the $CD7^+$ cells in the $CD34^+CD38^-$Thy-$1^{neg–lo}CD45RA^+$ compartment (fraction C) proposed before to be CLPs in cord blood[19] (not found in bone marrow; **Fig. 1**). Unexpectedly, their lineage output was identical to that of the $CD7^-$ MLPs, albeit at a lower cloning efficiency, with a similar proportion of lymphoid and lympho-myeloid colonies (**Fig. 2b** and **Table 2**; cloning efficiency, 11%). MLPs in fraction C did not form colonies in CFU assays (**Fig. 2e**), which indicated that standard colony assays may underestimate myeloid potential and provided an explanation of why this has not been detected in published reports[19]. Thus, $CD34^+CD38^-$Thy-$1^{neg–lo}CD45RA^+$ cells are MLPs regardless of their CD7 expression.

MLPs differentiate into B cells, NK cells and monocytes

We undertook a more rigorous analysis of human MLPs to confirm their myeloid potential. The finding that only half of MLP colonies showed bipotent myelo-lymphoid potential could have been due to inadequate myeloid support in our standard MS-5 assays. To improve detection of myeloid maturation, we cultured single MLPs on MS-5 stroma in the presence of myeloid cytokines, granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF). Clonal efficiency was improved under these conditions, with 21% of $CD7^+$ cord blood MLPs and 29% of $CD7^-$ cord blood MLPs giving rise to colonies (**Fig. 3a** and **Supplementary Table 3**). Inclusion of the monocytic cytokine macrophage colony-stimulating factor (M-CSF) further augmented cloning efficiency to 44% (**Fig. 3a**, **Supplementary Table 3** and **Supplementary Fig. 6**). However, when we took into account the 77% detection efficiency of the single-cell sorting protocol (**Supplementary Fig. 2**), these data suggested that 57% of the successfully seeded MLPs had myeloid

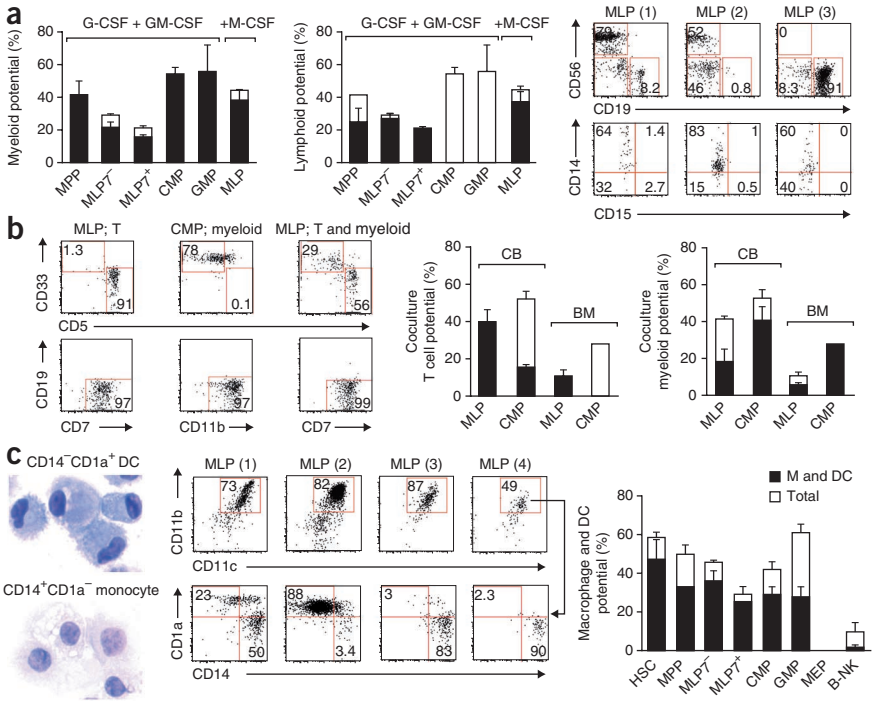

Figure 3 Clonal analysis of human MLPs. (**a**) Cloning efficiency of myeloid lineages (left) and lymphoid lineages (middle) of single cord blood progenitors deposited onto MS-5 stroma by flow cytometry sorting and cultured for 4 weeks with SCF, TPO, IL-7, IL-2, G-CSF and GM-CSF, with or without M-CSF. Bars indicate total cloning efficiency; filled portion indicates the proportion of myeloid potential (myeloid plus mixed colonies) or lymphoid potential (lymphoid plus mixed colonies). Right, marker profiles of MLP colonies (colony number in parentheses). (**b**) Cloning efficiency (right) of T lineages and myeloid lineages of single cord blood or bone marrow MLPs or CMPs deposited onto mixed MS-5 and MS-5–Delta-like 4 stroma by flow cytometry sorting and cultured for 4 weeks. Bars indicate total cloning efficiency; filled portion indicates the proportion of T cell (T cell plus mixed colonies) or myeloid potential (myeloid plus mixed colonies). Left, marker profiles of T cells, myeloid cells and mixed colonies. (**c**) Cloning efficiency (right) of monocyte and DC lineages of single cord blood progenitors deposited onto OP9 stroma by flow cytometry sorting and cultured for 2 weeks with GM-CSF, M-CSF, IL-4 and IL-6. Bars indicate total cloning efficiency; filled portion indicates the proportion of colonies containing both monocytes and DCs. Left, cell morphology of sorted Giemsa-stained $CD14^+$ and $CD1a^+$ cells. Original magnification, ×100. Middle, marker profiles of MLP colonies (colony number in parentheses). Numbers in quadrants (**a**, right) or outlined areas (**b**, left; **c**, middle) indicate percent cells in each. Data are representative of six (**a**) or three (**b**,**c**) experiments with three independent cord blood samples with over 12 wells for each fraction per experiment (mean and s.e.m.).

