Prospective isolation of human clonogenic common myeloid progenitors

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The hierarchical development from hematopoietic stem cells to mature cells of the hematolymphoid system involves progressive loss of self-renewal capacity, proliferation ability, and lineage potentials. Here we show the prospective isolation of early developmental intermediates, the human clonogenic common myeloid progenitors and their downstream progeny, the granulocyte/macrophage and megakaryocyte/erythrocyte progenitors. All three populations reside in the lineage-negative (lin^-) $CD34^+CD38^+$ fraction of adult bone marrow as well as in cord blood. They are distinguishable by the expression of the IL-3Rα chain, the receptor of an early-acting hematopoietic cytokine, and CD45RA, an isoform of a phosphotyrosine phosphatase involved in negative regulation of cytokine signaling. Multipotent progenitors, early lymphoid progenitors, and the here-defined myeloid progenitors express distinct profiles of hematopoiesis-affiliated genes. The isolation of highly purified hematopoietic intermediates provides tools to better understand developmental programs underlying normal and leukemic hematopoiesis.

Throughout life, the hematolymphoid system is regenerated from a small fraction of self-renewing hematopoietic stem cells (HSCs). Most of the knowledge about factors that initiate the maturation process of progenitor cells and induce restriction to one hematopoietic lineage is achieved in principle through two approaches: (*i*) the observation of natural occurring or artificially induced genetic alterations that lead to characteristic phenotypes, and (*ii*) the rigorous purification of HSC and/or progenitor cell populations that are tested at the clonal level for their full developmental capacity and in short-term developmental tests for their placement in a lineage. We recently have isolated by surface phenotype clonogenic common lymphoid progenitors (1) and clonogenic common myeloid progenitors (CMPs) and their myeloid downstream progeny, the granulocyte/macrophage (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs) (2) in mouse bone marrow. These findings together with data from other groups indicate that the commitment to either the lymphoid or myeloid lineage and downstream to the granulocyte/macrophage and megakaryocyte/erythrocyte lineages are exclusive events. In humans, multiple reports describe the enrichment of progenitor populations in bone marrow, mobilized peripheral blood, thymus, cord blood, fetal bone marrow, and fetal liver (3–16). However, to the best of our knowledge, thus far only early lymphoid lineage-restricted progenitors have been isolated and evaluated rigorously for all their developmental capacity on a clonal basis: An adult human bone marrow lineage-negative (lin^-) $CD34^+CD38^+CD10^+$ cell gives rise to B, natural killer (NK), and dendritic cells on a clonal level and to T cells at high frequencies (7), and a cord blood $CD34^+CD38^-CD7^+$ cell gives rise to B, NK, and dendritic cells (8), but both are devoid of myeloerythroid differentiation capacity. We therefore searched for distinct early myeloerythroid-committed progenitors, the human counterparts of the described murine CMPs, GMPs, and MEPs, in adult human bone marrow and cord blood.

Materials and Methods

Bone Marrow and Cord Blood Samples. Fresh human bone marrow (donor age, 20–47 years) was obtained with informed consent from healthy volunteers (AllCells, Berkeley, CA), allogeneic bone marrow donors (Department of Bone Marrow Transplantation, Stanford School of Medicine, Stanford, CA), and vertebral bodies of multiorgan cadaveric donors (Miami School of Medicine, Miami). Cord blood was drawn with informed consent after caesarian sections of full-term newborns (Department of Obstetrics, Stanford School of Medicine).

Cell Preparation, Flow-Cytometric Analysis, and Cell Sorting. $CD34^+$ cells were enriched from mononuclear cells by using immunomagnetic beads according to manufacturer instructions ($CD34^+$-selection kit, Miltenyi Biotec, Bergisch-Gladbach, Germany). For analysis and sorting of myeloid progenitors, cells were stained with phycoerythrin (PE)-Cy5-conjugated antibodies specific for lin markers CD3, S4.1; CD4, S3.5; CD7, CD7-6B7; CD8, 3B5; CD10, 5-1B4; CD14, TUK4; CD19, SJ25-C1 (Caltag, South San Francisco, CA); CD2, RPA-2.10; CD11b, ICRF44; CD20, 2H7; CD56, B159; GPA, GA-R2 (Becton Dickinson–PharMingen, San Diego), and FITC-conjugated anti-CD45RA, MEM56 (Caltag), PE-conjugated anti-IL-3Rα, 9F5 (Becton Dickinson–PharMingen), biotinylated anti-CD38, HIT2 (Caltag), and APC-conjugated anti-CD34, HPCA-2 (Becton Dickinson–PharMingen).

