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Broad Distribution of Colony-Forming Cells with Erythroid, Myeloid, Dendritic Cell, and NK Cell Potential Among CD34++ Fetal Liver Cells

Marcus O. Muench* and Alicia Bárcaña†

The generation of erythroid, myeloid, and lymphoid cells from human fetal liver progenitors was studied in colony-forming cell (CFC) assays. CD38− and CD38+ progenitors that expressed high levels of CD34 were grown in serum-deprived medium supplemented with kit ligand, flk2/flt3 ligand, GM-CSF, c-mpl ligand, erythropoietin, and IL-15. The resulting colonies were individually analyzed by flow cytometry. CD56+ NK cells were detected in 21.9 and 9.9% of colonies grown from CD38− and CD38+ progenitors, respectively. NK cells were detected in mostly large CD14+/CD15+ myeloid colonies that also, in some cases, contained red cells. NK cells were rarely detected in erythroid colonies, suggesting an early split between the erythroid and the NK cell lineages. CD1a+ dendritic cells were also present in three-quarters of the colonies grown from CD38− and CD38+ progenitors. Multilineage colonies containing erythrocytes, myeloid cells, and NK cells were present in 13.7 and 2.7% of colonies grown from CD38− and CD38+ progenitors, respectively. High proliferative-potential CFCs that generated multilineage colonies were also detected among both populations of progenitors. The total number of high proliferative-potential CFCs with erythroid, myeloid, and NK cell potential was estimated to be 2-fold higher in the CD38+ fraction compared with the CD38− fraction because of the higher frequency of CD38+ cells among CD34++ cells. The broad distribution of multipotent CFCs among CD38− and CD38+ progenitors suggests that the segregation of the erythroid, myeloid, and lymphoid lineages may not always be an early event in hemopoiesis. Alternatively, some stem cells may be present among CD38− cells. The Journal of Immunology, 2001, 167: 4902–4909.

Hemopoietic stem cells are classically defined as cells capable of extensive proliferation (self-renewal) and multilineage differentiation. A fundamental goal of research in hemopoiesis is to develop an understanding of the process of differentiation, as well as to ascertain the hierarchy of hemopoietic progenitors that arise from stem cells. Development of in vitro assays that identify stem cells by supporting long-term multilineage differentiation at the single-cell level has been elusive. Efforts toward this goal continue to better dissect the early events of human hemopoiesis. An impressive number of recent studies have reported the development of in vitro techniques for the clonal analysis of primitive hemopoietic progenitors and stem cells, the progeny of which comprise both myeloid and lymphoid hemopoietic cells (1–6). Most of these studies are based on the coculture of primitive progenitors/stem cells with murine stromal cell lines and cytokines that support the differentiation of myelocytes, dendritic cells (DCs), NK cells, and B cells. Simultaneous generation of myeloid and lymphoid progeny has also been achieved in bulk cultures. The lymphoid lineages (T cells, B cells, NK cells, and certain DCs) were generated together with myeloid cells from CD34++ progenitors in human fetal organ cultures (7). Furthermore, we recently reported the cytokine requirements for the generation of NK cells and B cells under stroma-free and serum-deprived conditions from early fetal liver progenitors (8).

The colony-forming cell (CFC) assay has been an important tool in the clonal analysis of the lineage and proliferative potentials of progenitors at various stages of differentiation. The CFC assay has also been instrumental in the discovery of many of the cytokines and molecules that regulate hemopoiesis. Owing to our limited knowledge of the factors that regulate hemopoiesis, initial use of the CFC assay was limited to the study of myelopoiesis and erythropoiesis (9, 10). The clonal development of lymphoid progenitors was not achieved until the discovery of cytokines such as IL-7, which supports the clonal growth of murine B cell progenitors (11–13), and IL-15, which supports the generation of NK cells (14). Techniques have advanced such that multipotent progenitors from murine tissues can form colonies with myeloid, erythroid, and lymphoid cells (15, 16). Recently, it has been reported that human NK cell colonies can be grown from intrathymic committed lymphoid progenitors (17). However, the generation of NK cells from multipotent, uncommitted progenitors has not been reported. In addition, human CFCs with DC potential have been detected among CD34+ adult bone marrow precursors. These CFCs are distinct from the well-characterized granulocyte-macrophage CFC and required GM-CSF and TNF-α to develop (18–20).

