Perspective

Deciphering developmental stages of adult myelopoiesis

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Abbreviations: CFU-E, colony forming unit-erythroid; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; G/M, granulocyte/monocyte; GMP, granulocyte monocyte progenitor; HSC, hematopoietic stem cell; IL7Ra, interleukin 7 receptor alpha; LSK, lineage negative, SCA1 positive, c-KIT positive; MEP, megakaryocyte erythroid progenitor; Mk/E, megakaryocyte/erythrocyte; MkP, megakaryocyte progenitor; MP, myeloid progenitors

Key words: hematopoiesis, hematopoietic stem cell, myeloid progenitor, myeloid precursor, lineage potential, lineage commitment, gene expression

The ability to subfractionate minor cellular subsets by multiparameter flow cytometry and to evaluate such cells for functional properties has been used to ascertain lineage relationships and detail developmental hierarchies in the hematopoietic system for more than 20 years. However, steady advances in technology combined with the use of novel cell surface markers continues to redefine the developmental landscape as novel subpopulations are purified and characterized. Although studies to date have failed to establish a definitive role for these receptors in regulating HSC function, their discovery has significantly enhanced their in vitro readout. Gene expression patterns of functionally opposing transcription factors that are known to play key roles for the appropriate development into separate myeloid lineages were associated with the functional activity of prospectively isolated subsets. Multiple genes traditionally associated with early lymphopoiesis were observed in early candidate granulocyte/monocyte, but not early megakaryocytic and/or erythroid progenitor cells. When functionally evaluated, such early granulocyte/monocyte precursors displayed a latent lymphoid activity, which was pronounced in subsets bearing high expression of the tyrosine kinase receptor FLT3.

Introduction

The formation of mature effector blood cells of at least 9 separate lineages is critically contingent on hematopoietic stem cells (HSC). HSC are operationally defined by their functional properties at the single cell level to be able to self-renewal and to differentiate into multiple lineages; the fundamental properties mediating lifelong hematopoiesis.

The strict functional requirements of self-renewal and multilineage differentiation have necessitated the use of experimental animal models to explore and characterize HSC function, with the mouse representing the most studied model for such endeavors. Pursuits to obtain an exact phenotypic identity of HSC based on their cell surface antigenic profile have been ongoing ever since the feasibility of such an approach was demonstrated in 1988.¹ No single marker has, to date, been identified as sufficient for defining HSC at a high degree of purity. Rather, the prospective identification of HSC requires a combination of markers, where the lack of markers found on mature blood cells (so called lineage negative cells) and expression of SCA1 and c-KIT (so called LSK cells) is often referred to as a population of phenotypic HSC. LSK cells represent a minor portion of BM cells (~0.1%) and contain all functional HSC activity, however, although substantially enriched for HSC activity, these cells do not represent a pure population of stem cells.² Using additional subfractionation procedures, Osawa et al separated LSK cells into a minor CD34⁺ fraction (~10% of LSK cells), and found such cells to be capable of reconstituting a very high number of recipient mice (>20%) at the single cell level, whereas the major CD34⁻ (~90% of LSK cells) subset lacked functional HSC activity.³

Since these seminal demonstrations, limited progress has been made to further develop purification strategies yielding higher functional HSC activity. However, recent studies using gene expression arrays with various HSC subpopulations have revealed several complementary markers found to be present or absent on HSC. Endoglin (CD105) and the Slam family member, Slamf1 (CD150), are two such additional cell surface receptors recently demonstrated to be highly expressed on long-term reconstituting HSC.⁴,⁵ Although studies to date have failed to establish a definitive role of these receptors in regulating HSC function, their discovery has markedly increased the ability to isolate HSC to high purity, and further reveal their in situ localization throughout the various hematopoietic tissues.⁵,⁶
Apart from representing a paradigmatic system for the direct investigation of somatic stem cell function, the blood system can also be viewed as a prototype system for cellular differentiation. The enormous demand of an adult organism such as the mouse to replace at least $10^{11}$ blood mature cells every hour makes it evident that blood cell production has to be appropriately coordinated. This is further underscored by the ability of the hematopoietic system to effectively balance the ratios of individual lineages, each with very different life spans, and to respond and revert to a normal state following hematopoietic stress such as bleeding or infections. Although HSC are the root source of all mature blood cells, they are very rare cells, and in addition, exhibit relative quiescence at any given time point in adult mice, making it evident that a significant level of control of mature blood cell production is mediated at the intermediate progenitor stages.

