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Coexistence of LMPP-like and GMP-like Leukemia Stem Cells in Acute Myeloid Leukemia

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SUMMARY

The relationships between normal and leukemic stem/progenitor cells are unclear. We show that in ~80% of primary human CD34+ acute myeloid leukemia (AML), two expanded populations with hemopoietic progenitor immunophenotype coexist in most patients. Both populations have leukemic stem cell (LSC) activity and are hierarchically ordered; one LSC population gives rise to the other. Global gene expression profiling shows the LSC populations are molecularly distinct and resemble normal progenitors but not stem cells. The more mature LSC population most closely mirrors normal granulocyte-macrophage progenitors (GMP) and the immature LSC population a previously uncharacterized progenitor functionally similar to lymphoid-primed multipotential progenitors (LMPPs). This suggests that in most cases primary CD34+ AML is a progenitor disease where LSCs acquire abnormal self-renewal potential.

INTRODUCTION

Prospective purification and characterization of stem/progenitor cells in normal hemopoiesis and leukemia has led to seminal observations in stem cell and cancer biology. In both human and mouse, multipotent hemopoietic stem cells (HSCs) generate a hierarchy of proliferative progenitor populations that progressively lose lineage potential as they pass through lineage restriction points. In mouse, the initial lineage division was originally suggested to be strictly into separate common myeloid-erythroid and

Significance

Understanding how normal stem/progenitor cells are transformed into cancer stem cells (CSCs) is a central biological and clinical question. Progress depends on defining relationships between CSCs and normal stem/progenitor cells. Seminal studies in AML initially suggested LSCs might be closely related to normal hemopoietic stem cells (HSCs) and not progenitors. Subsequent studies have not clarified the issue. We now define distinct, hierarchically organized LSC populations most closely related to normal progenitors, in most primary human AML patients. The more immature LSC is LMPP-like, providing support for the evolutionary conservation of hemopoietic lineage specification. Going forward, this work provides a platform for determining pathological LSCs self-renewal and tracking LSCs post treatment, both of which will impact on leukemia biology and therapy.

lymphoid lineages. But recent persuasive data suggest that multipotential progenitors (MPPs) initially differentiate into lymphoid-primed multipotential progenitors (LMPPs, with lymphoid and granulocyte-macrophage but no megakaryocyte-erythroid [MkE] potential) and those with MkE potential (Adolfsson et al., 2005; Arinobu et al., 2007; Lai and Kondo, 2006; Yoshida et al., 2006). More committed myeloid and lymphoid progenitors lie downstream (Akashi et al., 2000; Pronk et al., 2007).

In human, HSCs reside in the Lin-CD34+CD38-CD90+CD45RA- compartment and generate Lin-CD34+CD38-CD90-CD45RA- MPP with lymphomyeloid potential (Majeti et al., 2007). Downstream of MPP is an incompletely characterized Lin-CD34+CD38-CD90-CD45RA+ population (termed CD38-CD45RA+ hereafter). More committed Lin-CD34+CD38+ common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP) and megakaryocyte-erythroid progenitors (MEP) can be separated by differential expression of CD123 (IL3RA), CD110 (MPL), and CD45RA (Edvardsson et al., 2006; Manz et al., 2002). However, the transition and lineage restriction steps between early CD34+CD38- stem/progenitor populations and later CD34+CD38+ progenitors are poorly defined. More specifically a human LMPP-like population has not yet been identified.

Acute myeloid leukemia (AML) is the most common adult acute leukemia. It is genetically heterogeneous and molecular changes have been important in defining prognosis and therapy response (for review, see Dohner et al., 2010). Many patients still die of disease relapse after an initial therapy response, thought to be due to failure to eradicate chemoresistant leukemic stem cells (LSCs).

Seminal studies in AML (for review, see Dick, 2008) have led to the cancer stem cell (CSC) hypothesis, which postulates that cancers are organized in cellular hierarchies, like normal tissues. At the hierarchy apex are multipotent, largely quiescent, long-lived CSCs with marked self-renewal capacity that sustain disease and differentiate into "bulk cancer cells." Initial reports suggested that AML LSCs most closely resemble normal HSCs, sharing a common limited immunophenotype (CD34+CD38-) and in being rare populations (Bonnet and Dick, 1997; Ishikawa et al., 2007).

However, the notion that LSCs reside solely in the CD34+CD38- compartment has been challenged. Modeling of AML in mice has shown that more mature GMPs produce transplantable LSCs (Cozzio et al., 2003; Huntly et al., 2004; Krivtsov et al., 2006; So et al., 2003). Specifically, in blast crisis chronic myeloid leukemia candidate LSCs are GMP-like (Jamieson et al., 2004). Using more immunodeficient xenotransplant models, primary human AML cells from both CD34+38- and CD34+CD38+ compartments have LSC activity (Taussig et al., 2008; Yoshimoto et al., 2009). Finally, in ~20%–40% of primary AML, >80% of cells are CD34-; in some cases, where the percentage of CD34+ is <0.5%, LSC activity is present only in the CD34- compartment or in some cases where the percentage of CD34+ cells lies between 1%–20%, LSC activity has been detected in both CD34+ and CD34- compartments or only in CD34+ compartment (Martelli et al., 2010; Taussig et al., 2010).

Moreover, studies to date have not clarified the relationship between human AML LSCs and normal stem/progenitor populations. For example, not only does the normal CD34+CD38- compartment contain HSCs, but it also contains other progenitors (Majeti et al., 2007). Moreover, as cell surface marker expression can be aberrant in leukemia, it is not possible to decipher how LSCs map back onto normal HSCs/progenitors that share an immunophenotype. More broadly, questions have been raised about the rarity of CSCs (le Viseur et al., 2008; Quintana et al., 2008).

Therefore, we set out to clarify the cellular and molecular relationships between different AML LSC populations within a patient, between different patients, and finally, between AML LSC and normal HSC/progenitor populations to define molecular pathways important in transformation of normal HSCs/progenitors. We have focused on primary CD34+ AML (where >5% of cells are CD34+) that comprises the majority of AML. As human HSC/progenitor cells express CD34, we reasoned the stem/progenitor hierarchy in CD34+ AML might be more comparable to normal hemopoiesis.

RESULTS

Immunophenotype of Human CD34+ AML

We compared stem/progenitor-cell immunophenotypes in primary CD34+ AML and normal control samples. The immunophenotypic gating strategy for normal populations is illustrated in Figure S1A (available online) and was validated by post FACS-sort purity analysis (>99% pure) (Figure S1B). In vitro and in vivo assays confirmed the functional potential of sorted normal stem/progenitor cells (data not shown; see later in Figure 5). We immunophenotyped 100 primary AML samples (69 de novo, 20 secondary, 6 relapsed, and 5 refractory AML) spanning FAB subtypes, cytogenetic and molecular categories (Table 1; Table S1), and 8 age-matched control marrow samples.

There are two major immunophenotypic groups in CD34+ AML with respect to these markers (Figure 1and Table 1; Table S1). In 87.8% of 74 AML samples, most Lin-CD34+CD38-cells are Lin-CD34+CD38-CD90-CD45RA+ (CD38-CD45RA+) (>90% in most cases) and in 15 randomly selected cases have low/no CD10 and CD7 expression (data not shown). Hereafter, this group is named "CD38-CD45RA+ expanded" group (Figure 1Ab). In this group, most Lin-CD34+CD38+ cells are Lin-CD34+CD38+CD123+/loCD110-CD45RA+ (hereafter termed "GMP-like" as they share the immunophenotype of normal GMPs) (Figure 1Ae). Only CD38 is consistently expressed differently between CD38- CD45RA+ and the GMP-like populations (data not shown).

Less commonly, in 13.8% of 74 primary AML samples, there is a dominant Lin-CD34+CD38-CD90-CD45RA- population (>90% in most cases). Hereafter, this group is named "MPP-like expanded" group (Figure 1Ac). In this group, there is a corresponding major Lin-CD34+CD38+CD123+/loCD110-CD45RA- population that shares the immunophenotype of normal CMPs (Figure 1Af). The major difference in antigen expression between the two CD34+ populations was CD38. Segregation of AML cases into CD38-CD45RA+ expanded and MPP-like expanded groups did not correlate with morphology or genetic markers.