potential. B cells or NK cells were present in nearly all positive wells, which indicated that myeloid cytokines did not exert instructive effects on the lymphoid commitment of cord blood MLPs. Notably, 85% of positive wells with B cells or NK cells also contained $CD14^+CD11b^+$ monocytes or macrophages (**Fig. 3a** and **Supplementary Table 3**), which conclusively demonstrated that MLPs have the ability to give rise to both lymphoid and monocytic lineages. Of interest, exposure of bone marrow MLPs to myeloid cytokines instructed a myelomonocytic outcome (**Supplementary Fig. 7**), which demonstrated that cytokine signals are interpreted differently by neonatal and adult MLPs. None of the fractions we characterized had lineage potential consistent with a CLP; instead, all progenitors with multilymphoid output also retained macrophage lineage potential.

MLPs differentiate into T cells and myeloid cells

Because of the inability to 'read out' T cell potential in the same assay as that used for the other lineages, we could not rule out the possibility that T cells are produced from a different precursor in the MLP fraction. To address this possibility, we developed a coculture system in which we transduced MS-5 cells with the gene encoding Delta-like 4 and cultured those cells with untransduced MS-5 cells, which allowed T lymphoid and myeloid development in a single well. Single MLPs isolated from cord blood or bone marrow gave rise to $CD7^+CD5^+CD19^-$ T cell colonies and mixed T cell–$CD33^+CD11b^+$ myeloid colonies but not myeloid-only colonies (**Fig. 3b**). In contrast, CMPs from cord blood or bone marrow generated only myeloid colonies under the same conditions (**Fig. 3b**). These data confirm that MLPs can give rise to both T lymphoid and myeloid lineages.

MLPs differentiate into macrophages and DCs

DCs are potent antigen-presenting cells that share a common progenitor with macrophages (the macrophage-DC progenitor)[28–30]. Evidence of monocytic potential of MLPs prompted us to test whether these cells give rise to macrophages and DCs via a common intermediate. We seeded single cord blood MLPs on OP9 stroma, which supports myeloid differentiation but not B cell or T cell differentiation at a clonal level. We first expanded single cells into colonies with primitive-acting cytokines and then matured them into macrophages with M-CSF and IL-6 or into DCs with GM-CSF and IL-4. As expected, M-CSF cultures were composed mainly of $CD14^+CD11c^+CD1a^-$ macrophages, whereas GM-CSF cultures contained $CD14^-CD11c^+CD1a^+$ immature DCs (**Fig. 3c**, left). To investigate their combined macrophage and DC (MDC) potential, we cultured MLPs with both sets of cytokines (M-CSF plus GM-CSF and IL-6 plus IL-4). Over 45% of single $CD7^-$ MLPs gave rise to colonies under these conditions, consistent with the cloning efficiency of myeloid progenitors (**Fig. 3c**, right). Of those, 78% contained both macrophages and DC progeny (**Fig. 3c**), which suggested that MLPs have a combined MDC potential.

MLPs are the main source of DCs

Published studies have suggested that whereas DCs could arise from both human lymphoid and myeloid progenitors, the myeloid pathway represents the main source of DCs[31]. To investigate the potential of MLPs and myeloid progenitors to give rise to mature DCs, we expanded sorted MLP or GMP populations on OP9 stroma, differentiated them into immature DCs with GM-CSF and IL-4 and matured them by exposure to ligands for Toll-like receptors (TLRs)[29]. We compared those cells with standard DCs derived from $CD14^+$ peripheral blood monocytes (PBMs). Mature DCs that upregulated HLA-DR, the maturation markers CD40 and CD83 and the costimulatory molecules CD80 and CD86 were readily generated in a TLR-dependent manner (**Fig. 4a**,**b**). Stimulation of various TLRs differentiated MLPs into mature DCs more efficiently (up to 65% DCs) than GMPs (up to