The presence of DC progenitors among discrete subpopulations of primitive hemopoietic progenitors, such as CD38− CD34++ lineage− (CD38− CD34++ Lin−) candidate stem cells (21, 22), as well as the distribution of these DC progenitors among other CFCs with erythroid, myeloid, and NK cell potential, is presently unknown. To further the study of human stem cell differentiation and to better...
outlive the pathways leading to erythroid, myeloid, and lymphoid lineage commitment, a new multilineage colony-forming cell (CFC) assay was developed. We report on culture conditions that support the development of human CFCs with myeloid, erythroid, DC, and NK cell potentials. Using this assay, we investigated the presence and distribution of multipotent hematopoietic progenitors among the CD38− and CD38+ subsets of CD34+/Lin− fetal liver cells.

Materials and Methods

Isolation of fetal hematopoietic progenitors

Midgestation livers were obtained from elective abortions with approval of the Committee of Human Research at our institute. CD38− CD34+ Lin− (lineage = CD3, CD14, CD19, CD20, CD56, and CD235a) and CD38+ CD34+ Lin− fetal liver cells (23) were isolated by density separation, immunomagnetic bead depletion, and FACs as previously described (24, 25). For simplicity, these two populations of cells will be referred to as CD38− and CD38+ progenitors.

Colony assay and liquid culture conditions

Progenitors were grown in a serum-deprived medium (26, 27) supplemented with six recombinant human cytokines: kit ligand (KL) (50 ng/ml), flk-2/Flt-3 ligand (FL) (100 ng/ml), GM-CSF (20 ng/ml), c-mpl ligand (ML) (20 ng/ml), erythropoietin (EPO) (10 U/ml), and IL-15 (20 ng/ml). KL, FL, ML, and IL-15 were purchased from R&D Systems (Minneapolis, MN). EPO was purchased from Amgen (Thousand Oaks, CA) and GM-CSF (Leukine) from Immunex (Seattle, WA). This cytokine combination was used based on previous experiments that indicated that KL+FL+GM-CSF+IL-15 best supports the generation of NK cells from CD38+ progenitors among various cytokine conditions tested (8). EPO and ML were added to this cytokine combination to further support erythroid and megakaryocytic development. Attempts at generating colonies containing B and NK cells were made using the cytokine combination KL+FL+IL-15+IL-7 (8). IL-7 was used at 20 ng/ml and was purchased from R&D Systems.

Cells were cultured for 3 wk in parallel liquid cultures (8) and semisolid methylcellulose-based cultures (27). Liquid cultures were initiated at 1×104 to 1×105 cells/ml. CFCs were cultured at 50 or 100 cells/plate in three to seven replicate cultures. Colonies estimated to contain at least 50 cells were analyzed. Colonies were categorized into small (50−100 cells), large (500−10,000 cells), and high proliferative potential (HPP)-CFC derived (>10,000 cells) (28).

Analyses of lineage composition

Colonies containing erythrocytes were identified visually by the presence of hemoglobinized cells. The presence of myeloid (CD14+ and/or CD15+) cells and NK cells (CD56+ cells) was determined by flow cytometric analyses of live cells (8). Individual colonies were picked using a 200-μl pipette and dispersed in 100 μl of blocking buffer consisting of PBS supplemented with 0.01% NaN3 (Sigma; St. Louis, MO), 0.5% human γ-globulins (Sigma), and 5% normal mouse serum (Gemini Bio-Products, Woodland, CA). The blocking buffer also contained saturating amounts of CD14-PE (clone TUK4) (Caltag Laboratories, Burlingame, CA), CD15-PE (clone TUK4) (Caltag Laboratories, Burlingame, CA), CD36-PE (clone A6) (Beckman Coulter), CD4-PE (clone SK3; BD Immunocytometry Systems), CD1a-PE, CD2-FITC (clone MA66; Calbiochem, La Jolla, CA), CD3-FITC (clone 1D3; Beckman Coulter), CD56-PE (clone H12-2.1; Beckman Coulter), CD235a-PE (clone ITC-2; Beckman Coulter), CD33-PE (clone 5B11; Beckman Coulter), CD38-PE (clone 38-1; Beckman Coulter), CD45-PE (clone 2D1; Beckman Coulter), CD49d-PE (clone 2B11; Beckman Coulter), CD54-PE (clone H56; Beckman Coulter), HLA-DR-FITC (clone L243; BD Immunocytometry Systems) and CD56-PE (clone 5H5; Beckman Coulter) or nonfluorescent control mAbs. Alternatively, stained colonies were assayed for the presence of cytokine combination KL+FL+GM-CSF+ML+EPO+IL-15 was used to support multilineage hemopoiesis from CD38− and CD38+ progenitors. Progenitors grown in liquid cultures were used as controls for the flow cytometric analyses of colonies. Analysis of these control cultures indicated that the cytokines used supported the generation of CD56+ NK cells, CD14+ and CD15+ myeloid cells, CD36+CD235a+ erythroid cells, and CD41+CD42b+ megakaryocytes (Fig. 1). We have previously presented an extensive phenotypic profile of CD56+ cells, generated under similar culture conditions, and demonstrated the capacity of these cells to kill...
various tumor cell lines, such as K562, showing that the CD56+ cells generated are indeed NK cells (8).