Within these two groups, CD38 expression varies between AML samples (Figure 1B; Table S1) (Terstappen et al., 1992). Furthermore, within CD34+CD38- (Figure 1C) and CD34+CD38+ compartments (Figure 1D; Table S1), there are intersample differences of minor populations. Two possible

Table 1. Summary of Clinical, Cytogenetic, and Molecular Features of AML Samples Studied

Diagnosis		De Novo AML	Refractory AML	Relapsed AML	2° AML
Number of samples studied		69	5	6	20
Mean age (yrs) (range)		61 (20-85)	60 (47-68)	67 (57-74)	67 (42-85)
Mean blast count (%) (range)		70% (96-23)	58% (28-82%)	43% (20-75)	40% (20-94)
FAB type	Number FAB type available	58	5	6	NA[1]
	M0	6	0	0	
	M1	15	2	2	
	M2	11	1	1	
	M3	1	0	0	
	M4	14	2	2	
	M5	11	0	1	
	Other	10	0	0	0
Cytogenetics	Number analyzed	63	5	6	15
	Good risk[2]	8	0	0	0
	Intermediate risk[2]	42	3	2	11
	Poor risk[2]	13	2	4	4
FLT-ITD mutation	Number analyzed	54	5	5	15
	mutant (ITD/TK)[3]	14/1	0/0	0/0	2/0
	Wild-type	39	5	5	9
NPM mutation	Number analyzed	59	5	5	15
	Mutant	7	0	0	0
	wild type	52	5	5	15
c-kit mutation	Number analyzed	53	4	4	15
	mutant	1	0	0	0
	Wild-type	52	4	4	15
Dominant population in CD34+CD38- compartment	Number analyzed	58	2	1	13
	CD90-CD45RA+ (%)	50 (86.2)	2 (100)	1 (100)	12 (92.3)
	CD90-CD45RA-(%)	8 (13.8)	0	0	1 (7.8)
Dominant population in CD34+CD38+ compartment	Number analyzed	69	5	6	20
	CD110-CD45RA+ (%)	58 (84.1)	5 (100)	4 (66.7)	16 (80)
	CD110-CD45RA- (%)	11 (15.9)	0	2 (33.3)	3(15)
	CD110+CD45RA- (%)	0	0	0	1 (5)

FAB, French-American-British morphologic classification of AML (Bennett et al., 1976). Cytogenetic risk scores are based on the MRC classification (Grimwade et al., 2010). *FLT3*, *FMS-like Tyrosine kinase 3* gene. *ITD,* Internal tandem duplication mutation within *FLT3* gene. *TK*, tyrosine kinase domain mutation within *FLT3* gene. *NPM1*, *Nucleophosmin* gene. *KIT*: *KIT* gene. NA, not applicable.

reasons for these differences include (1) molecular heterogeneity between AML samples and (2) different degrees of admixture of normal and malignant cells. Despite this variability, 87.8% of a broad range of CD34+ AML samples have one dominant CD38-CD45RA+ population and a corresponding GMP-like population.

Leukemic Stem Cell Hierarchy in CD38-CD45RA+ Expanded AML

We focused on dissecting which populations had LSC activity within the major CD38-CD45RA+ expanded group. Previous data had shown that LSC activity resides in the CD34+ compartment in CD34+ AML (Blair et al., 1997; Bonnet and Dick, 1997). We assessed LSC activity in 12 patients where we FACS-sorted both CD38-CD45RA+ and GMP-like populations from each patient. Six of 12 gave primary engraftment; 5/6 primary and secondary engraftment (Figure 2), and 4/5 tertiary engraftment (Figure S2D). Samples details are in Table S2. Cells (10^5) of each leukemic population from each patient were intravenously injected into four NOD-SCID mice treated with anti-CD122 antibody to remove residual NK-cells (Figure 2A). Cells from both populations of the six engrafting samples were detected in marrow of all injected mice with similar levels of engraftment. FISH analysis from two AML patients with cytogenetic abnormalities, showed 90%–100% of engrafted human cells were leukemic (Figure 2B; Table S3).

FACS analysis confirmed that for both injected populations human CD34+ and CD34- cells were detected in mice (Figures 2Ca and 2Cd). In mice injected with CD38-CD45RA+ (Figures 2Cb and 2Cc) and GMP-like (Figures 2Ce and 2Cf) cells, nearly all human CD34+ and CD34- cells were CD33+CD19-, did not

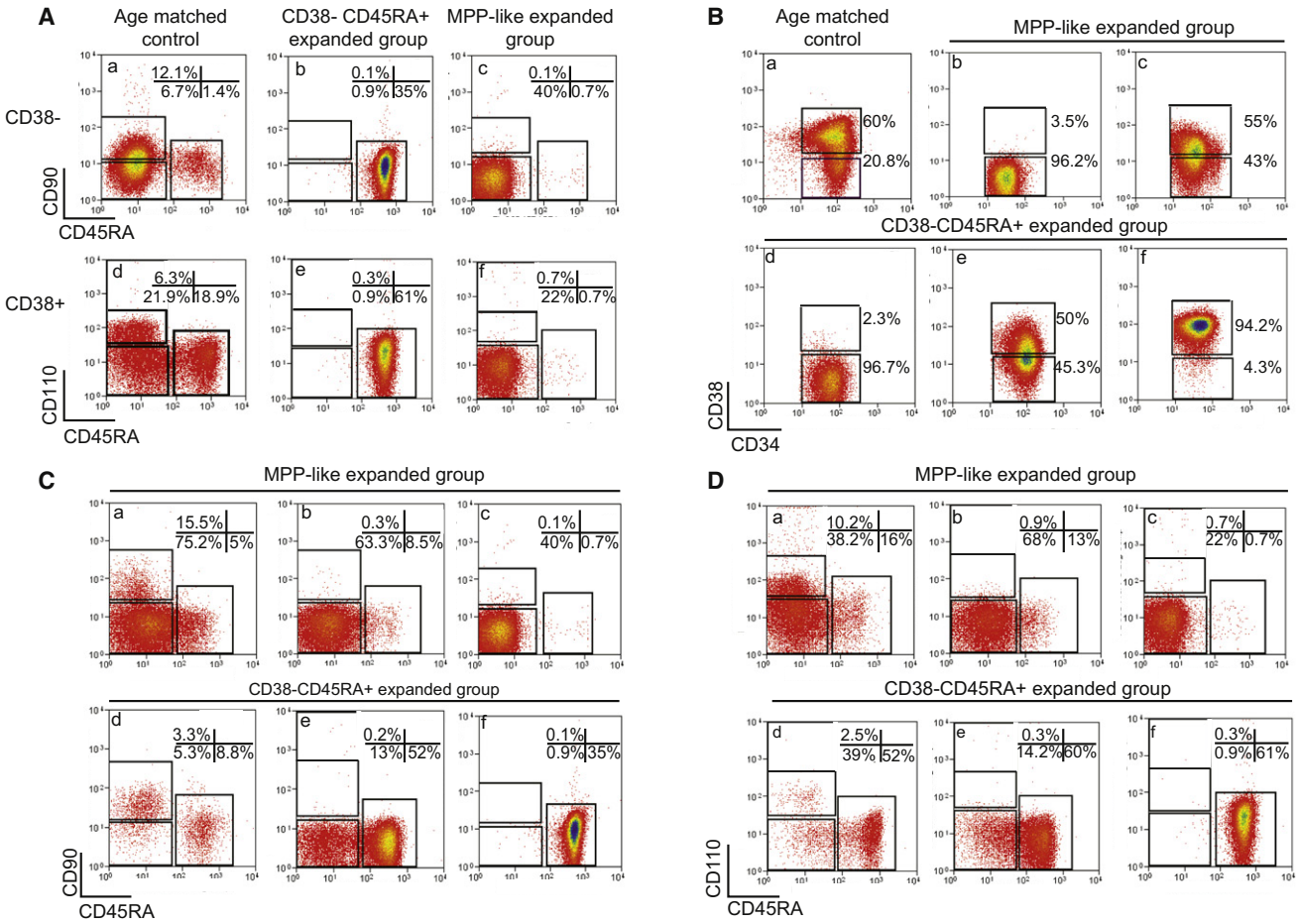


Figure 1. Human CD34+ AML Has Expanded Immunophenotypic Progenitor Populations

(A) CD90 and CD45RA expression in CD34+CD38- cells (top) and CD110 and CD45RA expression in CD34+CD38+ cells (bottom) from control sample (left), an AML sample where CD38-CD45RA+ population is expanded (center CD38-CD45RA+ expanded) and an AML sample where CD45RA- population is expanded (right MPP-like expanded). Positions of normal HSC, MPP, CD38-CD45RA, CMP, GMP, and MEP are shown.

(B) Patterns of CD38 expression in CD34+ cells in MPP-like expanded AML (top) and CD38-CD45RA+ expanded AML (bottom) with normal control shown (top left).

(C) Patterns of CD90 and CD45RA expression in CD34+CD38- cells in MPP-like expanded (top) and CD38-CD45RA+ expanded (bottom) AML.

(D) Patterns of CD110 and CD45RA expression in CD34+CD38+ cells in MPP-like expanded (top) and CD38-CD45RA+ expanded (bottom) AML.

Plots are representative examples. Percentage values in quadrants are the average of lin-CD34+ cells for all samples within the group.

See also Figure S1 and Table S1.

express T cell or erythroid markers (CD3 and CD235) and were blast-like (data not shown), consistent with leukemic myeloid-restricted engraftment. There was no LSC activity in the CD34- compartment in 4/4 samples where engraftment from CD34 compartments was noted (Figure S2A). In all four samples, the percentage of CD34+ cells was >20%. Thus, our data confirm previous reports showing engraftment from CD34- cells when >99% of the cells are CD34- (Martelli et al., 2010; Taussig et al., 2010).