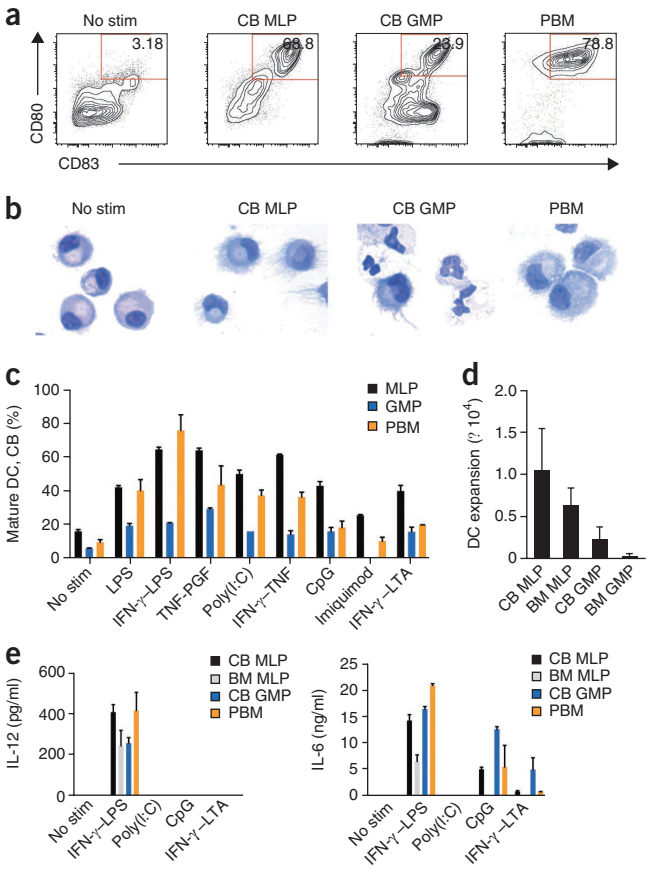

Figure 4 Differentiation of human progenitors into mature DCs. (**a**,**b**) Phenotypic (**a**) and morphological (**b**) characterization of DCs derived from differentiated cord blood MLPs, GMPs and PBMs isolated by leukopheresis and matured with IFN-γ and LPS or without TLR ligands (No stim). Numbers in outlined areas (**a**) indicate percent CD80$^+$CD83$^+$ cells. Original magnification (**b**), ×100. (**c**) Proportion of mature CD80$^+$ CD83$^+$ CD86$^+$ CD40$^+$ DCs in cultures of cord blood MLPs, GMPs and PBMs matured in the presence of various cytokines and TLR ligands (horizontal axis). TNF, tumor necrosis factor; PGE, prostaglandin E; poly(I:C), polyinosinic-polycytidylic acid; CpG, 2′-deoxyribo(cytidine-phosphate-guanosine); LTA, lipoteichoic acid. (**d**) Population expansion of cord blood– and bone marrow–derived MLPs and GMPs cultured in DC conditions. (**e**) Enzyme-linked immunosorbent assay of the secretion of IL-12 (left) and IL-6 (right) by DCs differentiated from MLPs, GMPs and PBMs. Data are representative of three independent experiments (mean and s.e.m. in **c**–**e**).

30% DCs) or unfractionated CD34$^+$ cells[32], whose output consisted mostly of other myeloid cell types (**Fig. 4c** and data not shown). With this protocol, a cord blood MLP yielded over 1 × 10^4 DCs, compared with ~1 × 10^3 DCs for a GMP (**Fig. 4d**). DCs derived from all fractions secreted IL-12, which is involved in the activation of cytotoxic T cells[33], as well as IL-6 and tumor necrosis factor and small amounts of IL-10 (**Fig. 4e** and **Supplementary Fig. 8**). Thus, at least *in vitro*, MLPs represent a more potent source of DCs than do myeloid progenitors and are thus suitable as a source for large-scale immune therapy applications.

In vivo potential of MLPs

To determine the lineage potential of MLPs *in vivo*, we injected a near-limiting dose of 1 × 10^3 cord blood MLPs or CMPs directly into the femurs of nonbese diabetic–severe combined immunodeficiency mice null for the common γ-chain (NSG mice) and analyzed the composition of the graft after 2 weeks and 4 weeks. CMPs gave rise to CD33$^+$CD19$^-$ myeloid grafts at 2 weeks in all recipients tested (**Fig. 5a**). However, by 4 weeks, the remaining myeloid cells were at or below the limit of detection (0.01%; data not shown). These data indicated that the myeloid output of progenitors in NSG mice peaked at 2 weeks and decreased thereafter. Transplanted cord blood MLPs (n = 4 mice) gave rise to grafts containing both CD19$^+$ B cells and CD33$^+$ myeloid cells at 2 weeks (**Fig. 5a**). The myeloid graft was much smaller at 4 weeks, consistent with the kinetics of myeloid output (data