Multilineage colony formation by CD38CD34+Lin− cells

Flow cytometric analysis of individual colonies was used to ascertain whether NK cells were present. The cell-surface phenotypic analysis was limited to two colors on our instrument with a third channel used for discrimination of live and dead cells, a prerequisite for reducing the number of background events. Myeloid cells were identified using anti-CD14 and anti-CD15 mAbs, and NK cells were identified using anti-CD56 mAb (Fig. 2). The presence of erythroid cells was determined visually. An overview of the lineage composition of all colonies analyzed is shown in Table I. Furthermore, more detailed analyses of the frequency and size distributions and lineage compositions of only those colonies containing CD56+ cells are presented in Tables II and III, respectively.

Analysis of 313 colonies, grown from CD38− progenitors isolated from five tissues, revealed the presence of NK cells in 24.6% of colonies (Table I). These same data represented as the mean ± SE of the results from individual experiments indicated the presence of NK cells in 21.9 ± 9.8% of colonies (Table II). NK cell colonies were more likely to be found among those colonies containing over 500 cells, although even a few colonies containing 50–100 cells were found to contain CD56+ cells. The greater likelihood of detecting CD56+ cells among large colonies may in part be due to the low frequency of NK cells detected. CD56+ cells represented a mean 1.7% (range = 0.003–20.4%) of cells within individual colonies. These observations are consistent with the low frequencies of NK cells detected in liquid cultures (Fig. 1).

Myeloid cells were detected in 98.1% of all colonies generated from CD38− progenitors (Table I). All colonies with NK cells contained myeloid cells and the majority, 55.8%, of these colonies further contained erythroid cells (Table III). The colonies with myeloid, erythroid, and NK cells comprised 13.7% of all colonies (Table I). Representative phenotypes of the different colony types are shown in Fig. 2. Pure myeloid colonies comprised 51.8% of all analyzed colonies (Table I). Colonies containing myeloid and NK cells but no erythroid cells constituted 10.9% of the total. Colonies containing myeloid and erythroid cells but not NK cells composed another 21.7%. No colonies comprised exclusively of erythroid and NK cells were observed, owing to the near ubiquitous presence of myeloid cells.

In addition to the heterogeneity in lineage composition of colonies derived from CD38− progenitors, there was also considerable variability in the size of these colonies. A high proliferative capacity is a marker of early progenitors and stem cells and, consequently, HPP-CFC are thought to be primitive progenitors and stem cells (29). We analyzed the lineage composition of 64 colonies generated from HPP-CFC (Table I). All but 1.6% of these colonies contained both myeloid and erythroid cells, and 59.4% of HPP-CFC were found to contain NK cells as well as myeloid and erythroid cells. Thus, the HPP-CFC fraction of CFCs is highly enriched for progenitors with lymphoid, myeloid, and erythroid potential.

Multilineage colony formation by CD38CD34+Lin− cells

NK cells were present in 9.9 ± 4.5% of 191 colonies grown from CD38− progenitors (Table II). Although the mean frequency of colonies with NK cells grown from CD38− progenitors was less than half that of colonies derived from CD38− progenitors (Table II), this difference was not significant (p = 0.22). All NK cell colonies derived from CD38+ progenitors also contained myeloid cells, and half of these colonies further contained erythroid cells (Table III). These multipotent progenitors represented 2.7% of all CFC (Table I), 5.1-fold less than found among CD38− progenitors.

In an analysis of 191 total colonies from four experiments, the CD38− progenitor population was found to contain 22.0% HPP-CFC. This frequency of HPP-CFC was higher than the 16.4%...
HPP-CFC found among 390 colonies analyzed from cultures of CD38<sup>−</sup> progenitors in six experiments (data not shown). The CD38<sup>+</sup> HPP-CFC generated colonies with erythroid and myeloid cells and 46.3% also contained NK cells (Table I). However, two large colonies had no detectable myeloid cells, and one of these did contain NK cells. Thus, the overall frequency of HPP-CFC with myeloid, erythroid, and NK cell potential found among CD38<sup>+</sup> progenitors was 10.2%, similar to the 9.8% measured among CD38<sup>−</sup> progenitors.