In mice injected with CD38-CD45RA+ cells, a minor fraction of human CD34+ cells were CD38-CD45RA+ (mean 1.3% for all patients), consistent with self-renewal of this population (Figures 2Da and 2Db). CD38-CD45RA+ cells also gave rise to GMP-like cells in vivo (Figure 2Dc). In contrast, when GMP-like cells are injected in mice, <0.2% of CD34+ cells are CD38-CD45RA+ (Figure 2Dd). Most injected GMP-like cells retain CD110-CD45RA+ phenotype (Figure 2De). Taken together, this suggests an in vivo immunophenotypic hierarchy where a minority of CD38-CD45RA+ cells self-renew and give rise to GMP-like cells whereas GMP-like cells cannot give rise to CD38-CD45RA+ cells.

Next, we confirmed that CD38-CD45RA+ and GMP-like AML populations have serial engraftment activity. Human cells (10^6) from primary recipients were injected into secondary recipients (Figure 2E). Engraftment was seen from five of six samples that showed primary engraftment, with similar secondary engraftment levels between cells taken from primary hosts transplanted with either CD38-CD45RA+ or GMP-like cells. Lower engraftment level was seen in secondary compared with primary hosts as previously reported (Bonnet and Dick, 1997). Immunophenotypic analysis of engrafted cells showed the fact that >99% of human cells were CD33+CD19- (Figures S2B and S2C). Most hCD34+ cells were GMP-like regardless of whether injected cells

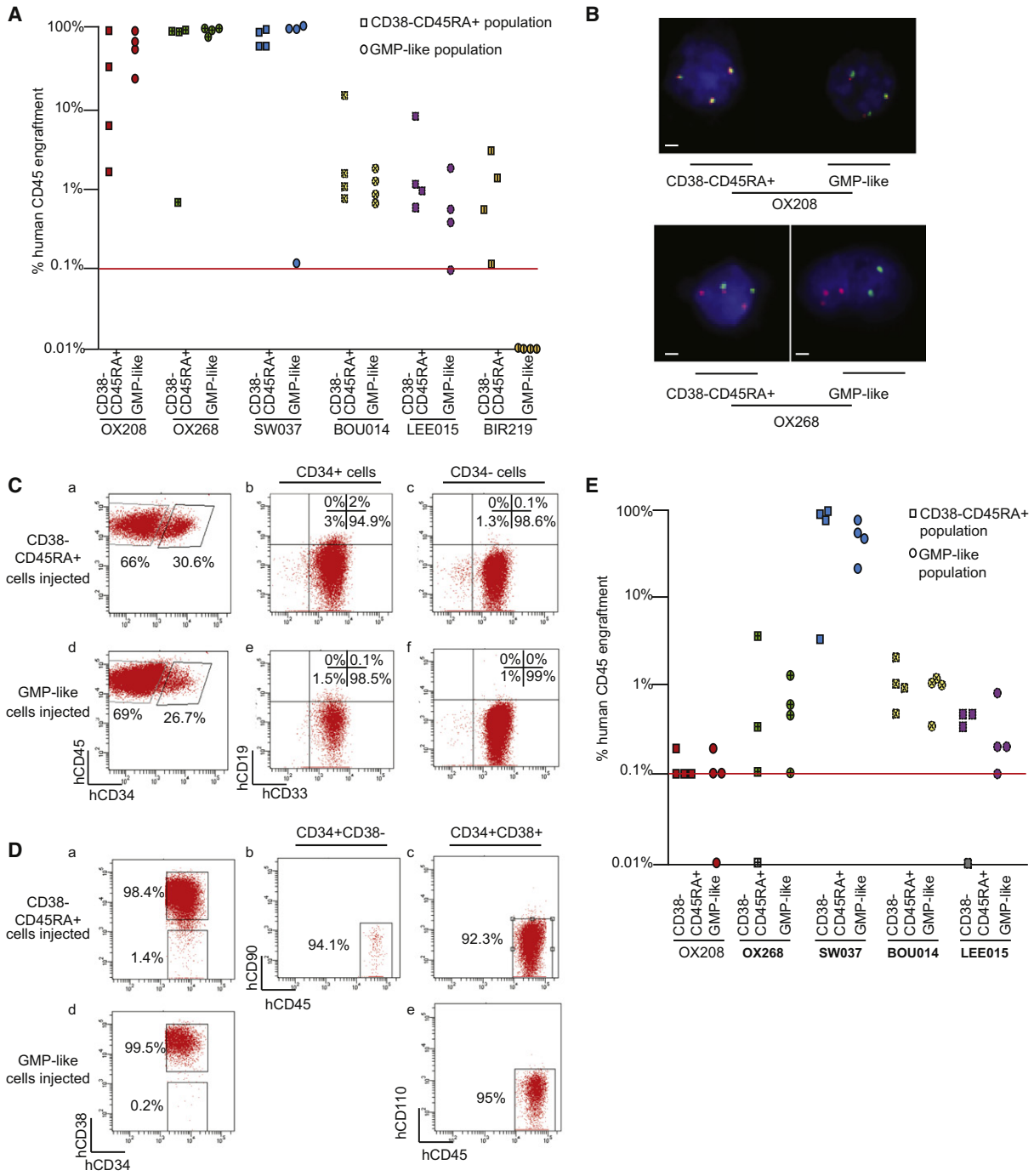


Figure 2. Two Classes of Hierarchically Ordered LSC Activity Coexist in Most AML Patients

(A) Percentage marrow engraftment of hCD45RA+ cells in individual NOD-SCID mice 12 weeks after transplantation from six different AML patient samples. CD38-CD45RA+ cells (squares) and GMP-like populations (ovals) from each patient were injected into four mice.

(B) FISH analysis of engrafted populations from patient OX208 (left) and OX268 (right). The cell on the left is from a mouse engrafted with CD38-CD45RA+ cells and that on the right engrafted with GMP-like AML cells. In patient OX 208, chromosome 14 is detected by a dual color IGH locus probe and in patient OX268, chromosomes 8 (red signal) and 12 (green) are detected by centromere probes. Cell nuclei are visualized by DAPI counterstain. Two micrometer scale bars are shown.

(C) A representative example of hCD45/hCD34 expression (left, a and d) hCD19/hCD33 expression in hCD34+(center, b and e), and CD34- (right, c and f populations from primary engrafted mice when transplanted with CD38-CD45RA+ (top) and GMP-like (bottom) AML cells. Mean percentage values from all six patients are shown.

(D) A representative example of hCD38 and hCD34 expression on engrafted hCD34+ cells (left, a and d), hCD90/hCD45RA expression in hCD34+CD38- cells (center, b) and hCD110/hCD45RA expression (right, c and e) in hCD34+CD38+ cells when CD38-CD45RA+ cells (top) and GMP-like AML (bottom) cells are injected into primary engrafted mice. Mean percentage values from all six patients are shown.

Table 2. Leukemia Initiating Cell Frequency in CD38-CD45RA+ and GMP-Like LSC-Containing Populations

		Cell Dose				
Sample	Fraction	1.00E+02	1.00E+03	1.00E+04	1.00E+05	LIC[a] frequency (95% confidence interval)
OX208	CD38-CD45RA+	1/4	1/4	3/3	3/3	1 in 1837 (495-6813)
	GMP-like	0/3	0/3	0/4	3/4	1 in 88550 (28014-279897)
OX268	CD38-CD45RA+	1/4	2/4	4/4	4/4	1 in 1064 (319-3543)
	GMP-like	0/4	0/4	3/4	4/4	1 in 8879 (2815-28005)
BIR219	CD38-CD45RA+	0/4	1/4	2/4	4/4	1 in 10777 (3252-35718)
	GMP-like	0/4	0/4	0/4	0/4	>1.00E+05

NOD/SCID mice were used as hosts for samples OX208 and OX268. NSG mice were used as hosts for samples from BIR219. Mice were sacrificed at 10 weeks. Data are reported as number of mice with AML/number of mice receiving transplant.
[a] LIC, leukemia-initiating cell.

were from animals initially injected with CD38-CD45RA+ or GMP-like LSCs, consistent with differentiation of CD38-CD45RA+ cells into GMP-like cells but not the reverse.

To assess leukemia initiating cell (LIC) frequency FACS-sorted CD38-CD45RA+ and GMP-like populations from three different primary AML samples were assayed (Table 2). The LIC frequency in the CD38-CD45RA+ compartment varied between 1/1064 to 1/10 777 and was much lower in GMP-like population (1/8879 to >1/100 000). This suggests the CD38-CD45RA+ population has more potent LSC activity.

Finally, we asked if leukemic cells expanded after injection into transplanted mice (Table S4). For samples where LIC frequency had been established, there was a 10^4–10^6-fold expansion of engrafted cell numbers in primary transplants from injected LSC pool. In cases where we could only calculate fold expansion of engrafted cells as a function of injected cells the fold expansion varied from 1.5–10^3 in both primary and secondary transplants.

In summary, both CD38-CD45RA+ and GMP-like populations, within the same patient, have LSC activity. CD38-CD45RA+ LSCs give rise to cells with a GMP-like phenotype but the converse does not occur.