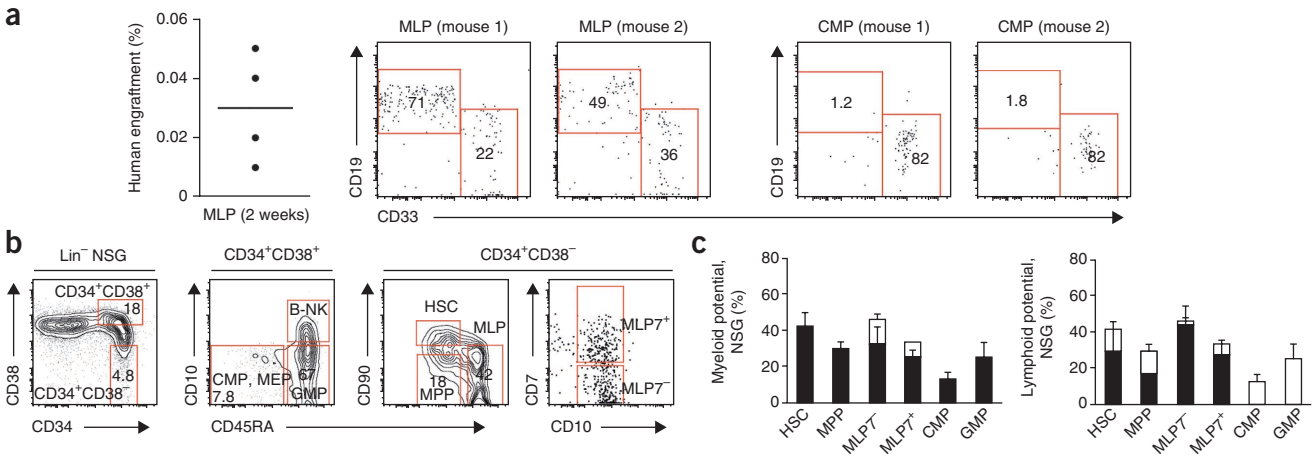

Figure 5 *In vivo* lineage potential of human progenitors. (**a**) Human cell engraftment (left) in the injected femur of NSG mice 2 weeks after intrafemoral transplantation of 1 × 10^3 cord blood MLPs (n = 4 mice) or CMPs (n = 4 mice); right, graft composition, gated on human CD45$^+$ events. (**b**) Analysis of the progenitor compartment in NSG mice (n = 4–8) 10 weeks after transplantation with 1 × 10^5 Lin$^-$ cord blood cells; human Lin$^-$ cells isolated by column purification from the marrow were stained with the marker panel in **Figure 1** without CD135 (thus, CMPs-MEPs appear as a single population). (**c**) Cloning efficiency of myeloid lineages (left) and lymphoid lineages (middle) of human progenitor fractions isolated from the bone marrow of NSG mice with human engraftment; single cells (population, horizontal axis) were deposited on MS-5 stroma by flow sorting and were cultured for 4 weeks with SCF, TPO, IL-7 and IL-2 (presented as in **Fig. 2b**). Numbers in outlined areas (**a**,**b**) indicate percent cells in each. Data are representative of three independent experiments with four to eight mice each and over 12 wells for each fraction per experiment (mean and s.e.m. in **c**).

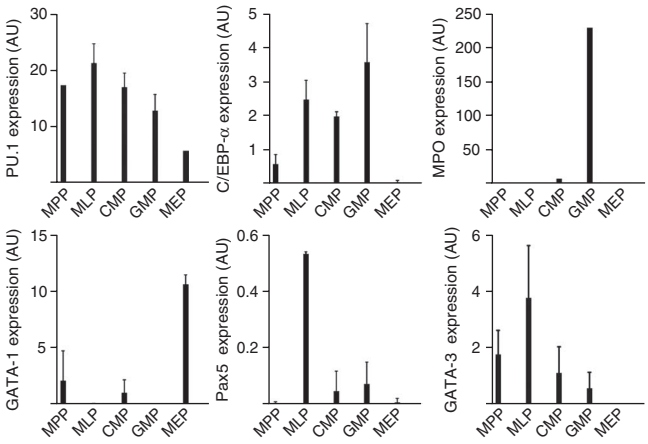

Figure 6 Lineage-specific gene expression in human progenitors. Quantitative PCR analysis of the expression of PU.1, C/EBP-α, myeloperoxidase (MPO), GATA-1, Pax5 and GATA-3 mRNA in progenitor fractions isolated from Lin⁻ cord blood by flow cytometry sorting; results are presented in arbitrary units (AU) relative to the expression of GAPDH (glyceraldehyde phosphate dehydrogenase). Data are combined from two independent experiments (mean and s.e.m.).

not shown). We detected no T cells, as MLPs generated only a transient graft in the injected femur and T cell development requires long-term engraftment (F.N. *et al.*, unpublished observations). Notably, of the MLP-derived myeloid cells, we detected $CD14^+$ monocytes but not $CD15^+$ granulocytes (data not shown). These data indicate that MLPs have bipotent lympho-monocytic potential *in vivo*.

Human HSCs regenerate progenitor hierarchy

To determine if the progenitor classes we identified were generated anew from HSCs, we analyzed the composition of the progenitor compartment in NSG mice stably repopulated by cord blood HSCs. Each of the seven progenitor fractions identified in cord blood and bone marrow, including $CD34^+CD38^-Thy\text{-}1^{neg–lo}CD45RA^+$ MLPs, were faithfully reconstituted by transplanted HSCs (**Fig. 5b**). Moreover, the developmental potential of each fraction isolated from NSG mice was identical to that of cord blood or bone marrow, as determined by clonal analysis on MS-5 stroma supplemented with SCF, TPO, IL-7 and IL-2. In particular, as for cord blood MLPs (**Fig. 2b**), every colony generated by $CD7^+$ and $CD7^-$ MLPs contained B lymphoid or NK lymphoid progeny, and 70% of colonies also contained myeloid cells (**Fig. 5c** and **Supplementary Table 4**; cloning efficiency, 45% ($CD7^-$ MLPs) and 34% ($CD7^+$ MLPs)). These results indicate that MLPs and other progenitors isolated from steady-state cord blood and bone marrow are intrinsic components of the human hematopoietic tree derived from HSCs.