**Lineage-committed CFC among CD38<sup>−</sup>/CD34<sup>+</sup>/Lin<sup>−</sup> cells**

The CD38<sup>−</sup> fraction of CFCs has been shown to contain various lineage-restricted progenitor populations defined by cell surface markers (30, 31). Although progenitors committed to myelopoiesis and erythropoiesis were detected, no pure NK cell colonies were detected (Table I). However, one NK cell colony containing CD56<sup>+</sup> cells and CD56<sup>−</sup> cells of undetermined lineage was observed among 110 colonies analyzed. Furthermore, pure erythroid colonies represented only 5.5% of total colonies, whereas pure myeloid colonies comprised 40.0% of all colonies.

We have previously shown the cytokine combination KL+IL-15 to be sufficient to support the generation of CD56<sup>+</sup> cells from CD38<sup>−</sup> progenitors (8). Therefore, we attempted to analyze the colony composition of CD38<sup>−</sup> CFCs grown in KL+IL-15, because such colonies are likely to consist only of NK cells and a few myeloid cells. The few colonies that grew under these conditions did not contain CD56<sup>−</sup> cells (data not shown). However, there were many clusters, with fewer than 50 cells, which were too small to analyze. These results are not surprising, considering that the mean number of CD56<sup>−</sup> cells measured was 24.9 ± 0.3/colony, in colonies grown in the presence of KL+FL+GM-CSF+ML+EP0+IL-15. Thus, without the growth factors that support myelopoiesis and erythropoiesis, most pure NK cell colonies are likely to be too small to be analyzed by flow cytometry. For likely similar reasons, we were unable to detect CD19<sup>+</sup> B cells in any colonies grown from CD38<sup>−</sup> progenitors in KL+FL+IL-7+IL-15, although these cytokines support both B and NK growth in liquid culture (8).

**Identification of CFC with NK cell and DC potential**

Because DCs have been detected within hemopoietic colonies (19), we analyzed our cultures for the presence of these cells. As shown in Fig. 3, two types of CD14<sup>+</sup> cells could be distinguished. One subset was characterized by a low side-scatter and low autofluorescence in the FL-1 (FITC) channel (Fig. 3, A and C). The other subset had a higher side-scatter and greater autofluorescence (Fig. 3, C and D). Using CD1a as a characteristic marker of DCs (Fig. 3E), it became apparent that most of the high side-scatter CD14<sup>+</sup> cells were DCs. Like the high side-scatter CD14<sup>+</sup> cells, the CD1a<sup>+</sup> cells also exhibited a high side-light scatter (Fig. 3F). Indeed, CD14 expression was observed on a subset of the CD1a<sup>+</sup> cells (data not shown). Furthermore, the high side-scatter cells in our cultures also expressed variable levels of CD2, CD80, CD83, and CD16, and HLA-DQ (Fig. 3G). High levels of CD40 and HLA-DR expression were also observed, consistent with the classification of these cells as DCs. Further phenotypic analysis of the CD1a<sup>+</sup> cells indicated that they also expressed CD4 and CD11a, and ~50% expressed CD11c, but they did not express CD8α or CD11b (data not shown). The lack of CD11b expression has been correlated with murine lymphoid DCs in the thymus (32) and bone marrow (33). However, the expression of CD14 by the DCs generated in our culture conditions might suggest a myeloid origin for these cells.

**Table I. Lineage composition of all colonies and colonies derived from HPP-CFC**

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>CFC Population</th>
<th>n</th>
<th>MyENk</th>
<th>MyE</th>
<th>MyNk</th>
<th>ENk</th>
<th>E</th>
<th>My</th>
<th>Nk</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>All CFC&lt;sup&gt;h&lt;/sup&gt;</td>
<td>313</td>
<td>13.7</td>
<td>21.7</td>
<td>10.9</td>
<td>0.0</td>
<td>0.6</td>
<td>51.8</td>
<td>0.0</td>
</tr>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>HPP-CFC</td>
<td>64</td>
<td>59.4</td>
<td>39.1</td>
<td>0.0</td>
<td>0.0</td>
<td>1.6</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>All CFC&lt;sup&gt;i&lt;/sup&gt;</td>
<td>110</td>
<td>2.7</td>
<td>39.1</td>
<td>2.7</td>
<td>0.0</td>
<td>5.5</td>
<td>40.0</td>
<td>0.9</td>
</tr>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>HPP-CFC</td>
<td>67</td>
<td>46.3</td>
<td>50.7</td>
<td>0.0</td>
<td>1.5</td>
<td>1.5</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>*My, CD14<sup>+</sup> and/or CD15<sup>+</sup> myeloid; E, erythroid; Nk, CD56<sup>+</sup> NK cells.</sup>