In Vitro Differentiation of AML LSC Populations

To complement the in vivo observations, we tested the differentiation potential of CD38-CD45RA+ cells and GMP-like populations in vitro. FACS-sorted CD38-CD45RA+ and GMP-like cells from five AML patients (ten populations) were cultured and cell output analyzed (Figure 3). After 4 days, most CD38-CD45RA+ cells remained CD38-CD45RA+ (Figure 3Bb). Remaining input CD38- cells gained CD38 expression and were GMP-like (Figure 3Bb). By day 8, most CD38-CD45RA+ cells were GMP-like and some cells had lost CD34 expression; consistent with differentiation to CD34- blasts (Figure 3Bc). Small numbers of cells remained CD38-CD45RA+.

After 4 days of culture of most GMP-like cells, retained their original immunophenotype. Some cells had already lost CD34 expression (Figure 3Bc). By day 8, more cells were CD34- and though GMP-like cells were still present (Figure 3Bc). There was no differentiation of CD34+CD38+ cells into CD34+CD38- cells. Thus, in vivo and in vitro data support the notion that the CD38-CD45RA+ AML population lies upstream of a GMP-like population at the apex of an LSC hierarchy in most AML samples.

Gene Expression Profiles of AML LSC and Normal Hemopoietic Stem/Progenitor Cells

We analyzed global mRNA expression profiles from 22 FACS-sorted CD38-CD45RA+ and 21 GMP-like populations from 27 AML patients. In 18/27 patients we compared expression profiles between the two populations within each patient, negating the effect of genetic/epigenetic changes between patients. We also obtained normal HSC, MPP, CMP, GMP, and CD38-CD45RA+ populations from four different age-matched marrow samples. We asked two questions: (1) Are the two AML LSC populations (CD38-CD45RA+ and GMP-like) molecularly distinct? (2) Which normal populations are the two AML LSC populations most similar to at a molecular level?

We used two approaches to determine if expression profiles for the two AML LSC populations were distinct. First, an unpaired standard t test (cutoff 0.01) gave a list of genes (360 genes - 458 probes) differentially expressed between 22 CD38-CD45RA+ and 21 GMP-like populations. Expression profiles of this gene set displayed by 3D Principal Component Analysis (PCA) (Figure 4A) shows 14/21 of the two populations well separated, but 7/21 GMP-like populations interspersed with CD38-CD45RA+ populations. This was confirmed by a paired t test (cutoff 0.01) applied to CD38-CD45RA+ and GMP-like cells from the subset of 18 AML cases where both populations were available from the same patient (917 probes; 748 mapped genes) (Figure S3A) and a moderated t test ($p = 0.05$) to correct for multiple testing (54 probes corresponding to 49 annotated genes) (false discovery rate of 0.05) (Figure S3B). We also examined the relationship between CD38-CD45RA+ AML and GMP-like AML populations by hierarchical clustering (Figure S3C). Most GMP-like and CD38-CD45RA+ AML populations cluster separately with 5/21 GMP-like and 1/22 CD38-CD45RA+ AML populations being interspersed.

(E) Percentage engraftment of hCD45RA+ cells in marrow of individual secondary transplanted mice transplanted with pooled hCD45+ cells from primary engrafted mice initially injected with either CD38-CD45RA+ (squares) or GMP-like (oval) cells (each primary leukemia is shown as a different symbol). Four secondary recipient mice were injected per population.
See also Figure S2 and Tables S2–S4.

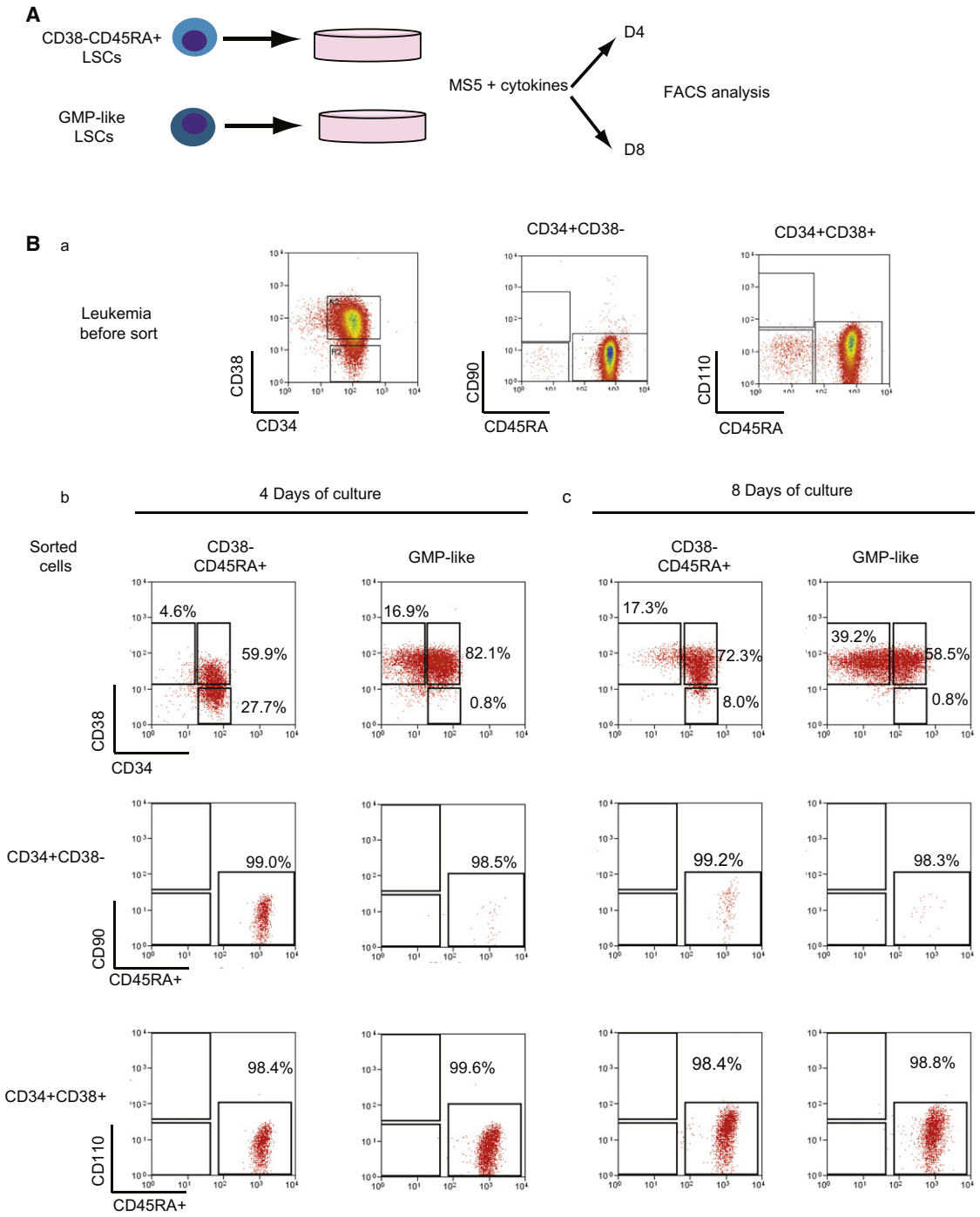


Figure 3. In Vitro Differentiation Hierarchy in CD38-CD45RA+ Expanded Group of AML

(A) FACS-sorted CD38-CD45RA+ or GMP-like populations from five AML samples were separately cultured for either 4 or 8 days, and then analyzed by FACS.
(B) (a) Top left, representative example of CD34 and CD38 expression in an AML sample. The purity of CD38-CD45RA+ (center) or GMP-like (right) populations post-FACS sort and prior to culture is shown.

Below, representative FACS plots of cells when CD38-CD45RA+ and GMP-like populations have been cultured for either 4 days (b) or 8 days (c). Top panel, CD34/CD38 expression; middle panel, CD90/CD45RA expression in CD34+CD38- cells; bottom panel, CD110/CD45RA expression in CD34+CD38+ populations. Percentage shown are mean for gated populations for all five AML samples studied.

To obtain a quantitative measure of the difference in gene expression between the two AML LSC populations, we compared expression between CD38-CD45RA+ and GMP-like populations from the same patient, by a non-parametric (rank product) method (Figure 4B). With a false discovery rate of 0.05 (pfp = 0.5), 524 mapped genes (567 probes) were

expressed more highly in CD38-CD45RA+ compared with GMP-like populations (Figure 4Ba). Similarly, 2054 genes (2201 probes) were more highly expressed in GMP-like compared with CD38-CD45RA+ populations (Figure 4Bb). CD38 was among the top twenty most differentially expressed genes. Taken together, CD38-CD45RA+ and GMP-like LSC populations are molecularly distinct in most cases.

To determine which normal populations the two AML LSC populations most closely resemble molecularly, we used ANOVA to establish a 2626 gene set (2789 probes) that maximally distinguish normal stem and progenitor populations. 3-D PCA was used to display profiles from the ANOVA curated gene set from normal populations (Figure 4C). The signature of normal HSCs was most closely related to the normal MPPs, with the normal CD38-CD45RA+, CMP and GMP populations more widely scattered. Individual expression profiles within normal immunophenotypic populations were tightly clustered.