Transcriptional program of human progenitors

To investigate the transcriptional program that underlies human progenitor development, we did quantitative PCR analysis of lineage-specific markers (**Fig. 6**), as their detection in uncommitted progenitors would be indicative of lineage potential[34]. The early myeloid transcription factors PU.1 and C/EBP-α were expressed in myeloid progenitors and also in MLPs. In contrast, we detected expression of myeloperoxidase, produced by mature myeloid cells, only in GMPs. GATA-1, an erythroid master regulator, was selectively expressed in MEPs. Finally, the key lymphoid transcription factors Pax5 and GATA-3 were expressed selectively in MLPs (**Fig. 6**). Thus, the expression of lineage markers in progenitors correlated with their functional potential, which provided an independent line of evidence to support the proposed hierarchical organization.

That conclusion was further supported by global gene-expression profiling. The expression of a set of annotated lymphoid genes by MLPs was different from that of multipotent progenitors (HSC or MPP; $P = 3.2 \times 10^{-5}$), myeloid progenitors (CMP; $P = 3.9 \times 10^{-7}$) and erythroid progenitors (MEP; $P = 5.8 \times 10^{-11}$; **Supplementary Table 5**). This gene signature included *LY96*, *SYK*, *LTB* and *MIST*, as well as major histocompatibility complex class I and II and immunoglobulin loci. To obtain a signature of lineage-specific gene expression in MLPs, we used MEPs as a reference population for the MLP-enriched gene set, excluding stem cell–specific transcripts. The resulting set of 392 genes had two distinct expression patterns (**Supplementary Fig. 9**). The set of MLP-specific genes included *LY96*, *SYK*, *LTB*, *MIST* and *LST1*, major histocompatibility complex loci, and genes encoding lymphoid transcription factors (*BCL6*, *BCL11A* and *NOTCH3*). A distinct cluster expressed by MLPs, GMPs and CMPs, but not by MPPs or MEPs, indicated a expression pattern shared by myeloid progenitors and MLPs. This set included genes encoding myeloid transcription factors (*CEBPA* and *SPI1*; **Fig. 6**), genes associated with innate immunity (*IFITM1*, *LILRA2*, *INFGR1*, *CLEC4A*, *ITGB2* and *CCL3*) and genes encoding transcription factors critical for development of macrophages and DCs (*IRF7* and *IRF8*)[35] (**Supplementary Fig. 9**). These results suggest that MLPs initiate the expression of lymphoid transcripts but maintain a shared gene-expression signature with myeloid progenitors. Together our findings prompt a revised model of human progenitor hierarchy (**Supplementary Fig. 10**).

DISCUSSION

Our findings have provided a comprehensive picture of early fate determination in human hematopoiesis. We found that myeloid commitment followed the classical model, with loss of lymphoid potential at the CMP stage and segregation of myeloid potential and erythroid potential in GMPs and MEPs, respectively. Myeloid potential and erythroid and megakaryocytic potential in the mouse have been found to segregate to distinct cells in the CMP fraction[36], and this remains a possibility in human hematopoiesis. In contrast, human MLPs are not lymphoid restricted but give rise to DCs and macrophages, in contrast to the classical model. MLPs can be uniquely identified as $Thy\text{-}1^{neg–lo}CD45RA^+$ cells in the immature $CD34^+CD38^-$ compartment of both cord blood and bone marrow that also contains $Thy\text{-}1^+CD45RA^-$ HSCs and $Thy\text{-}1^-CD45RA^-$ candidate MPPs[22]. In our assays, a large proportion of single cells in the MLP population gave rise to all the lymphoid and myelo-monocytic lineages but not the erythroid or granulocytic lineages. Thus, human early lymphoid development involves a previously unknown lineage choice between the canonical lymphoid B cell, T cell and NK cell fates and the MDC lineages traditionally viewed as myeloid restricted. We propose that the products of the MLP lineage choice in the bone marrow are the restricted B cell–NK cell precursors described here and MDC precursors, such as the macrophage-DC progenitor[30].