Lymphoid progenitors were stained with the same PE-Cy5-conjugated lineage mixture except for the omission of anti-CD7, anti-CD10, and the addition of anti-CD13, TUK1 (Caltag) followed by FITC-conjugated anti-CD10 (Ancell, Bayport, MN), PE-conjugated anti-IL-7Rα (Immunotech, Marseilles, France), and anti-CD38 and CD34 as described above. For additional analysis FITC-, PE-, Texas red-, or APC-conjugated or biotinylated monoclonal antibodies against the following antigens were used: CD45, HI30; CD13, TUK1; CD16, 3G8; CD71, T56/14; HLA-DR, TU36 (Caltag); CD3, HIT3a; CD4, RPA-T4, CD8, HIT8a; CD9, M-L13; CD19, HIB19; CD32, FLI8.26; CD33, WM53; CD41a, HIP8; CD45RA, HI100; CD56, B159; CD64, 10.1; CD71, M-A712; CD90, A59; CD117, YB5.B8; Glycophorin A (GPA), HIR2 (Becton Dickinson–PharMingen). Streptavidin-conjugated Texas red or Cy7-PE (GIBCO) was used for visualization of biotinylated antibodies. Dead cells were excluded by propidium iodide staining. Appropriate isotype-matched, irrelevant control monoclonal antibodies were used to determine the level of background staining. Cells were sorted and analyzed by using a highly modified dual-laser FACS

Abbreviations: HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; MEP, megakaryocyte/erythrocyte progenitor; NK, natural killer; lin^-, lineage-negative; PE, phycoerythrin; SCF, stem cell factor; FL, Flt3 ligand; Epo, erythropoietin; Tpo, thrombopoietin; LTC-IC, long-term culture initiating cell; CFU, colony-forming unit; GEMM, granulocyte/erythroid/macrophage/megakaryocyte.

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Vantage (Becton Dickinson Immunocytometry Systems, Mountain View, CA) and a dual-laser MoFlo cytometer (Cytomation, Fort Collins, CO), both equipped with a 488-nm argon laser and a 599-nm dye laser. All cells were double-sorted to obtain populations that were essentially pure for the indicated surface-marker phenotype. For single-cell and limiting-dilution assays, the re-sort was performed by using an automatic cell-deposition unit (ACDU) system (Becton Dickinson).

***In Vitro* Assays to Determine Differentiation Potential of Progenitors.** For myeloid colony formation, cells were cultured over 14 days in Iscove's modified Dulbecco's medium (IMDM, GIBCO) based methylcellulose medium (Methocult H4100, StemCell Technologies, Vancouver) supplemented with 20% FBS (lot no. 315853, ICN), 1% BSA, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, antibiotics, and human IL-3 (20 ng/ml), IL-6 (10 ng/ml), IL-11 (10 ng/ml), stem cell factor (SCF, 10 ng/ml), Flt3 ligand/(FL, 10 ng/ml), granulocyte/macrophage colony-stimulating factor (50 ng/ml), thrombopoietin (Tpo, 50 ng/ml), and erythropoietin (Epo, 4 units/ml) (all R & D Systems). FBS was selected from multiple sera to obtain significant megakaryocyte development. For short-term liquid cell culture, cells were cultured in IMDM with 10% FBS, IL-11 (10 ng/ml), SCF (10 ng/ml), FL (10 ng/ml), Tpo (50 ng/ml), and Epo (4 units/ml).

To support the formation of B and NK cells, progenitors were cultured for 3–5 weeks on irradiated (25 Gy) SyS-1 (Ac6) stromal cell monolayers in IMDM supplemented with 10% FBS (HyClone), SCF (10 ng/ml), FL (10 ng/ml), and IL-7 (50 ng/ml) (for B cell readout) or IL-2 (50 ng/ml, for NK cell readout), or IL-7 and IL-2 (for combined B and NK cell readout). Half of the medium was replaced weekly.

Long-term culture initiating-cell (LTC-IC) assays were performed in human long-term culture medium (Myelocult H5100, StemCell Technologies) supplemented with hydrocortisone on irradiated M2–10B4 monolayers according to manufacturer instructions. Cells were transferred to the above-described methylcellulose medium after 6 weeks. All cultures were incubated at 37°C in a humidified chamber under 7% CO_2.

Reverse Transcription–PCR Analysis. Total RNA was purified from 1,000 double-sorted cells from each population and amplified by reverse transcription–PCR as reported (17). Oligonucleotide primer sequences were as reported previously and can be provided upon request. PCR products were electrophoresed on an ethidium bromide-stained 2.0% agarose gel. PCR analysis was repeated at least twice for at least two separately prepared sets of samples.

Results

Identification of Three Distinct Cell Populations with High Myeloerythroid Colony-Forming Unit (CFU) Activity in Adult Human Bone Marrow. We first tested myeloid colony-forming potentials in $CD34^+$ and $CD34^-$ human bone marrow cells and confirmed previous findings that more than 95% of CFUs reside in the $CD34^+$ cell fraction (data not shown). Cells with multipotent, self-renewing hematopoietic capacity are $lin^-CD34^+CD38^-Thy1^+$, whereas $CD34^+CD38^+$ cells, the major fraction of all $CD34^+$ cells, are further differentiated and have lost both self-renewing capacity and bipotent lymphomyeloid potential (18–24). The coexpression of CD10 or CD7 on $CD34^+$ cells in adult bone marrow or cord blood defines the earliest described clonal human lymphoid-committed progenitors (7, 8). To exclude multipotent progenitors, lymphoid-restricted progenitors, and more mature lineage low cells, we therefore searched for the earliest myeloid-committed cells in the lin^- (CD2, CD3, CD4, CD7, CD8, CD10, CD11b, CD14, CD19, CD20, CD56, and GPA) $CD34^+CD38^+$ cell fraction of adult human bone marrow.