Next, using this same 2626 gene set, the expression profiles of 22 CD38-CD45RA+ and 21 GMP-like AML populations were distributed (Figure 4Ca and b). Both AML populations were more widely dispersed (presumably reflecting genetic/epigenetic heterogeneity in AML). CD38-CD45RA+ and GMP-like AML populations mainly clustered around their normal counterpart population. However, some GMP-like AML populations were located closer to normal CD38-CD45RA+ cells. Similar results were obtained with a 1338 Annova gene set (1519 probes) of differentially expressed genes after p-value correction (Figures S3D and S3E).

We used the same 2626 ANOVA gene set as a classifier to ask which normal population did each individual AML LSC populations most closely resemble (Figures 4D–4G). 17/22 (77%) CD38-CD45RA+ AML populations were classified as normal CD38-CD45RA+ cells and 5/22 (28%) as normal GMPs. Only 278 probes (272 genes) were required for the classifier. Likewise for the GMP-like AML populations, 13/21 samples were called as normal GMP (62%), 7 as normal CD38-CD45RA+ (33%), and 1 sample as normal CMP (5%) using 241 probes (235 genes). Similar results were obtained by hierarchical clustering using these same minimum gene sets (Figures S3F and S3G).

Taken together, the gene expression profiles of both AML LSC populations do not map most closely to normal HSCs but rather to normal progenitor populations. Given that both CD38-CD45RA+ and GMP-like LSC serially transplant, we asked if the expression profiles of these two LSC populations were enriched for genes suggested to mediate "self-renewal" (Krivtsov et al., 2006). Gene Set Enrichment Analysis (GSEA) shows significant enrichment for the expression of this "self-renewal gene" set in CD38-CD45RA+ LSCs compared with normal CD38-CD45RA+ population ($p = 0.014$) and GMP-like LSCs compared with normal GMPs ($p << 0.01$) (Figures S3H–S3K) arguing that LSC populations may have co-opted parts of a self-renewal-associated program not expressed in normal immunophenotypic counterpart progenitors.

Normal CD38-CD45RA+ Population Includes Cells with Lymphoid Primed Multipotential Potential

Global expression profiles of CD38-CD45RA+ AML most closely resemble normal CD38-CD45RA+ cells. The expression data also suggested that the normal CD38-CD45RA+ population is distinct from other stem/progenitor cells. Previous studies had not fully shed light on the function of normal CD38-CD45RA+ cells. To investigate the lineage potential of normal CD38-CD45RA+ cells, we performed colony assays (Figure 5A). Normal HSC, MPP, CMP, GMP, and MEP populations produced the expected lineage output. The CD38-CD45RA+ population had a cloning efficiency of ~30% and produced granulocyte, macrophage, and mixed granulocyte-macrophage colonies. Importantly, there was no erythroid output. When cells from the initial colony assay were replated, cells from CD38-CD45RA+ colonies had a replating potential intermediate between that of cells from MPP- and CMP-derived colonies (Figure 5B). CD38-CD45RA+ cells exhibited no megakaryocyte potential, in contrast to MEPs, CMPs, HSCs, and MPPs (Figure 5C).

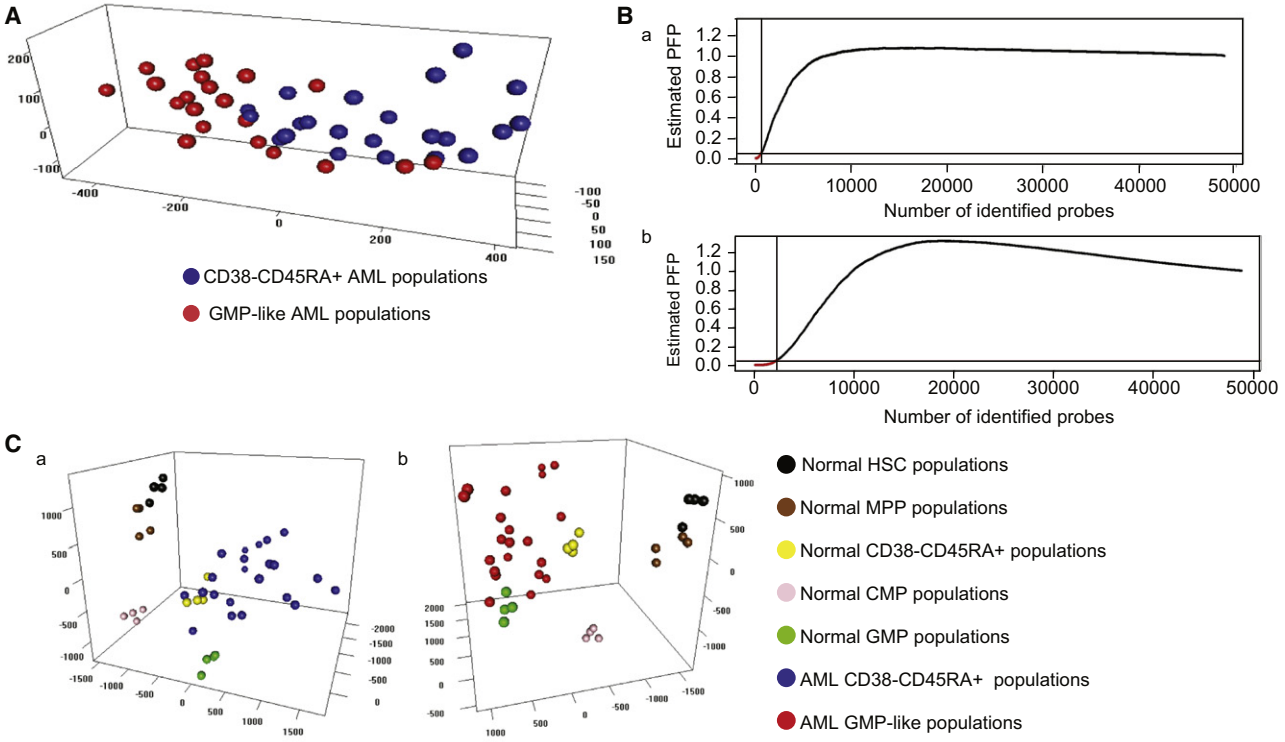
The B-lymphoid potential of normal CD38-CD45RA+ cells was tested in vitro. As a positive control, both HSC and MPP populations differentiated into myeloid (CD33-expressing) and B-lymphoid cells (CD19- expressing) cells, whereas CMP/GMPs only gave rise to CD33+ cells (Figure 5D). CD38-CD45RA+ cells differentiated into both CD33+ and CD19+ cells (Figure 5D). The frequency of cells with myeloid, B- and T-lymphoid potential in the CD38-CD45RA+ population was determined by limiting dilution analysis (Figures 5D–5H). On MS5 stroma, 1/3.79 CD38-CD45RA+ cells had myeloid potential (compared with 1/12.4 GMP cells) (Figure 5E). In conditions that promote both B cell and myeloid output, 1/6.38 CD38-CD45RA+ cells differentiated into CD19+ B cells and CD33+ myeloid cells, whereas 1/95.79 GMP cells showed similar potential (Figure 5F). B cell and myeloid potential was always seen together. The T cell potential of the CD38-CD45RA+ cells was tested in vitro. 1/12 CD38-CD45RA+ cells expressed established T cell markers CD1a, CD7, and CD3 (Awong et al., 2009) (Figures 5G and 5H) compared with 1/32 GMP cells. Finally, preliminary data show that CD38-CD45RA+ cells have NK cell potential.

We also searched for, and failed to find, early TCRδ VD, DD, or DJ and IgH DJ gene rearrangements in CD38-CD45RA+ cells, as compared with HSC, MPP, CMP, and GMP controls at either loci in CD38-CD45RA+ DNA or in control DNA (data not shown) consistent with CD38-CD45RA+ cells being upstream of the earliest committed lymphoid progenitor.

Expression of Lymphoid- and GM-Specific Genes in Normal CD38-CD45RA+ Cells

Murine multipotential stem/progenitor cells express low levels of multiple lineage-affiliated gene expression programs concordant with their lineage potentials (termed multilineage priming) (Akashi et al., 2003; Hu et al., 1997; Mansson et al., 2007; Miyamoto et al., 2002; Ng et al., 2009; Yoshida et al., 2006). As cells pass through lineage restriction points, losing lineage potential, there is gradual, concomitant, extinction of lineage-affiliated gene expression programs. In a refinement of this concept, it has been suggested that there is a cascade of lineage-affiliated transcriptional signatures, initiated in HSCs and propagated in a differential manner in lineage-restricted progenitors (Ng et al., 2009) (Figure 6A).