The identification of MLPs extends the findings of two published reports of human early lymphoid progenitors. The $CD34^+CD10^+CD24^-$ phenotype[18] is shared by MLPs and more mature progenitors, such as B cell–NK cell precursors. The $CD34^+CD38^-CD7^+$ phenotype[19,20] is more restrictive because only half of cord blood MLPs are $CD7^+$ and these cells are not found in adult bone marrow. The precise phenotypic identification of human MLPs, combined with improved clonal assays, allowed us to investigate their lineage potential at the single-cell level. Although published reports have detected only residual myeloid potential, consistent with the classical model, we have

shown here that under improved conditions, 57% of MLPs produced colonies on MS-5 stroma, and 85% of those contained B cell–NK cell and MDC lineages. Moreover, the proportion of myeloid, B cell and NK output was nearly 1:1:1, which indicated that these lineages were derived from the same cell. At least 45% of MLPs also generated T cells on OP9-DL1 stroma. Thus, it is most likely that this fraction contains a progenitor with combined B cell, T cell, NK cell and MDC potential. Those data and our survey of other progenitor populations provided no evidence of a lymphoid-restricted state (that is, a CLP) in human hematopoiesis. At present, it is believed that the CLP represents an obligate lymphoid intermediate in the mouse, despite reports that myeloid potential is retained even after B cell–T cell lineage restriction[10,12,13]. Human MLPs do not give rise to granulocytes *in vitro* or *in vivo* and have a low repopulating capacity, which suggests that they are also distinct from mouse lymphoid-primed multipotent progenitors. Reports of macrophage potential in mouse and human earliest thymic progenitors[13,37], CLPs[38] and the B cell–macrophage progenitors[39] support the idea that in mice, as in humans, macrophages may also arise in early lymphoid development.

Our results have also established that the $CD34^+CD38^-Thy\text{-}1^{neg–lo}$ $CD45RA^+$ phenotype identifies MLPs in both cord blood and bone marrow. The known differences between neonatal and adult cells, such as the requirement for IL-7 in lymphopoiesis[40], have given rise to speculation that early lymphoid progenitors in cord blood and bone marrow might be phenotypically and functionally distinct. However, the frequency and the B lymphoid, NK cell and MDC lineage potential of neonatal and adult human MLPs were similar. Thus, our data support the applicability of the proposed human hierarchy model to both neonatal and adult hematopoiesis. Still, there are differences between adult and neonatal MLPs in terms of the lower capacity to generate T lymphocytes and their capacity to be directed to the myeloid fate by cytokines. Concordant with those data, the output of mouse CLPs, earliest thymic progenitors and pro-B cells decreases with age[27], which suggests that age-related defects in immunity in mouse and human can be attributed in part to the function of lymphoid progenitors.

MLPs gave rise to B cells and monocytes after transplantation into NSG mice; however, it remains to be determined if MLPs contribute to the steady-state monocyte pool in humans. Primary monocytopenia is a rare disorder accompanied in some cases by B cell–NK cell cytopenia, with severe depletion of circulating B cells, NK cells and MDCs but normal hematocrit and neutrophil and platelet counts[41]. Analysis of the $CD34^+$ compartment in the bone marrow of one such patient has shown that $CD34^+CD38^-Thy\text{-}1^+$ HSCs and all progenitor populations were present, except the MLPs and the more committed B cell–NK cell precursors (V. Bigley *et al.*, unpublished observations). These observations suggest that the MLP may be an obligate intermediate in human steady-state B cell–NK cell and MDC development. Notably, T cell development was affected to a lesser extent, which suggests that in humans, as in mice, many different progenitor populations can contribute to thymopoiesis[42].

Monocytes, macrophages and DCs belong to a network of cells of the immune response called the mononuclear phagocyte system, and they share a common progenitor, the macrophage-DC progenitor[30,43]. Macrophages specialize in phagocytosis and innate immunity, whereas DCs specialize in antigen presentation to shape adaptive immune responses[44]. DCs arise from both myeloid and lymphoid progenitors, whereas monocytes and macrophages were thought to arise uniquely from myeloid progenitors, such as GMPs[45]. Our findings place the origin of MDC lineages in early human lymphopoiesis, which shows a redundancy in hematopoietic development that supports a version of the 'myeloid-based' model of hematopoiesis[46,47]. From an evolutionary standpoint, as macrophage-like phagocytes represent the earliest hematopoietic and immune cell type, we are tempted to envision it as an ancestral lineage program that was retained in the progenitors in which adaptive immunity arose during subsequent diversification of the hematopoietic tree. DCs have a potent ability to present antigens and stimulate T cells, which makes them useful tools for immune-therapy applications[48,49]. As MLPs can be readily isolated from patient cord blood, mobilized peripheral blood or bone marrow biopsies, expanded and differentiated to obtain large quantities of autologous T cells and DCs, they provide a useful platform for tailoring immunotherapy for research purposes and for ongoing immune therapy trials.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession code. GEO: microarray data, GSE21973.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