The myeloid colony-formation efficiency in methylcellulose

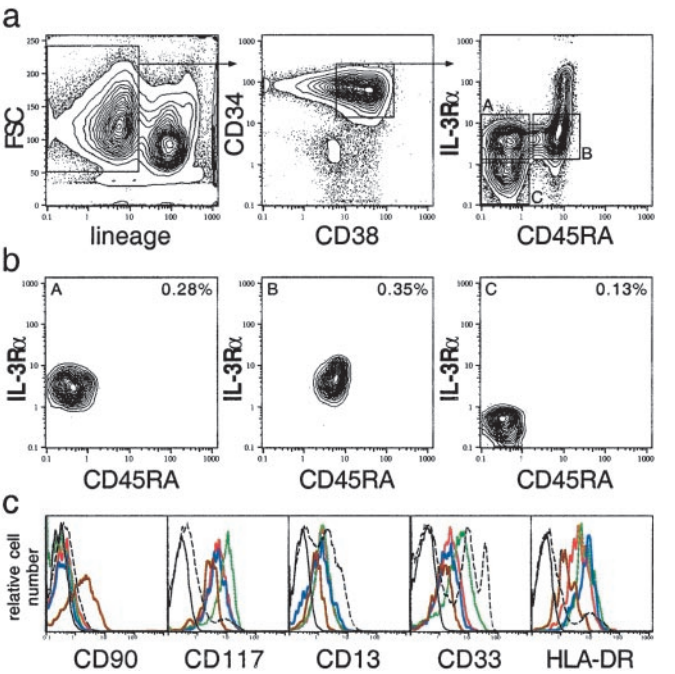

Fig. 1. Prospective isolation of myeloid progenitors in adult human bone marrow. (*a*) The $lin^-CD34^+CD38^+$ fraction was subdivided according to IL-3Rα and CD45RA expression (*a–c*). (*b*) Reanalysis of the sorted populations. Percentages relative to mononuclear bone marrow cells are shown. (*c*) Additional antigen expression profile on $CD34^+CD38^-$ (brown), IL-3Rα^{lo}CD45RA$^-$ (red), IL-3Rα^{lo}CD45RA$^+$ (green), and IL-3Rα^-CD45RA$^-$ (blue) populations. Isotype control gated on total $lin^-CD34^+CD38^+$ cells (black) and marker expression on $CD34^-$ bone marrow cells (dashed line) are shown.

was 52–73% for $lin^-CD34^+CD38^-$ cells, 67–93% for $lin^-CD34^+CD38^+$ cells, and 0–5% for $lin^-CD34^-CD38^-$ cells (Figs. 1 and 2 and data not shown). We tested the expression of multiple candidate surface antigens on the $lin^-CD34^+CD38^+$ fraction. With antibodies against CD45RA and the IL-3Rα, the $lin^-CD34^+CD38^+$ fraction is clearly separable in three distinct populations, the IL-3Rα^{lo}CD45RA$^-$ (candidate CMPs), IL-3Rα^{lo}CD45RA$^+$ (candidate GMPs), and IL-3Rα^-CD45RA$^-$ (candidate MEPs) fraction, accounting for $\approx$0.28, $\approx$0.35, and $\approx$0.13% of bone marrow mononuclear cells (Fig. 1 *a* and *b*). The three populations display relatively uniform levels of the SLF receptor c-Kit (CD117), CD13, CD33, and HLA-DR, but they are negative for Thy-1 (CD90), FcR III (CD16), FcR II (CD32), FcR I (CD64), CD41a, and CD9 (Fig. 1*c* and not shown).

The IL-3Rα^{lo}CD45RA$^-$, IL-3Rα^{lo}CD45RA$^+$, and IL-3Rα^-CD45RA$^-$ fractions gave rise to distinct colony types in methylcellulose CFU assays with the indicated cytokines (Fig. 2 *a* and *b*). To evaluate cloning efficiency and colony types precisely, single cells of each population from 18–27-year-old donors were sorted in 96-well plates (Fig. 2*c*). HSC-enriched $lin^-CD34^+CD38^-$ cells (288 cells, three donors), 336 $lin^-CD34^+CD38^+$IL-3Rα^{lo}CD45RA$^-$ cells, 384 $lin^-CD34^+CD38^+$IL-3Rα^{lo}CD45RA$^+$, and 384 $lin^-CD34^+CD38^+$IL-3Rα^-CD45RA$^-$ cells (four donors each) were plated. HSC-enriched $lin^-CD34^+CD38^-$ cells gave rise to all types of myeloid colonies including mixed colonies (CFU-GEMM), burst-forming units erythroid, CFU-megakaryocyte, CFU-megakaryocyte/erythroid, CFU-granulocyte/macrophage, CFU-granulocyte, and CFU-macrophage with a total cloning efficiency of 69%. Similarly, 84% of single IL-3Rα^{lo}CD45RA$^-$ cells gave rise to a comparable distribution of colony types. In contrast, 75% of $lin^-CD34^+CD38^+$IL-3Rα^{lo}CD45RA$^+$ cells gave rise exclusively to CFU-granulocyte, CFU-macrophage, and CFU-granulocyte/macrophage, whereas