Whether this also occurs in human hemopoietic stem/progenitor cells has not been previously reported. Therefore, we used quantitative RT-PCR to study expression of select genes shown in mouse to be representative of lineage-affiliated gene



E

	Predicted nearest normal populations for AML CD38-CD45RA+ (22 samples)					% recognised as normal CD38-CD45RA+
	HSC	MPP	CD38-CD45RA+	CMP	GMP	
Thr=5.4 (97probes)	3	2	12	0	5	54%
Thr=4.0 (278 probes)	0	0	17	0	5	77%
Thr=3.0 (661 probes)	0	0	17	0	5	77%
Thr=0 (2789 probes)	0	0	17	0	5	77%

G

	Predicted nearest normal populations for AML CD38-CD45RA+ (22 samples)					% recognised as normal CD38-CD45RA+
	HSC	MPP	CD38-CD45RA+	CMP	GMP	
Thr=5.4 (97probes)	0	3	7	0	11	52%
Thr=4.0 (278 probes)	0	2	6	0	13	62%
Thr=3.0 (661 probes)	0	0	7	1	13	62%
Thr=0 (2789 probes)	0	0	9	1	11	52%

Figure 4. Expression Profiling Shows AML LSC Populations Are Distinct and Most Closely Mirror Normal Progenitor and Not Stem Cell Populations

(A) 3D principle component analysis (PCA) displaying gene expression profiles using 458 differentially expressed probes (corresponding to 360 mapped genes) determined by a standard unpaired t test with a cutoff of 0.01 from 22 AML CD38-CD45RA+ (blue spheres) and 21 GMP-like LSC populations (red spheres) were available from the same patient.

(B) Rank product analysis of probe sets showing either increased expression in CD38-CD45RA+ compared with GMP-like populations (a and vice versa b) using gene expression profiles from 18 AML patients where both CD38-CD45RA+ and GMP-like population samples were available from the same patient. On the y axis, the false discovery rate; on the x axis the rank product of the probes. At a false discovery rate of 0.05, numbers of differentially expressed probes are shown as red lines.

(C) 3-D PCA of expression profiles of four FACS-sorted normal populations (HSCs, black spheres, MPPs, brown spheres, CD34+CD38-CD90-CD45RA+, yellow spheres, CMPs, pink spheres and GMPs, green spheres) using a 2629 ANOVA gene set (2789 probes) of differentially expressed genes in normal populations. Positions of 22 CD38-CD45RA+ AML populations (blue spheres) (a, left) and 21 GMP-like AML populations (red spheres) (b, right) are shown.

expression programs, in 10 and 100 FACS-sorted normal HSC, CD38-CD45RA+, GMP, and MEP cells (Figure 6B; Figure S4and Table S5). The aim was 2-fold. First, to establish if lineage-affiliated patterns of gene expression seen in mouse held true in human. Second, to determine if CD38-CD45RA+ cells expressed a lineage-affiliated transcriptional program similar to murine LMPP cells. The gene set chosen for study was based on previous published data (Akashi et al., 2003; Hu et al., 1997; Mansson et al., 2007; Miyamoto et al., 2002; Ng et al., 2009; Pronk et al., 2007; Shojaei et al., 2005; Yoshida et al., 2006) and our own results.

Consistent with previous data, we found that in ten FACS-sorted cells, *MPL* (Buza-Vidas et al., 2006) and *HLF* (Shojaei et al., 2005) were most highly expressed in HSCs (Figure 6Ba) (representative of stem-cell only genes). Using our own expression data, we confirmed that the stem/progenitor regulator genes *BMI1* and *MEIS1* were expressed in HSC and CD38-CD45R+ cells, with markedly lower expression in GMP and MEP. Similarly, *KIT*, *IKZF1 (*IKAROS*)* and *RUNX1* were also expressed in HSC and CD38-CD45RA+ cells but also were expressed in GMP and MEP. Interestingly, *HOXA9* and, to a lesser extent, *IL3RA*, were expressed principally only in CD38-CD45RA+ cells.

Turning to myeloid lineage affiliated genes, the early myeloid genes *CEBPA*, *CSF3R*, *SPI1* (*PU.1*) are all expressed in HSCs with expression retained in CD38-CD45RA+ and GMP cells but extinguished in MEP (Figure 6Bb). These are reminiscent of stem cell/myelolymphoid genes (Ng et al., 2009). The next pattern of myeloid gene expression is seen primarily in CD38-CD45RA+ and GMP cells (*CSFR2A* and *GFI1*) (i.e., restricted myelo-lymphoid). Finally, the last layer of myeloid gene expression is exemplified by the late myeloid-specific genes (*MPO* and *CSFR1*), which were mainly expressed in GMPs only (differentiated myeloid). The pattern of early lymphoid gene expression is more focused; *CD79A*, *ETS1*, *VPREB1*, sterile *IGHM*, *FLT3*, *NOTCH1*, and *RUNX3* all maximally expressed in CD38-CD45RA+ with little expression in other cell types (Figure 6Bc). Finally, early stem-erythroid-megakaryocyte gene expression is epitomized by *VWF*, *TAL1*, and *GATA2* (Figure 6Bd) (all expressed in HSCs and MEPs). Both *VWF* and *TAL1* were expressed at much lower levels in CD38-CD45RA+ cells. *GATA2* continues to be expressed in these cells, consistent with its broader role in progenitor biology (including GMP) (Rodrigues et al., 2008). The erythroid-specific cytokine receptor gene *EPOR*, the erythroid-megakaryocyte transcription factor *GATA1* and late erythroid transcription factor *KLF1* show restricted expression (principally in MEPs). Importantly, none of these three genes was expressed in CD38-CD45RA+ cells. Identical patterns of gene expression were obtained when either 10 or 100 FACS sorted cells where studied (Figure S4).

Our gene expression data are consistent with multilineage priming in primary human stem/progenitors analogous to that in mouse (Mansson et al., 2007; Ng et al., 2009). Moreover, these data are consistent with the lympho-myeloid (GM), but not erythroid-megakaryocyte, lineage potential of CD38-CD45RA+ cells. Finally, by Gene Set Enrichment Analysis, genes more highly expressed in human CD38-CD45RA+ cells (Figure 6) are enriched in murine LMPP (Ng et al., 2009; data not shown).

DISCUSSION

We present the immunophenotype, LSC potential and global gene expression profiles of a broad range of CD34+ AML samples using normal stem/progenitor cells as a comparator. The aim was to dissect the relationship between normal stem/progenitor cells and leukemic populations with LSC potential. The key findings are (1) There are two stem/progenitor immunophenotypic patterns in CD34+ AML and 80% of cases are accounted for by one pattern with expansion of CD38-CD45RA+ and CD38+CD45RA+ GMP-like cells. (2) Within this dominant pattern, both populations coexist in most patients and have LSC potential. (3) These two AML LSC populations are molecularly distinct though there is overlap in global gene expression profiles suggesting further purification of these populations may be possible. (4) In vivo and in vitro, CD38-CD45RA+ AML cells give rise to a GMP-like population but not the converse. Analysis of CD38-CD45RA+ and GMP-like LSC expression profiles suggests that the former population is enriched for genes upregulated in more immature FAB type AMLs (M0, M1), whereas the latter for genes expressed in more mature FAB type AMLs (M2, M4, and M5) (data not shown) consistent with CD38-CD45RA+ LSCs being more immature than GMP-like LSC (Figures 2D and 3). (5) Gene expression profiles of both LSC populations are most similar to their normal immunophenotypic counterpart progenitor populations and not normal HSCs. (6) The previously poorly characterized normal CD38-CD45RA+ progenitor population functionally most closely approximates to a murine LMPP. This population is CD10- (data not shown) and distinct from the recently described CD10+ MLP (myeloid-lymphoid progenitor) (Doulatov et al., 2010) that lacks granulocyte potential, an attribute of the murine LMPP.

Recent work abrogating residual immunity in NOD-SCID mice showed that LSCs were present in both CD34+CD38- and CD34+CD38+ populations (Taussig et al., 2008). However, it was unclear how homogeneous LSCs were and what the relationship was between LSCs and normal stem/progenitor cells within and between patients.

Therefore, we tested LSC function by intravenously injecting AML cells in NOD-SCID mice treated with anti-CD122 antibody and NSG mice. Our data provide compelling evidence that CD34+CD38- LSCs are most similar, molecularly and immunophenophenotypically, to a LMPP-like progenitor and not HSCs. We also pinpoint that CD34+CD38+ LSCs are most like a GMP-like progenitor. In most CD34+ AML patients, both progenitor-like LSCs coexist and experimentally are hierarchically ordered. The CD38-CD45RA+ AML population has a higher LSC frequency compared with GMP-like LSC, consistent with

(D–G) Classifier analysis using the 2789 ANOVA selected differentially expressed set of probes that separates normal populations. This probe set was used to call the identity of the 22 CD38-CD45RA+ AML populations (D and E) and 21 GMP-like AML populations (F and G). On the x axis more probes (from the most differentially expressed to the least differentially expressed) are used in the classifier from right to left. Top x axis shows numbers of probes used. Bottom x axis depicts corresponding threshold values. y axis, number of AML samples called.
See also Figure S3.