Various subsets of granulocytes (G; neutrophils, eosinophils and basophils), monocytes/macrophages (M), erythrocytes (E), megakaryocytes (Mk) and mast cells, collectively constitute what is referred to as the myeloid blood cell lineages while cells of the B, T and NK cell lineages jointly make up the lymphoid blood cell subsets. Dendritic cells have been proposed to arise from both the myeloid or lymphoid arms, and therefore by definition do not adhere to the simplistic myeloid and lymphoid separation of blood cells. The prospective identification of a class of progenitors restricted in lymphoid developmental potential, otherwise known as common lymphoid progenitors (CLP), suggested the possibility that early erythromyeloid differentiation might be mediated by a precursor with combined myeloid but limited lymphoid differentiation potential (so-called common myeloid precursors, or CMP). Indeed, by searching murine BM for myeloid precursor activity, Akashi et al reported the identification of CMP within a minor lineage negative, IL7Rα negative, c-KIT positive and SCA1 negative compartment (referred to here as myeloid progenitors; MP), as a compartment containing the vast majority of the total myeloid colony forming potential in a mouse. When further subfractionated based on expression of CD16/32 and CD34, the myeloid lineage potentials were suggested to be hierarchically structured such that clonal CD16/32^low^CD34^+^ CMP could give rise to both CD16/32^low^CD34^+^ Mk/E restricted precursors (MEP) or CD16/32^high^CD34^+^ G/M restricted precursors (GMP).

This model has gained widespread use in studies aimed at elucidating the functional consequence of conditions affecting early myeloid development, including the characterization of various transgenic or knockout mice. However, whereas the progenitor activity of MEP and GMP appears mutually exclusive, a misunderstood feature of CMP is their relatively rare combined MEP/GMP activity. It is possible that although CMP have the ability to develop along all myeloid lineages, their ability to do so is either random or guided to some extent by the culture conditions used to develop their developmental potential, i.e., that a high degree of stochastic characterizes their clonal in vitro output. This would subsequently result in a high fraction of clonal output belonging to either the Mk/E or G/M lineages, while rarely containing both. In support of this argumentation are results from simultaneous studies of candidate HSC, which by definition should have the capacity to differentiate into all blood cell lineages, but that displayed very similar clonal lineage output as those observed for CMP when evaluated under identical conditions. A complication of such argumentation is however that candidate HSC utilized for comparative purposes has later been demonstrated to also contain other transiently reconstituting subsets (multipotent progenitor cells), where at least a portion of such cells most likely have undergone some degree of lineage commitment. Thus, it is important to highlight for future studies regarding the isolation and identification of hematopoietic stem and progenitor subsets that there is the ever-present need for highly purified HSC subsets, and their clonal parameters be carefully assessed in the context of the newly identified progenitor subpopulations when interpreting such new data.

Separation of cells based on the use of a reporter gene knocked into a particular endogenous locus or transgenic mice carrying a reporter gene in the context of a bacterial artificial chromosome (BAC) are powerful experimental approaches. Such strategies are especially useful for the study of intracellular proteins such as transcription factors and signalling molecules, where detection by other means would result in loss of cellular viability. PU.1 (Sfpi1) is a transcription factor of the Ets family members that strongly influence early differentiation events towards the lymphoid and GM lineages, despite being largely dispensable for differentiation into the Mk/E lineages. Using a transgenic reporter mouse strain engineered to express the green fluorescence protein (GFP) under the control of the endogenous PU.1 regulatory elements, Nuff et al further explored the concept of LSK CD16/32^low^CD34^+^ CMP, and were able to demonstrate that PU.1^high^ CMP failed to produce Mk/E offspring. These studies also revealed that expression of tyrosine kinase receptor FLT3 on CMP was associated with very limited Mk/E developmental potential. Additional studies exploring the functional capacity of LSK cells based on the expression of FLT3 reached similar conclusions, i.e., that expression of FLT3 is associated with only limited developmental potential for the Mk/E lineages, although harbouring robust combined lymphoid and G/M potential. However, it is possible that these FLT3-expressing cells might possess residual Mk/E potential at a very low frequency, with limited in vivo significance. Regardless, most current data suggest that Mk/E developmental potential segregates, to a large extent, prior to that of adaptive and innate immune system components, without excluding the possibility of a composite differentiation model in which G/M output could originate from multiple precursor types.