S.D. and F.N. designed and did experiments; S.D. wrote the manuscript; K.E. analyzed microarray data; L.T.N. did DC population expansion experiments; and P.S.O. and J.E.D. supervised the study and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Sample collection and sorting. Cord blood samples were obtained with informed consent according to the procedures approved by the institutional review boards of the University Health Network and Trillium Hospital. Lin^- cord blood cells were purified by negative selection with a StemSep Human Progenitor Cell Enrichment kit according to the manufacturer's protocol (StemCell Technologies). $CD34^+$ cells isolated from bone marrow and mobilized peripheral blood by positive selection were from Lonza. Lin^- cells were thawed and then were stained at a density of 1×10^6 cells per 100 µl with the following antibodies (all from BD, unless stated otherwise): fluorescein isothiocyanate–conjugated anti-CD45RA (HI100; 4 µl), phycoerythrin-conjugated anti-CD135 (4G8; 8 µl), phycoerythrin-indodicarbocyanine–conjugated anti-CD7 (8H8.1; 2 µl; Coulter), allophycocyanin-conjugated anti-CD10 (HI10a; 4 µl), phycoerythrin-indotricarbocyanine–conjugated anti-CD38 (HB7; 3 µl), allophycocyanin-indotricarbocyanine–conjugated anti-CD34 (4 µl) and biotin-conjugated anti-CD90 (5E10; 4 µl; secondary staining with Qdot 605 conjugated to streptavidin; 2 µl; Invitrogen). Cells were sorted by flow cytometry (1–10 cells per well in single-cell or limiting-dilution format) directly into 96-well plates preseeded with stroma by a single-cell deposition unit coupled to a FACSAria sorter (BD); this provided the correct number of cells in 88% of wells, as assessed by counting of cells deposited into empty wells (**Supplementary Fig. 2**). The purity of single-cell sorting was routinely assessed by recovery of sorted cells and was >99%. CFU assays were done as described[16].

Clonal assays on MS-5 stroma. MS-5 stroma was seeded in 96-well plates (Nunc) coated with 0.2% (wt/vol) gelatin at a density of 5×10^3 cells per well in H5100 medium (StemCell Technologies) supplemented with the following cytokines (all from R&D Systems): SCF (100 ng/ml), IL-7 (20 ng/ml), TPO (50 ng/ml), IL-2 (10 ng/ml) and, in some experiments, GM-CSF (20 ng/ml), G-CSF (20 ng/ml) and M-CSF (10 ng/ml). After 48 h, single-sorted progenitor cells were sorted onto stromal monolayers. MS-5 cultures were maintained for 4 weeks with weekly changes of half the media. Cells were resuspended by physical dissociation, filtered through Nitex membranes and stained with anti-CD45 (Immu19.2; Coulter, or 2D1; BD), anti-CD19 (4G7; BD), anti-CD14 (RMO52; Coulter), anti-CD15 (MMA; BD), anti-CD33 (PG7.6; BD), anti-CD56 (B159; BD) and anti-CD11b (ICRF44; BD) and then were analyzed by high-throughput flow cytometry. Myeloid colonies were identified as $CD33^+CD11b^+$ wells negative for CD19 or CD56. Lymphoid colonies were identified as $CD19^+$ wells (B cells), $CD56^+$ wells (NK cells) or $CD19^+CD56^+$ wells (B cells and NK cells) negative for myeloid markers. Mixed colonies contained both myeloid and lymphoid cells (B cells and/or NK cells). For coculture experiments, MS-5 and MS-5-DL4 cells were mixed at a ratio of 4:1 and were cultured with SCF, IL-7, TPO, Flt3 (10 ng/ml) and GM-CSF. Cultures were maintained as described above and were analyzed with anti-CD5, anti-CD7, anti-CD33, anti-CD11b and anti-CD19. T cell colonies were identified as $CD5^+CD7^+$ wells negative for CD33, CD11b or CD19. Myeloid colonies were identified as $CD33^+CD11b^+$ wells negative for CD7, CD5 or CD19. Clones were required to have over ten $CD45^+$ cell–gated events (of the appropriate cell-surface phenotype) to be considered positive.

DC assays on OP9 stroma. OP9 stroma was seeded in 96-well plates (Nunc) at a density of 5×10^3 cells per well in α-MEM (Gibco) with 20% (vol/vol) FBS. Sorted progenitor populations were expanded for 9 d with SCF (100 ng/ml), TPO (50 ng/ml), IL-7 (10 ng/ml) and Flt3 (10 ng/ml), then were differentiated for 7 d into DCs with GM-CSF (50 ng/ml) and IL-4 (20 ng/ml) or into macrophages with M-CSF (20 ng/ml) and IL-6 (20 ng/ml) or a combination of these cytokines. Cells in wells were resuspended by physical dissociation and were stained with anti-CD45, anti-CD11b, anti-CD11c (B-ly6; BD), anti-CD14, anti-CD15 and anti-CD1a (BL6; Coulter) and analyzed by flow cytometry. Myeloid colonies were identified as $CD11b^+CD11c^+$ wells, which were further distinguished with anti-CD14 (monocytes) and anti-CD1a (DCs).

T cell assays on OP9-DL1 stroma. OP9-DL1 stroma was seeded in 96-well plates at a density of 5×10^3 cells per well in α-MEM (Gibco) and 20% (vol/vol) FBS (previously tested for T cell support), plus Flt3 (5 ng/ml) and IL-7 (5 ng/ml). Cells were filtered through Nitex membranes and were transferred onto fresh stromal monolayers twice a week or as needed and were analyzed with anti-CD45, anti-CD3 (SK7; BD), anti-CD5 (L17F12; BD), anti-CD7, anti-CD4 (13B8.2; Coulter) and anti-CD8 (HIT8a; BD). Positive wells from cord blood were assigned scores for $CD4^+CD8^+$ T cells at 8 weeks; positive wells from bone marrow were assigned scores for $CD3^+$ cells at 4 weeks.