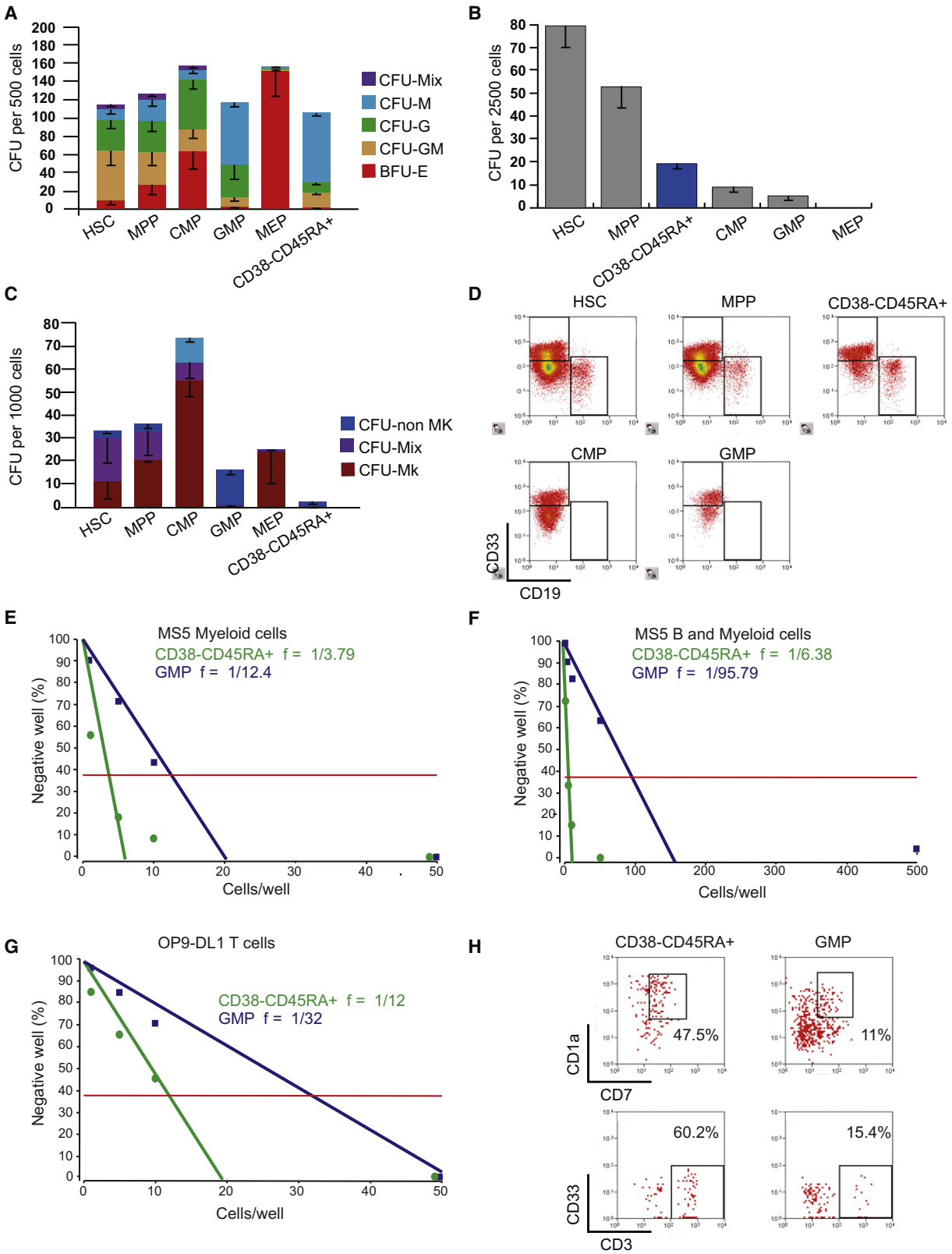


Figure 5. Normal CD38-CD45RA+ Cells Have Lymphoid Primed Multipotential Myeloid Potential

(A) Myeloid/erythroid colony growth of FACS sorted normal marrow HSC, MPP, CMP, GMP, MEP, and CD38-CD45RA+ populations from five normal samples. The number of colonies/500 cells plated was scored (mean −1 SD). Colony lineage affiliation is shown by colored bars (right).

higher self-renewal potential of CD38-CD45RA+ LSCs (Ishikawa et al., 2007) and CD38-CD45RA+ LSC lying at the apex of an LSC hierarchy. Thus, we confirm previous findings and suggest potential resolution to previous open questions.

Our data pointing to the progenitor nature of LSCs are also consistent with an early report of t(8:21) AML where the leukemic CD34+CD38- compartment was CD90- (i.e., of progenitor immunophenotype) in contrast to HSC (CD34+CD38-CD90+) (Miyamoto et al., 2000). Interestingly, HSCs in remission samples contained preleukemic mutations suggesting that though LSC have a progenitor phenotype, disease-initiating mutations may occur in HSC to produce a preleukemic state.

One important question is whether LSC potential genuinely exists in more than one population (i.e., both CD38-CD45RA+ and GMP-like populations or other CD34+ populations), or if it resides in only one population that has not yet been purified to homogeneity. There are conflicting previous reports from current immunodeficient mouse models about where LSC potential resides; some suggest that the LSC potential resides in just the CD34+CD38- compartment (Ishikawa et al., 2007), whereas others suggest it is present in both CD34+CD38- and CD34+CD38+ compartments (Taussig et al., 2008). Though our gene expression data argue that the two LSC populations are distinct, the overlap in expression profiles in some patients, and the fact that CD38 is expressed as a continuous variable, suggest that additional purification of these two populations may allow one discrete LSC population to be identified. Our expression profiles will provide additional testable cell-surface markers for use in fractionating LSC to homogeneity. Separating CD38-CD45RA+ and GMP-like AML populations in all patients is important before re-evaluating their hierarchical relationship, LSC potential (including LSC frequency by limit dilution) and molecular constitution.

Our data suggest that the previously uncharacterized normal CD38-CD45RA+ population contain cells with LMPP-like potential in vitro. A previous report showed these cells had 5% GM plating potential in colony assays and limited growth in vitro (Majeti et al., 2007). We saw more extensive colony potential only when freshly isolated cells were used or, if previously frozen, cells were exposed to FLT3L (data not shown). Moreover, our liquid culture assays were conducted on MS5 stroma. Despite our in vitro data, there are two caveats. First, B- and T cell and GM potential needs to be demonstrated from single cells. Second, we have been unable to demonstrate that human CD38-CD45RA+ cells have GM and T cell, B cell, and NK potential in vivo. Normal CD38-CD45RA+ cells engraft very poorly both in neonatal NOG mice (Majeti et al., 2007) and NOD-SCID mice treated with anti-CD122 antibody (N.G., P.V., unpublished data). We find <0.1% engraftment when up to 10^4 normal CD38-CD45RA+ adult marrow or cord blood cells are assayed from 1 to 8 weeks after intravenous or intraosseous injection. In contrast murine LMPP engraft both marrow and thymus. Most likely this reflects a limitation of the xenotransplant model and improvements in this assay will be required before robust engraftment from CD38-CD45RA+ cells is seen. Further experiments will also be required to show that single CD38-CD45RA+ cells prime both GM and lymphoid expression programs. Though we could show this in ten cells, we were unable to technically assess this at a single cell level (N.G., P.W., A.M., T.E., S.E.J., and P.V., unpublished data). We suspect this reflects the relative paucity of mRNA at the single cell level in human CD38-CD45RA+ cells.

Nevertheless, in vitro data in support of a functional equivalent to the murine LMPP within human CD38-CD45RA+ cells are compelling. It is likely that LMPP populations will be widely conserved in lower vertebrate models and confirmation of this will allow a careful dissection of mechanisms that allow the erythroid-megakaryocyte lineage program to separate from the other hemopoietic lineages through evolution.

An important observation from global expression studies is that the LSC populations most closely resemble normal progenitor populations rather than stem cells or MPPs. This raises the hypothesis that LSCs may have hybrid characteristics, retaining the proliferative potential of progenitors, not seen in quiescent stem cells, and specifically acquiring pathological stem-cell like self-renewal potential. Support for this comes from expression profiling of GMP-like LSCs derived either from ectopic MLL oncoprotein expression or CEBPα mutant mice that possess a GMP-like expression signature with a superimposed self-renewal associated (stem-cell like) signature (Kirstetter et al., 2008; Krivtsov et al., 2006). Interestingly, the self-renewal associated gene expression signature from MLL-transformed murine GMP is enriched in primary human AML LSC profiles (Figure S5). As a next step in highlighting key deregulated self-renewal associated pathways in LSCs, careful comparison of primary human AML LSC profiles and normal stem/progenitor cells from our study with different leukemia-associated self-renewal associated signatures may be helpful. Ultimately, an important future goal will be to determine global expression signatures from LSC and normal stem/progenitor populations purified to homogeneity.

(B) Cells from colonies in primary platings (A) were plated in a secondary replating assay. Number of colonies/2500 cells plated from each of the cell type is illustrated (mean −1 SD).