**Staging Murine Myelopoiesis using Multiparameter Flow Cytometry**

Reporter knockin approaches do not always reveal the expected patterns of gene expression across cell populations and tissues. It has been demonstrated that this gene targeting approach can frequently affect endogenous levels of the gene they were knocked into, leading to non-physiological expression levels. Alternatively, the expression pattern of the target gene may not be faithfully recapitulated in whole tissues or at the cell population level. As suggested by previous work and our own unpublished observations, we attempted to enhance the purity of candidate CMP without having to utilize genetically engineered transgenic mice. We recently reinvestigated the lineage potentials of the MP compartment by developing a polychromatic FACS isolation strategy based on differential expression of CD16/32, CD41, Slamf1 and Endoglin (Fig. 1). Our rationale for using these
markers was that high-level expression of CD16/32 is associated with almost exclusive differentiation potential towards the G/M lineages. Furthermore, expression of CD41 had previously been demonstrated to be associated with Mk differentiation potential, and since these cells co-purify with candidate CD16/32lowCD34+ CMP, such work directly shows that the originally described CMP is a heterogeneous cell population. We next hypothesized that Slamf1 and Endoglin might be selectively preserved or down-regulated upon HSC differentiation, as is the case for instance SCA1, which is expressed at high levels on HSC but downregulated on lineage committed precursors.

**Mixed Clonal Myeloid Potential is a Rare Property of Cells within a Candidate Myeloid Progenitor Compartment and is only Marginally Enhanced by Preventing Apoptosis**

Using the isolation scheme described in Figure 1, we functionally evaluated the six indicated cellular subsets at the clonal level for their ability to form myeloid progeny in vitro (Fig. 1A and ref. 20). As expected, due to their significant overlap with the previously described MkP and GMP, CD41high cells within the MP compartment (which in addition are characterized by a Slamf1+ phenotype) give rise exclusively to Mk, whereas CD16/32high cells effectively give rise to only G/M colonies. Virtually all such CD16/32high cells are EndoglinlowSlamf1low, and CD16/32lowEndoglinlowSlamf1low cells similarly give rise to G/M but not Mk/E containing colonies, although rare mixed colonies is occasionally observed from these cells (Fig. 2B). Expression of Slamf1 is associated with robust Mk/E developmental potential. High-level expression of Endoglin is associated exclusively with erythroid developmental potential; EndoglinhighSlamf1+ cells displayed a higher proliferative potential than EndoglinhighSlamf1+ cells. Overall, the results from our studies contrasted the interpretations from original studies of CMP, since we observed very few clones with mixed myeloid lineage potential from any of the subpopulations comprising the more heterogeneous populations ascribed with CMP potential. Importantly, we were able to establish that specific myeloid fates segregated with distinct cell surface phenotypes.

Original studies of CMP were largely based on data obtained from cells with transgenic overexpression of the anti-apoptotic protein BCL2. It therefore remained formally possible that our newly identified myeloid subsets would also display an increased frequency of CMP activity if isolated from BCL2 transgenic mice. To test this, we compared the clonal activity of WT and BCL2 transgenic CD16/32lowCD41Slamf1lowEndoglinlow (Pre MegE) and CD16/32lowCD41Slamf1lowEndoglinlow (Pre GM) cells, which together with lineage committed MkP constitute the previously described CMP fraction. These experiments revealed that while expression of the BCL2 transgene enhanced the overall clonogenic activity of these subsets (Fig. 2A), we nevertheless only observed a very modest increase in the frequency of clones with mixed myeloid composition (Fig. 2B). These experiments demonstrate that decreasing the threshold for apoptosis does not dramatically alter the lineage potential of early progenitors within the MP compartment, although clonal readouts are significantly enhanced.