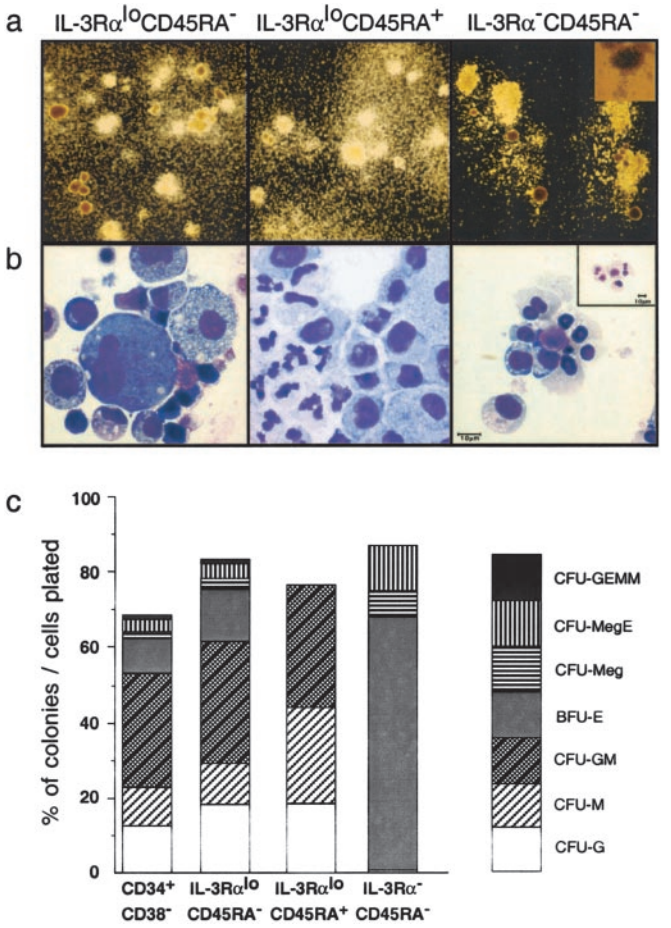

Fig. 2. Colony formation in methylcellulose. (*a*) Morphology of day-14 colonies derived from sorted progenitors (inverted light microscope, ×4). (*Right Insert*) CFU-megakaryocyte/erythroid (×10). (*b*) Cellular morphology form single-picked colonies: CFU-granulocyte/erythroid/macrophage/megakaryocyte (GEMM, *Left*), CFU-granulocyte/macrophage (*Center*), burst-forming units/erythroid (*Right*), and CFU-megakaryocyte (*Right Insert*) (Giemsa, ×100 oil). (*c*) Clonogenic myeloid colony formation in methylcellulose. Scored were 288 wells receiving lin$^-$CD34$^+$CD38$^-$ cells (three donors), 336 wells receiving lin$^-$CD34$^+$CD38$^+$IL-3Rα^{lo}CD45RA$^-$ cells, and 384 wells each receiving lin$^-$CD34$^+$CD38$^+$IL-3Rα^{lo}CD45RA$^+$ and lin$^-$CD34$^+$CD38$^+$IL-3Rα^-CD45RA$^-$ cells (four donors each). CFU-MegE, CFU-megakaryocyte/erythroid; CFU-GM, CFU-granulocyte/macrophage; BFU-E, burst-forming units/erythroid; CFU-Meg, CFU-megakaryocyte; CFU-M, CFU-macrophage; CFU-G, CFU-granulocyte.

87% of lin$^-$CD34$^+$CD38$^+$IL-3Rα^-CD45RA$^-$ cells produced burst-forming units erythroid, CFU-megakaryocyte, CFU-megakaryocyte/erythroid, and two CFU-granulocyte (0.5%). In analogy to the defined mouse progenitors (2) we therefore termed the IL-3Rα^{lo}CD45RA$^+$ cells GMPs and the IL-3Rα^-CD45RA$^-$ cells MEPs.

Morphological features of cells in each colony are shown in Fig. 2*b*. In our culture condition, megakaryocytes were still immature on day 14; a majority displayed only one or two nuclear lobes (Fig. 2*b Left*), whereas scattered mature megakaryocytes possessed lobulated polyploid nuclei (Fig. 2*b Right Insert*).