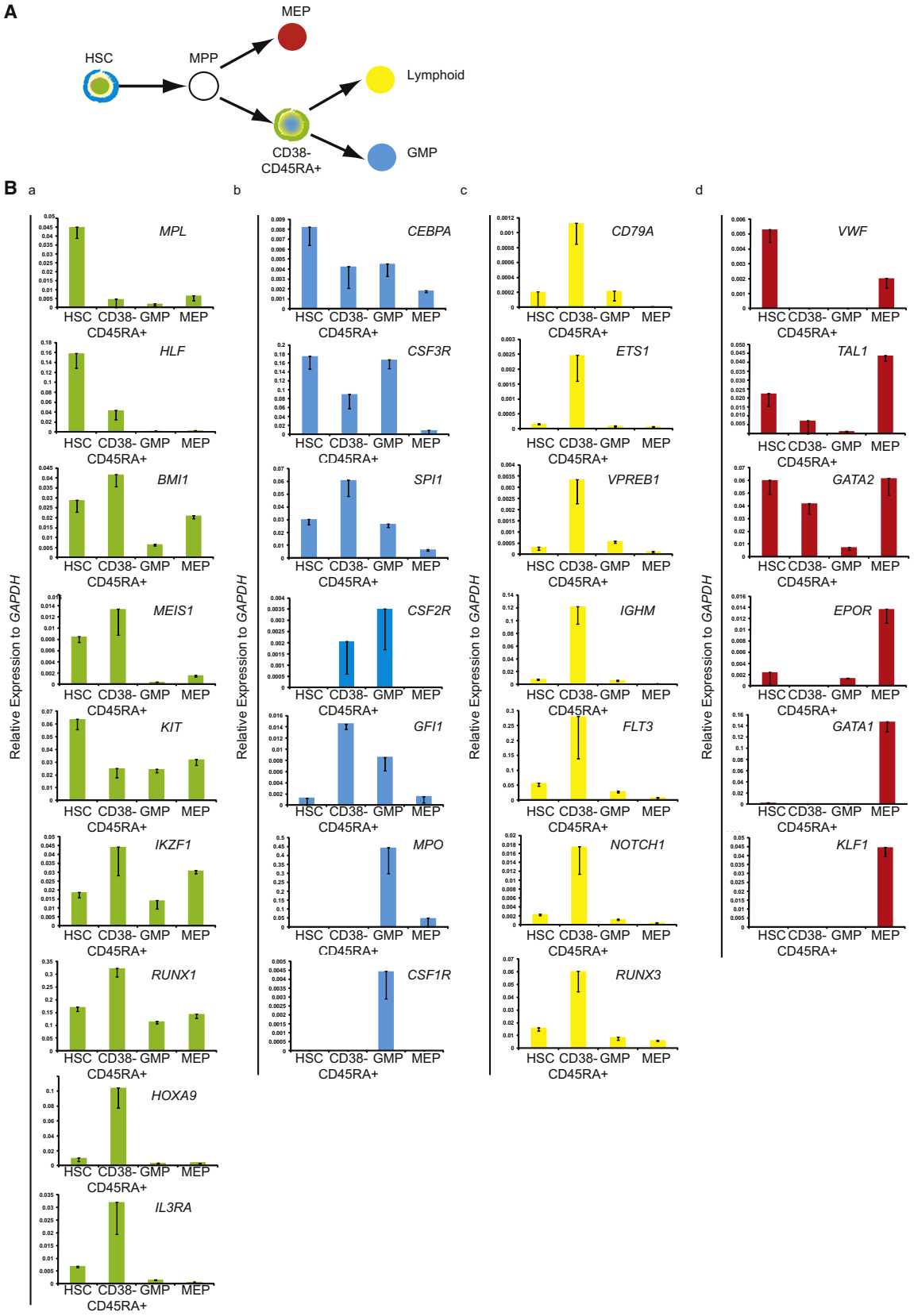
(C) Megakaryocyte colony growth from FACS sorted normal marrow HSC, MPP, CMP, GMP, MEP, and CD38-CD45RA+ populations from three normal individuals. Mean number (−1 SD) of colonies/1000 cells plated is depicted. Colony lineage affiliation is shown by different colored bars (right).

(D) Representative FACS analysis of CD19/CD33 expression in cells produced from bulk culture of FACS sorted HSC, MPP, CD38-CD45RA+, CMP, and GMP cells from four normal marrows.

(E and F) Limit dilution analysis to determine frequency of cells with myeloid (CD33+) (E) and mixed B cell (CD19+)/myeloid (CD33+) potential (F) in CD38-CD45RA+ (CD45RA+) (green line) and GMP cells (blue line). One to 500 sorted cells from 4 normal marrow samples were tested in individual wells in MS5 stroma/cytokine coculture (144 replicates for each condition).

(G) Limit dilution analysis to determine T cell potential frequency in CD38-CD45RA+ (green line) and GMP cells (blue line). One to 500 sorted cells from 3 marrow samples were tested in individual wells in OP-DL1 stroma/cytokine coculture (36 replicates for each condition). T cell potential was defined as cells expressing CD1a, CD7, and/or CD3.

(H) Representative FACS analysis plots of CD1a, CD7 (top) and CD33 and CD3 expression in cells produced from bulk culture of FACS sorted CD38-CD45RA+ and GMP cells from three marrows.


A
HSC
MPP
MEP
Lymphoid
CD38-
CD45RA+
GMP
B
a
b
c
d
Relative Expression to GAPDH
MPL
HLF
BMI1
MEIS1
KIT
IKZF1
RUNX1
HOXA9
IL3RA
CEBPA
CSF3R
SPI1
CSF2R
GFI1
MPO
CSF1R
CD79A
ETS1
VPREB1
IGHM
FLT3
NOTCH1
RUNX3
VWF
TAL1
GATA2
EPOR
GATA1
KLF1
HSC CD38- GMP MEP
CD45RA+

What implications do our observations have for AML patients? As many patients die of disease relapse it will be important step to monitor the size of distinct leukemic stem/progenitor populations by combining multi-color immunophenotyping with genetic analysis to look at clonal architecture. This critical advance can then be applied to sequential pre- and posttreatment samples to identify therapy resistant leukemic populations that drive relapse. One can then ask if such populations have functional LSC activity in xenografts. Accurate identification of small, residual, posttherapy relapse-driving populations will be of prognostic value, allowing one to direct patients at high risk of relapse to alternative therapy and, conversely, spare patients likely to be cured, from further toxic therapy. Finally, comparison of such leukemic populations with normal stem/progenitor cells at a molecular level could provide novel rational targeted therapies, for example, either monoclonal antibodies directed against relapse driving population (Majeti et al., 2009) or against pathways of abnormal self-renewal.

EXPERIMENTAL PROCEDURES

Patient Samples

Bone marrow samples from AML patients and patients undergoing orthopedic surgery with normal blood counts/films were obtained with informed consent (protocols 06/Q1606/110 and 05/MRE07/74 approved by Oxford Ethics Committee B). Mononuclear cells were isolated by Ficoll density gradient within 24–48 hr of collection. CD34+ progenitors were purified using CD34 Microbead Kit and MACS separation columns (Miltenyi Biotec, Bisley UK). CD34+ and deplete fractions were frozen in 90% FCS/10% DMSO in liquid nitrogen. Prior to manipulation, frozen CD34+ cells were thawed and left overnight in Stemspan (StemCell Technologies, Sheffield UK) supplemented with 100 ng/ml of SCF, TPO, and FLT3-L (Peprotech, London).

Xenograft Assay

Experiments were performed both in Universities of Lund and Oxford. In Lund, they were approved by the University of Lund Animal Welfare Committee. In Oxford work was conducted in accordance with UK Government Home Office approved Project License 30/2465. Ten to 14-week-old NOD/SCID or NSG mice were irradiated 100–125 cGy twice 4 hr apart, 24 hr before 10^2–10^5 cells were injected intravenously. Mice were killed 10–12 weeks later. To abrogate antibody-mediated cell clearance, NOD/SCID mice were injected intraperitoneally with 200 μg of anti-CD122 antibody and NSG mice with either anti-CD122 antibody or IVIG (1 mg/gram body weight) after irradiation. Anti-CD122 antibody generated from TM-β1cell line was purified using High Trap Protein G Columns (Amersham Pharmacia, High Wycombe UK). Normal multilineage engraftment was defined by separate CD45+CD33+ and CD45+CD19+ populations with appropriate scatter characteristics. AML engraftment was defined by a single CD45+CD33+ population.

ACCESSION NUMBERS

Micorarray data have been deposited in ArrayExpress (www.ebi.ac.uk/arrayexpress/) with accession number E-TABM-978.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and five tables and can be found with this article online at doi:10.1016/j.ccr.2010.12.012.

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Figure 6. Granulocyte-Monocyte And Lymphoid but Not Erythroid-Megakaryocyte Gene Expression Priming in CD34+CD38-CD90-CD45RA+ Cells

(A) Schematic diagram of cellular hierarchy of human stem/progenitor cells and cascade of lineage-affiliated gene signatures. HSC- and CD38-CD45RA+-affiliated gene signature (green); myeloid (GM) lineage-affiliated gene signature (blue); lymphoid lineage-affiliated gene signature (yellow); ME lineage-affiliated gene signature (red).

(B) Mean mRNA expression (five replicates ±1 SD) from two pooled normal bone marrow samples of indicated genes relative to *GAPDH* determined by Quantitative Real-Time RT-PCR in ten FACS-sorted HSC, CD38-CD45RA+, GMP and MEP cells. Data from genes affiliated with (a) HSC and CD38-CD45RA+ cells (green bars); (b) granulocyte-monocyte lineage cells (blue bars); (c lymphoid lineage cells (yellow bars); (d) erythroid-megakaryocyte lineage cells (red bars). See also Figure S4 and Table S5.

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