Most evidence suggests that growth factors at early multipotent precursor levels typically do not act in a deterministic fashion. However, it is tempting to speculate that individual growth factors can dramatically alter clonal composition via effects on proliferation and survival independent of BCL2; a feature evidenced in studies investigating the synergy between BCL2 and...
growth factor-mediated anti-apoptosis. We therefore compared the ability of various growth factor combinations to stimulate mixed myeloid colony formation of the pre-GM subpopulation (Fig. 2C). These experiments showed that using cytokine cocktails with limited growth factor combinations (c-KIT ligand, Interleukin-3 and Erythropoietin), or a multifactor cytokine combinations (c-KIT ligand, Interleukin-3, Erythropoietin, Thrombopoietin, Interleukin-11 and FLT3 ligand), did not change the mixed myeloid lineage output of CD16/32 Endoglin $^\text{low}$ Slamf1 $^\text{low}$ (pre-GM) cells (Fig. 2C), which remained very marginal as previously determined (Fig. 2A and Pronk et al. 20).}

The Transcriptome of Early Myeloid Progenitors; Differential Expression of Lineage Associated Transcription Factors

We speculated that the transcriptional profiles of the newly identified progenitors could reveal insights into the gene expression programs underlying myeloid lineage fate determination. To investigate this hypothesis, we performed microarray analyses using the Affymetrix 430.2 platform, which permitted gene expression analysis at the whole genome level. For comparative purposes, we also examined the expression profile of candidate CLP (originally defined CLP additionally gated on FLT3$^+$ CLP$^{\text{FLT3}^+}$), reasoning that this popula-
tion would represent a gene expression signature of early lymphoid development. These studies revealed distinct transcriptional signatures within the progenitor subsets we prospectively identified from the MP fraction, that were predictive of the lineage potential read out in functional evaluations. For example we observed differential expression of numerous well-characterized regulators with known roles in lineage specification in the newly fractionated populations. We also identified many additional genes enriched in each of the progenitor subsets that may play important roles in lineage specification. However, at the same time, our studies also caution against the predictive validity of gene accessibility models and their association to lineage potential merely based on low-level transcription. It was previously reported that more than 60% of CMP co-expressed G/M and Mk/E affiliated genes at the clonal level. Such data postulated that the developmental potential of CMP is reinforced by the transcriptional accessibility of genes associated with both G/M and Mk/E development. A pre-requisite of the feasibility of such work is that low-level transcription is an indicator of true locus accessibility, but also that such investigated genes truly reflect lineage specificity. In other words, since the vast majority of CMP fail to generate mixed lineage outcomes, it is no longer clear as to whether low-level transcription reliably predicts the developmental potential of a single cell. Consistent with this, HSC exhibit transcripts of many 'non-hematopoietic' genes, yet are restricted to the production of hematopoietic offspring. Instead, it might be that the stages of differentiation described herein are characterized by a continuous, and likely asynchronous specification process, where transcriptional "remnants" can be occasionally found in early lineage committed cells. This concept was supported by experiments using single cell multiplex analyses of the MP subsets in which we noted simultaneous expression of MPO (a myeloid-associated gene) with the megakaryocytic- and erythroid-affiliated genes, Mpl and EpoR, in only a few cells, whereas the vast majority of the cells analyzed did not simultaneously express genes involved in specifying these divergent fates. Again, our molecular data does not support the existence of a substantial progenitor pool with mixed myeloid potential such as CMP within the Lin^SCA1^-c-KIT^+ myeloid progenitor compartment, consistent with our functional data.