<sup>†</sup> All colonies with ≥50 cells were analyzed. The colony composition of 1.3% of CD38<sup>−</sup>/CD34<sup>+</sup>/Lin<sup>−</sup> and 9.1% of CD38<sup>−</sup>/CD34<sup>+</sup>/Lin<sup>−</sup> could not be classified because no hemoglobinized cells were observed and no myeloid or NK cell Ags were detected.

**Table II. Frequency and size of colonies containing CD56<sup>+</sup> cells**

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Expt.</th>
<th>CFC/100 Cells</th>
<th>n</th>
<th>% CD56&lt;sup&gt;+&lt;/sup&gt;</th>
<th>&gt;1 × 10&lt;sup&gt;3&lt;/sup&gt;</th>
<th>100-500</th>
<th>&gt;500</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>1</td>
<td>15</td>
<td>88</td>
<td>15.9</td>
<td>ND</td>
<td>78.6</td>
<td>21.4</td>
</tr>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>2</td>
<td>41</td>
<td>164</td>
<td>60.4</td>
<td>32.32</td>
<td>37.6</td>
<td>8.1</td>
</tr>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>3</td>
<td>25</td>
<td>75</td>
<td>16.0</td>
<td>58.3</td>
<td>33.3</td>
<td>8.3</td>
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<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>4</td>
<td>14</td>
<td>49</td>
<td>10.2</td>
<td>20.0</td>
<td>40.0</td>
<td>20.0</td>
</tr>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>5</td>
<td>2</td>
<td>14</td>
<td>7.14</td>
<td>0.0</td>
<td>0.0</td>
<td>100</td>
</tr>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Combined&lt;sup&gt;h&lt;/sup&gt;</td>
<td>19 ± 6.5</td>
<td>390</td>
<td>21.9 ± 9.8</td>
<td>34.2</td>
<td>53.8</td>
<td>8.5</td>
</tr>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>1</td>
<td>1.9</td>
<td>13</td>
<td>23.1</td>
<td>0.0</td>
<td>0.0</td>
<td>66.7</td>
</tr>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>2</td>
<td>21</td>
<td>64</td>
<td>3.1</td>
<td>50.0</td>
<td>50.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>3</td>
<td>11</td>
<td>33</td>
<td>6.1</td>
<td>50.0</td>
<td>50.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>4</td>
<td>54</td>
<td>81</td>
<td>7.4</td>
<td>83.3</td>
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<td>16.7</td>
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<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Combined&lt;sup&gt;h&lt;/sup&gt;</td>
<td>22 ± 11</td>
<td>191</td>
<td>9.9 ± 4.5</td>
<td>53.8</td>
<td>15.4</td>
<td>33.1</td>
</tr>
<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>5&lt;sup&gt;i&lt;/sup&gt;</td>
<td>ND</td>
<td>30</td>
<td>46.7</td>
<td>50.0</td>
<td>50.0</td>
<td>ND</td>
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<tr>
<td>CD38&lt;sup&gt;−&lt;/sup&gt;/CD34&lt;sup&gt;+&lt;/sup&gt;/Lin&lt;sup&gt;−&lt;/sup&gt;</td>
<td>6&lt;sup&gt;i&lt;/sup&gt;</td>
<td>ND</td>
<td>34</td>
<td>67.7</td>
<td>100</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>h</sup> Results are presented as the mean ± SE of the results of individual experiments or as the combined results from all experiments.

<sup>i</sup> Only colonies containing erythroid and myeloid cells with >500 cells were analyzed.