IL-3Rα^{lo}CD45RA$^-$ Cells Give Rise to GMPs and MEPs and Contain Clonal Progenitors of Granulocyte/Macrophage and Megakaryocyte/Erythrocyte Colony-Forming Cells. To test whether the oligopotent IL-3Rα^{lo}CD45RA$^-$ cells are capable to give rise to GMPs and MEPs, 2,000–5,000 IL-3Rα^{lo}CD45RA$^-$ cells were cultured on Sys-1 stromal cells with different cytokine combinations. Development of both GMPs and MEPs was achieved with SCF, IL-11, FL, Epo, and Tpo after 72 h of culture (Fig. 3*a*). Of note, IL-3Rα and CD45RA expression levels were always higher in cultured cells than in freshly isolated bone marrow cells. In four experiments with different donors, cultured cells were re-sorted on methylcellulose (in total 250 IL-3Rα^{lo}CD45RA$^-$ cells and 300 GMPs and MEPs each). As with the freshly isolated populations, albeit with lower cloning efficiency, IL-3Rα^{lo}CD45RA$^-$ cells gave rise to all types of colonies (but CFU-GEMM), GMPs exclusively gave rise to granulocyte/macrophage colonies, and MEPs gave rise to megakaryocyte/erythrocyte colonies and four (1.6%) granulocyte and macrophage colonies (Fig. 3*b*). Therefore, the IL-3Rα^{lo}CD45RA$^-$ cells can give rise to functional GMPs and MEPs and contains likely the physiologic progenitor of both populations.

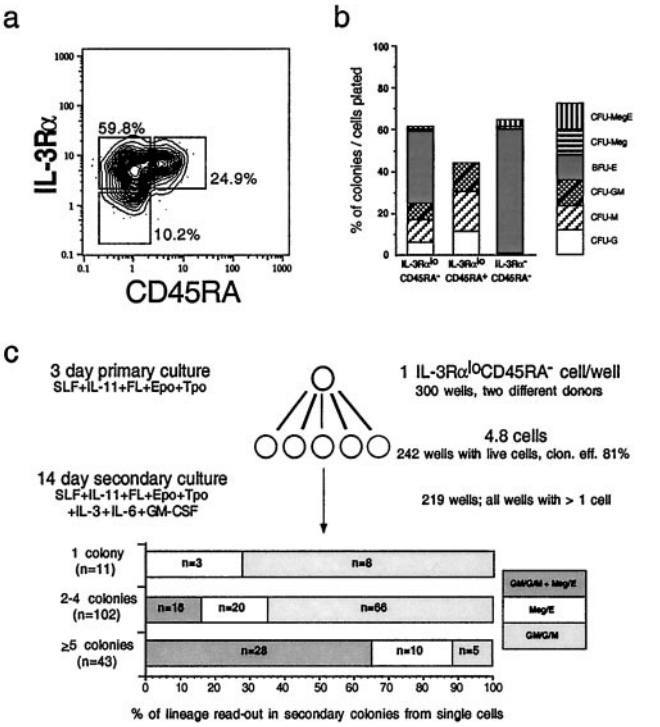

Fig. 3. Lin$^-$CD34$^+$CD38$^+$IL-3Rα^{lo}CD45RA$^-$ cells are progenitors of IL-3Rα^{lo}CD45RA$^+$ and IL-3Rα^-CD45RA$^-$ cells and have clonal bipotent granulocyte/macrophage and erythrocyte/megakaryocyte potential. (*a*) IL-3Rα^{lo}CD45RA$^-$ cells give rise to both IL-3Rα^{lo}CD45RA$^+$ and IL-3Rα^-CD45RA$^-$ cells after 72-h culture on Sys-1stromal cells in the presence of SCF, IL-11, FL, Epo, and Tpo. (*b*) Colony readout of sorted cells from primary cultures (gates in *a*) in methylcellulose. CFU-MegE, CFU-megakaryocyte/erythroid; CFU-Meg, CFU-megakaryocyte; BFU-E, burst-forming units erythroid; CFU-GM, CFU-granulocyte/macrophage; CFU-M, CFU-macrophage; CFU-G, CFU-granulocyte. (*c*) Myeloid differentiation potential of single IL-3Rα^{lo}CD45RA$^-$ cells. Single cells were expanded in primary cultures and then transferred to methylcellulose (see *Materials and Methods*).

To test whether the IL-3Rα^{lo}CD45RA$^-$ cell fraction contains cells that are either granulocyte/macrophage- or erythrocyte/megakaryocyte-committed, or whether single IL-3Rα^{lo}CD45RA$^-$ cells have both granulocyte/macrophage and megakaryocyte/erythrocyte potential, 300 single IL-3Rα^{lo}CD45RA$^-$ cells from two donors were sorted into Terasaki wells and cultured for 72 h. At this time, 242 wells contained live cells with on average 4.8 cells per well. All wells with >1 cell (219) were transferred into secondary methylcellulose cultures (Fig. 3*c*). When only 2–4 secondary colonies were formed, 15.6% of single IL-3Rα^{lo}CD45RA$^-$ cells gave rise to both granulocyte/macrophage and megakaryocyte/erythrocyte