### A Subset of Candidate Granulocyte/Monocyte Progenitors with a Robust Lymphoid Differentiation Capacity

An unexpected observation that emerged from our global gene expression analyses following hierarchical clustering of gene sets was that cells with G/M potential (pre GM) and lymphoid destined progenitors, CLP^FLT3^, shared many transcriptional similarities, in contrast to that observed for progenitors destined for Meg/E fates. When we evaluated the lymphoid potential of cells within the CD16/32^low^Endoglin^low^Slamf1^low^ (pre GM) fraction using stromal OP9 cultures, we noted a substantial B lymphoid activity of FLT3^+ but not FLT3^+ cells, although such FLT3^+ cells still exhibited a substantially reduced lymphoid activity as compared to CLP^FLT3^ (Table 1). Slamf1^+ myeloid progenitors cells were simultaneously assessed in such experiments, and were found to completely lack lymphoid differentiation capacity (Table 1). These data suggest that early G/M precursors and CLP^FLT3^ have a close lineage relationship, whereby the transcriptional programs for specification of the downstream maturing G/M and lymphoid subsets are maintained in these early progenitor cell types. Overall, we interpret these findings to indicate that Mk/E differentiation potential segregate earlier than entry into the G/M and/or lymphoid lineages, a conclusion that has gained support by recent demonstrations of residual G/M but not Mk/E developmental potential of CLP, as well as circumstantial evidence that certain acute myeloid leukemias can be derived from precursors characterized by several lymphoid features.

A second approach to reveal developmental relationships of these committed progenitor subsets is to identify highly or selectively expressed genes associated with a particular cellular subset and subsequently explore their expression patterns in the other progenitor fractions. Hierarchical clustering analysis using this approach revealed that a vast majority of lymphoid specific genes are expressed at selectively high levels in the pre GM fraction (Fig. 3A). However, it should be noted that some CLP specific genes do not adhere to this pattern. For instance, the B lineage commitment factor, EBF1, where mRNA is expressed at high levels in CLP, was not associated with lymphoid potential in pre GM cells (Fig. 3B). When investigating the myeloid subsets for more specific expression of individual transcription factors, our data is in agreement with models of counteracting transcription factors to underlie hematopoietic cell fate determination processes. In the case of most transcription factors, it appears likely that it is their interplay with other regulatory factors rather than their mere absence or presence, that constitute critical thresholds for their activity. A well-characterised model is based on the interplay of transcription factors GATA1 and PU.1, which physically form transcriptional complexes and functionally oppose each other at gene promoters. The outcome of such interactions is the resolution of G/M and erythroid cell fates as mediated by via PU.1 and GATA1, respectively. Consistent with this mechanism in related lineages, transcriptional antagonism has also been observed for PU.1 and C/EBPα for macrophage and neutrophil cell fate specification. Furthermore, there is suggested interplay

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**Table 1** B-lineage potential of cultured myeloid and lymphoid cellular subsets

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cells plated</th>
<th>Wells with B cells (OP9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lin^IL7Ra^+FLT3^+SCA1^low^cKIT^low^</td>
<td>10</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>(CLP)</td>
<td>5</td>
<td>6/6 (100%)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>23/23 (100%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>32/45 (71%)</td>
</tr>
<tr>
<td>Lin^IL7Ra^+FLT3^+SCA1^+cKIT^+Slamf1^+</td>
<td>10</td>
<td>3/12 (23%)</td>
</tr>
<tr>
<td>(Pre GM&amp;GMP: FLT3^+)</td>
<td>5</td>
<td>0/12 (0%)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1/12 (8%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0/12 (0%)</td>
</tr>
<tr>
<td>Lin^IL7Ra^+FLT3^+SCA1^+cKIT^+Slamf1^+</td>
<td>10</td>
<td>7/12 (58%)</td>
</tr>
<tr>
<td>(Pre GM&amp;GMP: FLT3^+)</td>
<td>5</td>
<td>7/12 (58%)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5/12 (42%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1/12 (8%)</td>
</tr>
<tr>
<td>Lin^IL7Ra^+FLT3^+SCA1^+cKIT^+Slamf1^+</td>
<td>10</td>
<td>0/48 (0%)</td>
</tr>
<tr>
<td>(Pre MegE&amp;Pre CFU-E)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Indicated cell types and cell numbers were sorted by FACS into 96-well plates on OP9 stroma cultures, supplemented with FL and IL-7. 14 days after initiation of cultures, cells were harvested, stained with antibodies against B220 and CD19 and evaluated by flow cytometry to identify number of wells containing B220^+^CD19^+^ B-cells.
between GATA1 and GATA2 in regulating the commitment to the erythroid lineages from primitive hematopoietic progenitors, yet these findings remain to be validated using primary hematopoietic cells. There is further suggestion that NOTCH1 signalling inhibits commitment to the erythroid pathway, while PAX5 is thought to antagonize myeloid commitment via the ability to oppose activity of the myeloid transcription factors, C/EBPα and GATA-containing complexes. It remains to be determined whether transcription factor antagonism is a general mechanism employed for the divergence or resolution of all early hematopoietic lineage relationships. Thus, through the identification of early lineage-restricted precursor subsets such as those described herein, it should be possible to further illuminate the molecular pathways governing transcriptional priming in early hematopoiesis.