<sup>j</sup> Only HPP-CFC-derived colonies with >1 × 10<sup>3</sup> cells were analyzed.
Table III. Lineage composition of colonies containing CD56+ cells

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Expt.</th>
<th>Myeloid</th>
<th>Erythroid</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD38+CD34+Lin-</td>
<td>1</td>
<td>28.6</td>
<td>0.0</td>
<td>71.4</td>
</tr>
<tr>
<td>CD38+CD34+Lin-</td>
<td>2</td>
<td>51.1</td>
<td>0.0</td>
<td>48.9</td>
</tr>
<tr>
<td>CD38+CD34+Lin-</td>
<td>3</td>
<td>25.0</td>
<td>0.0</td>
<td>75.0</td>
</tr>
<tr>
<td>CD38+CD34+Lin-</td>
<td>4</td>
<td>60.0</td>
<td>0.0</td>
<td>40.0</td>
</tr>
<tr>
<td>CD38+CD34+Lin-</td>
<td>5</td>
<td>100.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CD38+CD34+Lin-</td>
<td>Combinedb</td>
<td>44.2</td>
<td>0.0</td>
<td>55.8</td>
</tr>
<tr>
<td>CD38+CD34+Lin-</td>
<td>1</td>
<td>66.7c</td>
<td>0.0</td>
<td>0.0</td>
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<td>4.3</td>
<td>95.7</td>
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* Results are from the same experiments listed in Table I.
* Results are presented as the combined results from all experiments.
* Results do not add up to 100% owing to the presence of an NK cell colony lacking myeloid or erythroid elements.

Discussion

Human multipotent CFCs were successfully grown into colonies containing erythrocytes, myelocytes, NK cells, and DCs. Because the potential to generate erythroid, myeloid, and lymphoid cells is a property of stem cells, the broad distribution among CD34+Lin- cells of CFCs capable of generating cells from each of these lineages was surprising. Colonies containing erythrocyt, myeloid, and NK cells represented nearly 14% of those grown from CD38 progenitors and nearly 3% of those grown from CD38 progenitors. Because a high-proliferative potential is another property of stem cells, we also studied the lineage composition of the large colonies derived from HPP-CFC. These large colonies derived from CD38 progenitors contained DCs (data not shown). Representative analyses of colonies stained with CD14-FL, CD15-PE, and CD56-FITC for cells with DC characteristics. Only HPP-CFC-derived colonies with DCs were detected among 70.3% of colonies with erythroid progenitors and nearly 3% of those grown from CD38 progenitors as well as 80 colonies grown from CD38 progenitors for the presence of CD11c+DCs (data not shown). Representative analyses of colonies stained with CD11c and CD56 are shown in Fig. 4. Respective1,74.1 and 77.5% of colonies derived from the CD38 progenitors (n = 5 experiments, 313 colonies). In contrast, only 43.7 ± 7.1% of colonies grown from CD38 progenitors contained DCs (n = 3 experiments, 110 colonies).

Because CD11c is a better marker of DC than CD14, we further analyzed 81 colonies grown from CD38 progenitors as well as 80 colonies grown from CD38 progenitors for the presence of CD11c+DCs (data not shown). Representative analyses of colonies stained with CD11c and CD56 are shown in Fig. 4. Respective1,74.1 and 77.5% of colonies derived from the CD38 progenitors contained CD11c DCs. Among those colonies that contained NK cells, 83.3% derived from CD38 progenitors also contained DCs, whereas all NK cell colonies derived from CD38 progenitors contained CD11c DCs. Indeed, a few colonies from both progenitor populations appeared to consist of primarily DCs and NK cells as exemplified by the colony shown in Fig. 2F. There was less of an association between the erythroid and DC lineages. CD11c DCs were detected among 70.3% of colonies with erythrocytes derived from CD38 progenitors and only 56.7% of erythropoietin DCs were observed which differed in their levels of auto fluorescence (C) and light scatter profiles (A and D). E and F, CD11c+ DCs had a similar high autofluorescence and moderate to high side light scatter profile as some CD11c+ DCs. These representative results were obtained from CD38 progenitors+Lin- cells cultured for 3 wk in KL+FL+G-CSF+IL-15. G, Coexpression of various DC markers was analyzed on moderate to high side-scatter cells generated in cultures containing KL+FL+GM-CSF+IL-15. ML and EPO were not added to the culture to enrich the final cell population for DCs owing to the lack of erythroid cells.

FIGURE 3. Generation of DCs in bulk liquid cultures. Two populations of CD14+ cells were observed which differed in their levels of autofluorescence (C) and light scatter profiles (A and D). E and F, CD11c+ DCs had a similar high autofluorescence and moderate to high side light scatter profile as some CD11c+ DCs. These representative results were obtained from CD38 progenitors+Lin- cells cultured for 3 wk in KL+FL+G-CSF+ML+EPO+IL-15. G, Coexpression of various DC markers was analyzed on moderate to high side-scatter cells generated in cultures containing KL+FL+GM-CSF+IL-15. ML and EPO were not added to the culture to enrich the final cell population for DCs owing to the lack of erythroid cells.