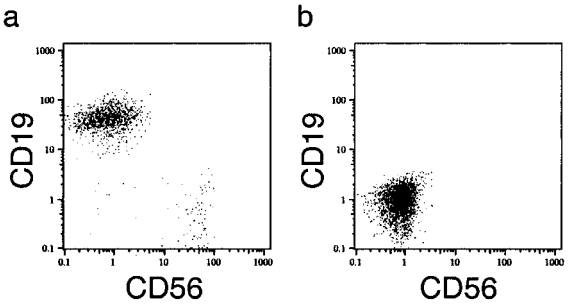

Fig. 4. IL-3Rα^{lo}CD45RA$^-$ cells contain no lymphoid-committed progenitor cells. (*a*) Development of CD19$^+$ and CD56$^+$ cells from 33 lin$^-$ CD34$^+$CD38$^+$IL-7Rα^+CD10$^+$ cells after 4 weeks of culture. (*b*) No CD19$^+$ or CD56$^+$ cells develop from 5,000 IL-3Rα^{lo}CD45RA$^-$ cells under the same conditions. Plots show cell fractions gated on human CD45$^+$ cells.

colonies; when ≥5 secondary colonies were formed, 65% of single IL-3Rα^{lo}CD45RA$^-$ cells gave rise to both types of colonies. Therefore, at least 28% (44 of 156) single IL-3Rα^{lo}CD45RA$^-$ cells that give rise to colonies in this assay have bipotent granulocyte/macrophage and megakaryocyte/erythrocyte potential. As with the cells plated from primary short-term stromal cell culture, no CFU-GEMMs were observed, in contrast to 1.4% CFU-GEMMs from IL-3Rα^{lo}CD45RA$^-$ cells directly plated in methylcellulose, indicating the loss of multipotent differentiation capacity of single cells in the primary culture. Based on these data, IL-3Rα^{lo}CD45RA$^-$ cells represent the CMP population.

CMPs, GMPs, and MEPs Contain No B or NK Cell-Restricted Progenitors. To test B and NK cell developmental capacity of the three progenitor populations, cells were cultured for 3–5 weeks on Sys-1 stromal layers with SCF, FL, IL-7, and IL-2. Under these conditions 100 lymphoid lin$^-$CD34$^+$CD38$^+$CD10$^+$IL-7Rα^+ progenitors reliably gave rise to B and NK cells, but from up to 5,000 CMPs, MEPs, or GMPs no B or NK cell readout could be detected (Fig. 4). However, up to 100 lin$^-$ CD34$^+$CD38$^-$ cells tested did not give rise to B or NK cells either, indicating that only lymphoid-committed cells were able to readout into those lineages under the given culture conditions. Therefore, although we cannot exclude a possibility that the myeloid progenitors harbor cells with some lymphoid developmental potential *in vivo*, none of the three populations contains cells readily committed to the B or NK lineage.

Myeloid Progenitors Have Limited or No Self-Renewing Capacity. To test limited self-renewal or self-renewal activity, HSC-enriched cells (lin$^-$CD34$^+$CD38$^-$), CMPs, GMPs, and MEPs were plated in limiting dilution (10–5,000 cells) in LTC-IC assays and transferred to methylcellulose after 6 weeks of culture to evaluate myeloid colony formation. Three experiments with different donors were performed. The estimated frequency of LTC-IC was 1 in 20 for lin$^-$CD34$^+$CD38$^-$ cells, 1 in 1,700 for CMPs, 1 in 4,600 for GMPs, and 1 in 5,800 for MEPs (data not shown). Therefore, myeloid progenitor cells have only limited or no self-renewing capacity in this assay.

Progenitor Populations Show Distinct, Lineage-Specific Gene Expression Profiles. To evaluate whether distinct progenitors express specific transcription factors, cytokine receptors, enzymes, and other functional proteins, we tested the expression of several genes in HSC-enriched lin$^-$CD34$^+$CD38$^-$ cells, myeloid progenitors, lin$^-$CD34$^+$CD38$^+$CD10$^+$ lymphoid progenitors, and whole CD34$^+$ cells by reverse transcription–PCR (Fig. 5). The results show expression profiles generally consistent with the distinct *in vitro* readout capacities of the different progenitors.