**Summary**

We anticipate that the discovery of the lineage-committed subsets described herein will be valuable tools in future investigations aiming to enhance our understanding of events dictating and establishing monolineage hematopoietic cell commitment. The ability to prospectively identify cellular subsets at defined developmental stages and with defined lineage potentials in vivo permits direct investigations of the features of such cells both in normal developmental and in hematopoietic disease. Whereas such work is relatively straightforward for HSC, for which there exists a functional long-term in vivo readout as a defining property, non-self renewing subsets are more problematic since their in vivo behaviour in transplantation settings is more limited. This is further complicated by the requirement of an appropriate homing process, which is intimately associated with HSC, but perhaps not necessarily with other hematopoietic progenitor cell subsets. Therefore, the lineage potential of progenitors downstream of HSC has and continues to rely on surrogate clonal in vitro assays. In our hands, such assays appear to faithfully reveal the lineage potential of most progenitor cells, although these assays have limitations; for example they cannot simultaneously assay for all combined myeloid and lymphoid potentials. Future studies will reveal whether G/M progeny are formed exclusively following a Mk/E versus G/M/CLP branching, or whether true CMP activity can arise at some developmental stage and act complementary to other developmental pathways. Critical for such experiments is the development of a highly effective assay system allowing for the simultaneous investigations, at a clonal level, for myelopoiesis, erythropoiesis, and lymphopoiesis. This would fully rule out that progenitor cells with mixed myeloid colony forming ability totally

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**Figure 3.** Pre GM cells have a higher transcriptional association to CLP than other myeloid progenitor subsets. (A) RNA was extracted from 5,000–10,000 cells of each indicated fraction, followed by linear amplification, and hybridized to Affymetrix 430A 2.0 arrays. A heat-map is displayed for genes that were 2-fold or higher differentially expressed between CLP compared to all other indicated cell types, on which overall clustering was next performed (following elimination of genes whose expression levels did not reach a baseline of 50 in either of the cell types displayed). Red indicates high expression, white intermediate and blue low-level gene expression. (B) Expression levels in indicated subtypes (derived from microarrays) of individual transcription factors previously implicated to act in opposing fashions.
lack the potential for lymphoid development, rather than representing a subset of fully multipotent hematopoietic progenitors whose lymphoid output is merely very low.

Material and Methods

Mice. 2–3 months H2K-BCL-2 or wild type C57BL/6 were used throughout these studies. Mice were maintained at the Lund University animal facility. All mice procedures were performed with consent from the local ethics committee.