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colonies were nearly always composed of both myeloid and erythroid cells. NK cells were also present in 59% of HPP-CFC-derived colonies grown from CD38<sup>+</sup>/H11002<sup>-</sup> cells (27). Thus, the CD38<sup>+</sup> progenitors of CD38<sup>+</sup>/H11002<sup>-</sup> cells may be the progeny of CD38<sup>-</sup> cells. Fig. 5A illustrates this possibility in a model that assumes hemopoiesis to be an orderly process of progressive lineage restriction. In such a model, CD38 expression must fluctuate to account for the presence of the marker on progenitors with erythroid, myeloid, and NK cell potential. The progenitor-progeny relationship between CD38<sup>-</sup> and CD38<sup>+</sup> cells is in most part based on the higher frequency of progenitors with the properties of stem cells being observed among the CD38<sup>-</sup> fraction. Thus, the existence of infrequent CD38<sup>-</sup> stem cells remains a possibility not at odds with previous findings.

Other models of hemopoietic differentiation can be envisioned that would preserve the progenitor-progeny relationship between

![Flow cytometric detection of DCs and NK cells in colonies.](image)

**FIGURE 4.** Flow cytometric detection of DCs and NK cells in colonies. Colonies grown from CD38<sup>+</sup>/CD34<sup>-</sup>/Lin<sup>-</sup> cells were visually scored for the presence of erythrocytes, isolated, and then stained with mAbs recognizing DCs (CD1a) and NK cells (CD56). A, Large colony that contained erythrocytes and myeloid cells but neither DCs nor NK cells (shown as a negative control). B, Nonerythroid colony containing CD1a<sup>+</sup> DCs. C, Example of an HPP-CFC-derived colony that contained erythrocytes and DCs, but not NK cells. D, HPP-CFC-derived colony that contained erythrocytes, DCs, and NK cells.

![Hypothetical models of hemopoietic differentiation.](image)

**FIGURE 5.** Hypothetical models of hemopoietic differentiation. Two models are presented. A, In the first model, it is assumed that hemopoietic differentiation undergoes an orderly process of progressive lineage restriction. Note, to account for the different types of bipotent progenitors observed, lineage restriction is represented as the progressive loss of lineage potential. Thus, two types of myeloid progenitors are generated, one with NK cell potential and the other with erythroid potential. For this model to fit the data, CD38 expression must modulate resulting in some committed progenitors being CD38<sup>-</sup>/CD34<sup>+</sup> and some early progenitors/stem cells being CD38<sup>-</sup>/CD34<sup>+</sup>. B, In the second model, it is assumed that CD38<sup>-</sup> CD34<sup>+</sup> progenitors are the precursors of all CD38<sup>-</sup> CD34<sup>+</sup> progenitors. For this model to fit the data, multiple pathways of hemopoietic differentiation must exist. Note the weights of the arrows are approximately proportional to the likelihood of the differentiation pathway, based on the frequencies of the different types of progenitors that we detected. These models are meant to represent two extreme possibilities. A number of other models can be envisioned by combining elements of both models.
CD38− and CD38+ cells and fit our findings. Hemopoietic differentiation has been described as a stochastic process (38). This is primarily based on the analysis of the lineage composition of colonies generated from paired daughter cells. The paired colonies would often differ in lineage composition, indicating that differentiation occurred randomly (39, 40). Furthermore, no orderly pattern of myeloid/erythroid differentiation could be discerned from these reports. A model that assumes that CD38− cells are the precursors of CD38+ cells and that also accounts for the different types of progenitors we detected in our cultures is shown in Fig. 5B. Multiple pathways of differentiation are required in this model, consistent with a stochastic model of hemopoiesis. This model would account for the loss of erythroid potential by some CD38+ progenitors, whereas such lineage restriction may also occur at a later, CD38+, stage of differentiation for other progenitors. Moreover, some CD38− stem cells may rapidly commit to the myeloid lineages while others proliferate and develop into CD38+ progenitors still capable of forming erythroid and lymphoid cells. The two models shown in Fig. 5 represent extreme possibilities, and other models can be envisioned that allow for multiple pathways of differentiation as well as some fluctuation of CD38 expression during differentiation.