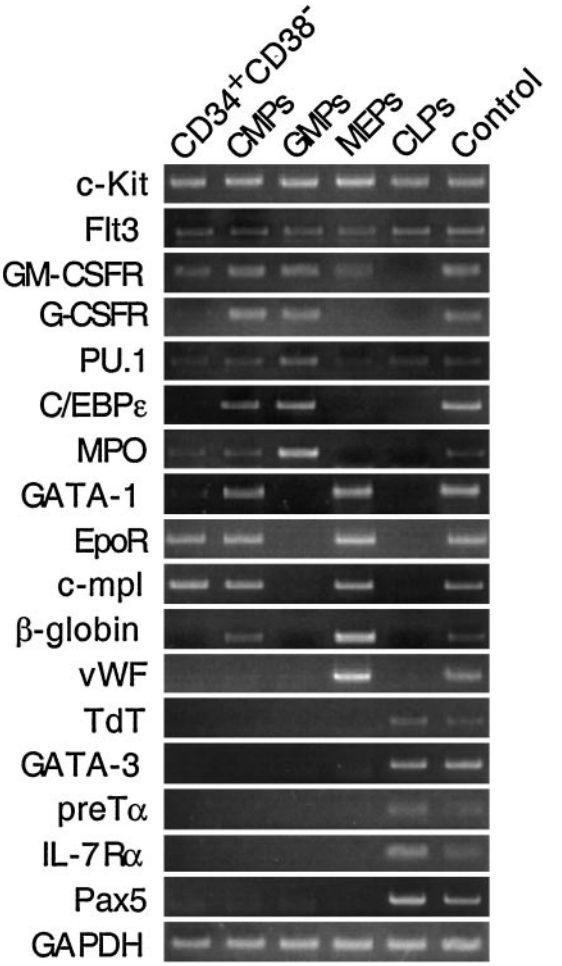

Fig. 5. Differential mRNA expression of hematopoiesis-affiliated genes in primitive progenitors (CD34$^+$CD38$^-$), myeloid-committed progenitors, and lymphoid-committed progenitors. Total CD34$^+$ bone marrow cells were used as controls. GM-CSFR, granulocyte/macrophage colony-stimulating factor receptor; G-CSFR, granulocyte colony-stimulating factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; vWG, von Willebrand factor; C/EBPε, CCAAT-enhancer-binding protein ε; MPO, myeloperoxidase; EpoR, erythropoietin receptor; TdT, terminal deoxynucleotidyl transferase.

Whereas granulocyte colony-stimulating factor receptor, C/EBPε, and MPO were expressed by GMPs and not by MEPs, GATA-1, EpoR, c-mpl, β-globin, and von Willebrand factor were detected in MEPs but not in GMPs. Interestingly, some gene transcripts such as PU.1, MPO, and GATA-1 were detectable in HSC-enriched CD34$^+$CD38$^-$ cells. It shall be important to test whether these transcripts result in protein production that is involved in cell fate decision at this early developmental stage. It shall also be important to determine whether these transcripts are present in lin$^-$CD34$^+$CD38$^-$Thy1$^+$ HSC or in Thy1$^-$ progenitors (17). Of note, none of the myeloid progenitors expressed detectable levels of genes relevant in lymphoid development as TdT, GATA-3, preTα, IL-7Rα, and Pax-5, which were expressed in the lymphoid-committed lin$^-$CD34$^+$CD38$^+$CD10$^+$ progenitors. Therefore, the gene-expression profile supports the exclusive separation of the lymphoid and myeloid and further the downstream granulocyte/macrophage and megakaryocyte/erythrocyte developmental pathway.

CMPs, GMPs, and MEPs Can Be Isolated from Cord Blood. Two cord blood samples were evaluated for the above surface-marker expression, and 100 cells of each population were tested for

myeloid colony-formation activity. Although the distinct surface-marker expression profile was similar to adult bone marrow, percentages of the myeloid progenitor populations were slightly different: IL-3Rα^{lo}CD45RA$^-$ CMPs account for ≈0.4%, IL-3Rα^{lo}CD45RA$^+$ GMPs for ≈0.3%, and IL-3Rα^-CD45RA$^+$ MEPs for ≈0.05% of the mononuclear cell fraction (data not shown). HSC-enriched lin$^-$CD34$^+$CD38$^-$ cells and CMPs formed all types of colonies with cloning efficiencies of 68 and 83%, respectively. GMPs formed exclusively granulocyte/macrophage colonies (cloning efficiency 41%), and MEPs formed megakaryocyte/erythrocyte colonies (cloning efficiency 88%) with only 4% granulocyte/macrophage colony readout (data not shown). Therefore, myeloid progenitor cells with similar lineage restrictions can be found in cord blood.

Discussion

In this study we dissect by prospective isolation human multipotent hematopoietic progenitor cells from the earliest committed CMPs and further the downstream GMPs from MEP-committed stages. We directly show that CMPs are clonogenic progenitors of both GMPs and MEPs. The phenotype of these populations does not overlap with that of HSCs, and the defined populations have only very low LTC-IC activity. Furthermore, none of these myeloid progenitors exhibit *in vitro* differentiation activity for B or NK cells. We did not test the T cell potential of these populations; however, myeloid progenitors express neither early lymphoid markers such as CD7 and CD10 (7, 8) nor IL-7Rα (not shown), which is expressed in human T cell progenitors and transduces indispensable signals in human T cell development (25). In two experiments we transplanted 1.5×10^5 CD34$^+$CD38$^-$ HSC-enriched cells, 3.8×10^5 CMPs, and 1.5×10^5 MEPs into sublethally irradiated NOD/SCID/β_2m$^{-/-}$ recipients. Three weeks after transplantation, mouse bone marrow contained 21, 30, and 3% human CD45$^+$ and/or CD71$^+$ nucleated cells derived from CD34$^+$CD38$^-$ cells, CMPs, and MEPs, respectively. CD34$^+$CD38$^-$ cells and CMPs gave rise to both glycophorin A$^+$ erythroid cells and CD15$^+$ myelomonocytic cells, whereas MEPs exclusively gave rise to glycophorin A$^+$ erythroid cells (not shown). Thus, considering their high cloning efficiency, expansion capacity, and restricted differentiation potentials, these populations are likely the human counterparts of mouse myeloid progenitors. Although B cell potentials are undetectable in myeloid progenitors *in vitro*, *in vivo* progeny of 3.8×10^5 CMPs contained a small number (0.1%) of CD19$^+$ cells (not shown). It is unclear whether the minor B cell progeny is derived from contaminants of lymphoid precursors among a high number of CMPs transplanted or whether putative bipotent B cell/myeloid progenitors share their surface phenotype with CMPs. It shall be important to clarify this issue by future studies, because minor B cell potentials were observed similarly in both fetal and adult murine CMPs (2, 26).