Purification of hematopoietic stem and progenitor cells. Hematopoietic stem and myeloid progenitor cells were isolated by staining of unfractoned bone marrow cells using unconjugated antibodies against CD4 (GK1.5), CD8 (53–6.7), B220 (RA3-6B2), Gr1 (RB6-8C5), Mac1 (M1/70), all Biologic and visualized with fluorochrome-conjugated goat and rat antibodies. Lineage stained cells were next c-kit-enriched by using c-KIT-conjugated magnetic beads (Miltenyi, Bergisch Gladbach, Germany). c-KIT enriched cells were subsequently stained with antibodies against Ter119 (own preparation), SCA1 (D7, Biologic), c-KIT (2B8, Ebioscience), CD16/32 (FcγRII/III; 2.4G2 Pharmingen), CD41 (Igta2b; MWReg30, Pharmingen), Slamf1 (CD150; TC15-12F12.2, Biologic) and Endoglin (Eng/CD105; M7/18, Ebioscience). Propidium Iodide (Molecular Probes) was used to exclude dead cells.

Common lymphoid progenitor cells were obtained by sorting cells with a lineage negative, SCA1 low, c-KIT low, IL7Rα positive, FLT3 positive phenotype. Cells were maintained on ice when possible through all procedures and were sorted on a FACS Aria cell sorter (Becton Dickinson). All flow cytometry and FACS data were analyzed with FlowJo software (Treestar, Ashland, OR).

In vitro culture assays. Freshly isolated and sorted hematopoietic progenitor cellular subsets were cultured using liquid and solid-solide culture conditions (at 37°C, 98% humidity and 5% CO2) to evaluate clonogenic activity and lineage potentials of isolated cell populations. For liquid stroma supported cultures, indicated cell types and numbers (using a single cell donor coupled to a FACS Aria) were sorted into 96-well plates containing in OP9 stroma cells and analyzed for clonogenic and B-lineage potentials. For solid culture conditions (at 37°C, 98% humidity and 5% CO2), cultures were performed as described with some minor modifications. In short, 0.6% BactoAgar 2X (Becton Dickinson) was mixed with preheated DMEM 2X (Chemicon), supplemented with sodium pyruvate (Sigma) and sodium bicarbonate (Sigma), FCS and cytokines as above and kept at 37°C. Sorted cells were added and 1 ml was plated in duplicates or triplicates in 25 mm Petri dishes. After 7 days, cultures were fixed with glutaraldehyde (Fluka) and thereafter floated in a water bath and dried onto a glass slides and subsequently stained for (I) 4 hour for acetylcholinesterase [components: trisodium citrate (Sigma), copper sulphate pentahydrate (Sigma), potassium ferricyanide (Sigma), di-sodiumhydrogen orthophosphate (Fluka), sodiumdihydrogen orthophosphate (Fluka) and acetylthiocholine iodide (Sigma)], (II) 1.5 hour luxol fast stain [Luxol fast blue powder (Sigma), Urea (Fluka), 70% alcohol], followed by (III) 2 minutes of hematoxylin staining [hematoxylin (Sigma), sodium iodide (Sigma), potassium aluminium sulphate (Sigma), chloral hydrate (Sigma) and citric acid (Sigma)]. Slides were mounted with coverslips using DePex. Thereafter, numbers and types of generated colonies were determined using light microscopy.

Affymetrix gene expression and data analysis. RNA was extracted from purified adult BM subsets using RNaseasy mRNA purification kit (Qiagen). Subsequent handling was performed at the SweGene Affymetrix unit at Lund University (http://www.swegian.org/microarray). Briefly, RNA (arrays in triplicate for all progenitor fractions except for pre MegE for which 5 arrays were produced) was labeled and amplified according to AffymetrixTM; Small Sample Labelling Protocol v.2, with the exception that the second round of in vitro transcription (IVT) was performed using AffymetrixTM GeneChipTM Expression 3’ amplification kit. Hybridization and washing was performed according to AffymetrixTM GeneChipTM Expression analysis technical manual. Chips were scanned using an AffymetrixTM GeneChipTM Scanner 3000 and scaled to a median intensity of 100. For subsequent analysis, probe level expression values were extracted using RMA and subsequent analyses was performed using dChip software (http://biosun1.harvard.edu/complab/dchip/) following filtering (0.5 < SD/mean < 1,000).

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Multiparameter flow cytometric subfractionation of a myeloerythroid progenitor cell hierarchy


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