Although our findings lend support to a stochastic process involved in lineage differentiation, a purely random control of lineage differentiation was not observed. Colonies containing NK cells nearly always contained myeloid or myeloid and erythroid cells. Only a single colony containing erythroid and NK cells, but no identifiable myeloid cells, was observed in a culture of CD38+ progenitors. Therefore, suggesting that the segregation of erythroid and NK cell lineage potentials is an event that takes place at an earlier stage of hemopoiesis than the segregation of myeloid and NK cell potential. Alternatively, it is worth considering the possibility that the presence of erythroid or myeloid cells within colonies affects the differentiation of NK cells, leading to the preferential development of NK cells within myeloid colonies. However, we have not found evidence to suggest that erythroid cells inhibit NK cell development or that myeloid cells efficiently support NK cell development in our cultures (Ref. 8 and our unpublished observations). Furthermore, our previous findings that GM-CSF and IL-3, growth factors that support the growth of early myeloid progenitors, also support the growth of NK cell progenitors lends support to a close relationship between the myeloid and NK cell lineages (8). NK cells are considered a type of lymphocytic cell that is developmentally close to T cells (41), because they share numerous cell-surface markers and functional activities. Indeed, a common T/NK progenitor has been described to exist in the human fetal thymus (42). Our data suggest the possibility of multiple pathways of NK cell differentiation. This is a more complex and flexible scenario than the traditional models, which contemplate an absolute split between lymphoid- and myeloid-committed progenitors. One pathway could be represented by a common lymphoid progenitor, contained among CD38− cells, and restricted to lymphoid lineages (43). An additional pathway of NK differentiation could occur very early in development, suggested by the presence of bipotent NK/myeloid progenitors and the absence of NK/erythroid progenitors at the CD38+ stage. The greater plasticity afforded by a stochastic model of hemopoiesis would accommodate multiple pathways of development for the various hemopoietic lineages.

DCs were present in approximately three-quarters of the colonies generated from CD38− and CD38+ progenitors. This frequency was notably higher than the 17% rate measured in clonal cultures of adult bone marrow CD38+ progenitors (44). The use of fetal tissue or the cytokines we used to support our cultures may have contributed to the higher cloning efficiency that we observed. GM-CSF, FL, KL, and IL-15 have all been shown to support the growth of DCs (20, 45, 46). Previous reports have demonstrated the generation of DC colonies from CD34+ bone marrow cells cultivated in the presence of GM-CSF and TNF-α (19, 20). Several different types of DCs are thought to exist (47). The DCs generated in our cultures expressed the CD1a Ag and displayed a typical DC phenotype: CD40+HLA-DR+HLADQ+CD80+CD83+/−CD86+. In addition, they expressed CD2, CD4, and CD11a. Some CD11c+ cells also expressed CD11c and CD14, but they were negative for CD8a or CD11b expression. The lack of CD11b expression has been correlated with murine lymphoid DCs in the thymus (32) and bone marrow (33). Furthermore, the expression of lymphoid-associated markers such as CD2 and CD4 has been reported on human thymus-derived DCs and DC progenitors (48, 49). A recent study showed that CD2+CD14+ cells in the peripheral blood are the precursors of DCs, and they seem to constitute a distinct subpopulation of CD14+ monocytes, functionally and phenotypically different from monocyte-derived DCs (50). However, the expression of CD14 by DCs has also used as an indication of the myeloid origin of these cells (34, 51). In the absence of defining phenotypic or functional characteristics, the lymphoid or myeloid origin of the DCs produced under our culture conditions is presently unclear.

Both a lymphoid and a myeloid DC progenitor have been described to exist among CD38+ progenitors (43, 52). Although some of the DC colonies we observed may have arisen from one of these progenitors, more than half of the colonies analyzed also contained erythrocytes and could not have been derived from either of these committed progenitors. Indeed, only a few colonies had the appearance of nearly pure DC colonies, whereas most colonies containing DCs contained other cell populations. The CD10+ lymphoid-committed progenitor characterized by Galy et al. (43) would possibly generate colonies containing exclusively NK cells and DCs in our cultures. Over 80% of colonies that we analyzed that contained NK cells also contained DCs. Furthermore, colonies containing erythrocytes were less likely to contain DCs than colonies containing NK cells, suggesting a closer relationship between the DC and NK cell lineages than the DC and erythroid lineages. Indeed, some colonies containing predominantly NK cells and DCs were observed, including colonies from cultures of CD38+ progenitors. Because the lymphoid progenitor described by Galy et al. is CD38+, the possibility that differentiation toward the DC and NK cell lineages occurs at an earlier stage of hemopoiesis would indicate that several pathways of differentiation may lead to the formation of these cell types. Certainly further evidence is required to prove the existence of more than a single pathway of lymphoid differentiation. The ability to now measure CFC with erythroid, myeloid, and lymphoid potential will likely aid in the in vitro identification and study of stem cells and early progenitors.

Acknowledgments

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References