The defined myeloid progenitors exhibit distinct surface-expression patterns of IL-3Rα and CD45RA. IL-3 is an early cytokine that supports proliferation and differentiation of HSCs as well as primitive multipotent and myeloid progenitors (27–29), whereas the receptor tyrosine phosphatase CD45, and presumably its CD45RA isoform, can negatively regulate at least some classes of cytokine-receptor signaling (30). Several previous studies have been communicated concerning the surface-antigen expression of early myeloid progenitor cells in humans (3, 6, 9–12). It was shown that CD34$^+$CD45RA$^+$ cells are devoid of erythroid and enriched for granulocyte and macrophage progenitor cells (6, 10). Also, different levels of IL-3Rα expression on CD34$^+$ cells reportedly are associated with preferential lineage readout, as IL-3Rα^- cells are enriched for erythroid, IL-3Rα^{lo} cells for multipotent, and IL-3Rα^+ cells for granulocyte/macrophage colony-forming cells (9). However, in those studies early lymphoid cells, lineage low or positive cells, and/or HSCs and multipotent progenitors within the CD34$^+$ fraction were not excluded by using negative or positive markers, likely resulting in heterogeneous populations. When reported, the populations exhibited relatively low cloning efficiencies (6, 12) or contained multipotent progenitors and HSCs (9). In our study, the relationship between the expression pattern of CD45RA/IL-3Rα and lineage potentials within the myeloid progenitor fraction is consistent with previous reports. The additional use of a panel of markers enabled us to highly purify lineal populations of CMPs and downstream GMPs and MEPs, which represent developmental checkpoints for erythroid/megakaryocyte versus granulocyte/macrophage lineages.

It was reported also that CD64 (FcγR I)-expressing cells in the human CD34$^+$ bone marrow fraction (≈23% of fetal bone marrow CD34$^+$ cells) contained granulocyte/macrophage-committed progenitors at a relatively low frequency (cloning efficiency 26–50%; refs. 11 and 12). We could not find CD64 expression on the lin$^-$CD34$^+$CD38$^+$ cells by using antibodies from hybridoma clone 10.1, which also were used in these previous studies (11). The most likely explanation for this finding is that the CD64$^+$ cells were gated out in our study by using CD11b as a lineage marker, because the vast majority of CD64$^+$ cells reportedly coexpress CD11b (11). Therefore it is possible that the CD64$^+$ progenitors may reside in a lineage low to positive fraction, which is likely to be downstream of GMPs. Indeed, although the progenitor populations described here cover most of the colony-forming cells, we found a low frequency of both erythroid and myeloid colony-forming cells in the CD34$^+$CD38$^+$lin$^+$ fraction. We propose that most if not all myeloid hematopoiesis is developing through the here-described progenitors; however, we cannot exclude the existence of other, parallel-developing, minor progenitor populations that express lineage markers.

In mouse hematopoiesis, CMPs, GMPs and MEPs all are contained in the lin$^-$c-Kit$^+$Sca-1$^-$ population and are distinguished further based on the expression profile of CD34 and the FcγR II/III (CD16/CD32; ref. 2). In contrast, all the human myeloid progenitor populations described here are positive for CD34 and do not express detectable levels of CD16/CD32. We therefore in turn looked for IL-3Rα and CD45RA expression on murine myeloid progenitors (2). Interestingly, in mouse hematopoiesis, IL-3Rα as well as CD45RA expression both were negative to low in MEPs, intermediate in CMPs, and positive in GMPs, without providing sufficient discrimination to clearly set a cut-off level between the different cellular fractions (not shown). Thus, a number of markers expressed in progenitor or stem cells seem to be differentially regulated in early human and mouse myelopoiesis (31).

The prospectively purified, lineage-restricted human progenitors at defined myeloid developmental stages shown here should provide tools to study transcriptional regulation as well as the role of intrinsic and/or extrinsic signals in lineage commitment (32). Also, common developmental pathways or lineage origins of rare hematopoietic cells such as dendritic cells can be evaluated directly (33, 34). Finally, these progenitors might be useful in determining stages at which leukemogenic events occur (17, 35–38).

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