Quantitative PCR. RNA extracted from ~2×10^4 sorted progenitors with TRIzol reagent (Invitrogen) was treated with DNAse I and reverse transcribed with SuperScript II (Invitrogen). Applied Biosystems 7900HT was used for real-time PCR with SYBR Green PCR Master Mix (Applied Biosystems), 200 nM primers (Qiagen) and >20 ng cDNA; reactions were done in triplicate. Gene expression was quantified with SDS software (Applied Biosystems) based on the standard curve method.

Microarray analysis. Total RNA was extracted with TRIzol (Invitrogen) from 5×10^3 to 10×10^3 cells from HSC, MLP, CMP, GMP and MEP populations (**Table 1**) and was amplified, hybridized to Illumina HT-12 microarrays and analyzed with GeneSpring GX 10.0.2 software (Agilent Technologies) after quantile normalization. Probes with differences in expression were assessed by analysis of variance followed by the Benjamini-Hochberg correction (false-discovery rate, 0.05). The MLP-specific gene-expression signature was generated by probes showing a pattern of expression in MLPs greater than expression in MEPs after an initial filter for probes with differences in expression of at least twofold between any two populations, except between HSC and MPP. Cluster analysis was done with MultiExperiment Viewer of the TM4 Microarray Software Suite.

Mouse transplantation. NSG mice (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ; Jackson Laboratory) were bred and housed at the Toronto Medical Discovery Tower–University Health Network animal care facility. Animal experiments were done in accordance to institutional guidelines approved by University Health Network Animal care committee. Mice were sublethally irradiated (200–250 cGy) 24 h before transplantation. Cells were transplanted intrafemorally into anesthetized mice. A 27-guage needle was used to drill the right femur, and cells in a volume of 25 µl were transplanted with a 28.5-guage insulin needle. Mice were killed after 2 or 4 weeks for progenitor analysis or after 10 weeks for HSC analysis. Marrow was isolated by flushing of bone cavities with 2 ml Iscove's modified Dulbecco's medium, and 100 µl marrow was stained for the surface markers CD45, CD19, CD33, CD14, CD15 and CD56. For analysis of HSC-derived hierarchy, human progenitors were isolated from pooled bone marrow with the Mouse/Human Chimera Enrichment kit according to the manufacturer's protocol (StemCell Technologies), with the addition of StemSep Human Hematopoietic Progenitor Cell Enrichment Cocktail (100 µl/ml; StemCell Technologies).

DC culture. OP9 stroma cells were seeded in six-well plates at a density of 1×10^6 cells per well in α-MEM with 20% (vol/vol) FBS, plus SCF (100 ng/ml), Flt3 (100 ng/ml), TPO (50 ng/ml) and IL-7 (20 ng/ml). Human progenitors were sorted from cord blood, bone marrow or mobilized peripheral blood and were seeded on OP9 stroma at a density of 1×10^2 to 1×10^3 cells per well. Cells were cultured for 2 weeks, with exchange of half the medium twice a week. Cells in wells were resuspended by physical dissociation and filtered through Nitex membranes, and $CD45^+$ cells were sorted into suspension cultures with α-MEM and 20% FBS, plus GM-CSF (50 ng/ml) and IL-4 (20 ng/ml). Cells were cultured for 5 d with one change of medium. Cells were collected and 2×10^5 cells per well were matured for a total of 24 h (except where indicated otherwise) in RPMI medium, 2% (vol/vol) human serum and L-glutamine, plus the following combinations of TLR ligands and times: IFN-γ (1,000 U) for 4 h plus LPS (10 ng/ml) for 20 h; LPS (10 ng/ml); tumor necrosis factor (10 ng/ml), IL-1β (10 ng/ml), IL-6 (1,000 IU) and prostaglandin E_2 (10 µM); polyinosinic-polycytidylic acid (10,000 ng/ml); CpG (10 µM); imiquimod (1,000 ng/ml); lipoteichoic acid (1,000 ng/ml); or IFN-γ (1000 U ng/ml) for 4 h and lipoteichoic acid (1,000 ng/ml) for 20 h. Cells were stained with anti-CD14, anti-CD80 (L307.4; BD), anti-CD86 (FUN-1; BD), anti-CD83 (HB15e; BD), anti-CD40 (5C3; BD) or anti-CD14, anti-HLA-DR (L243; BD), anti-CD11c, anti-CD1a and anti-CD11b (all from BD) and were analyzed by flow cytometry. Cytokine secretion was measured by enzyme-linked immunosorbent assay.

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Statistics. Clonal data are based on single-cell or limiting-dilution experiments. For single-cell experiments, clonogenic efficiency is reported as percent positive wells. Limiting-dilution data are presented as the estimated limiting dilution frequency ± 95% confidence interval. Limiting-dilution analysis was done with the Extreme Limiting Dilution Analysis software provided by the Walter and Eliza Hall Institute of Medical Research Bioinformatics.

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