

Lineage specification and plasticity in CD19⁻ early B cell precursors

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We describe here three CD19⁻ B cell precursor populations in mouse bone marrow identified using 12-color flow cytometry. Cell transfer experiments indicate lineage potentials consistent with multilineage progenitor (MLP), common lymphoid progenitor (CLP), and B lineage-restricted pre-pro-B (Fr. A), respectively. However, single cell in vitro assays reveal lineage plasticity: lymphoid/myeloid lineage potential for CLP and B/T lineage potential for Fr. A. Despite myeloid potential, recombination activating gene 2 reporter activation is first detected at low levels in most MLP cells, with 95% of CLPs showing 10-fold increased levels. Furthermore, single cell analysis shows that half of CLP and 90% of Fr. A cells contain heavy chain DJ rearrangements. These data, together with expression profiles of lineage-specific genes, demonstrate progressive acquisition of B lineage potential and support an asynchronous view of early B cell development, in which B lineage specification initiates in the MLP/CLP stage, whereas myeloid potential is not lost until the pre-pro-B (Fr. A) stage, and B/T lymphoid plasticity persists until the CD19⁺ pro-B stage. Thus, MLP, CLP, and Fr. A represent progressively B lineage-specified stages in development, before the CD19⁺ B lineage-committed pro-B stage.

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Abbreviations used: CLP, common lymphoid progenitor; DL1, Delta-like 1; FTOC, fetal thymic organ culture; LSK, LIN⁻Sca1⁺cKit⁺; MLP, multi-lineage progenitor; SCF, stem cell factor.

B cell development in the mouse occurs in the fetal liver before birth and shifts shortly thereafter to the bone marrow, where it continues throughout life (1). The production of B cells is a highly ordered process, mediated by several transcription factors that regulate expression of a set of lymphoid- and B lineage-specific genes at well-defined developmental stages (2). Thus, Ig heavy chain D_HJ_H rearrangements occur on both chromosomes in pro-B cells, followed by V_H to D_HJ_H rearrangement to yield a functional heavy chain protein in pre-B cells. Heavy chain protein then associates with surrogate light chain components to form a pre-B cell receptor that signals events required for development to later stages, where Ig light chain rearranges and associates with heavy chain, allowing its expression on the surface of a newly formed B cell (3). Although such development from pro-B to pre-B and B cell is relatively well characterized (4), the very early B lineage stages, before CD19 expression, are less well understood (5–8).

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Differentiation from hematopoietic stem cells to early B lineage cells proceeds through a series of intermediate steps during which cells are thought to become progressively more restricted in their developmental potential (9). In this model of development, hematopoietic stem cells produce multilineage progenitors (MLPs) that are capable of developing into erythroid, myeloid, and lymphoid lineage cells. Then these MLPs generate progeny populations restricted to either lymphoid (common lymphoid progenitor [CLP]) or erythroid/myeloid (common myeloid progenitor) cell lineages (10, 11). CLP stage cells eventually generate CD19⁺ pro-B cells. Immediately before the CD19⁺ pro-B stage, cells that appear B lineage restricted have been identified (5, 7, 8, 12) based on expression of CD45R/B220 and are hereafter referred to simply as B220. These cells rapidly generate CD19⁺ pro-B cells in vitro and so we have referred to them as pre-pro-B cells (5, 7, 13), a stage presumed to be intermediate between the CLP and CD19⁺ stages of development.

On the other hand, clear identification of these early CD19⁻ stages, defining the point at which they become committed to the B lineage (14)

and lose the capacity to generate alternate hematopoietic cell types, has been difficult and remains in dispute (15–17). B cell developmental stages in mouse bone marrow have been subdivided previously based on a diverse set of cell surface proteins, including B220, CD19, CD43, CD24/HSA, CD25/IL2R α , CD117/cKit, and CD127/IL-7R α (13, 18–20). Differential expression of steel factor (stem cell factor [SCF]) receptor CD117/cKit and the IL-7R CD127 has been used to distinguish MLPs (CD117^{hi}CD127⁻) from CLPs (CD117^{med}CD127⁺) among lineage-negative bone marrow cells (10). Although CLPs were initially described as generating lymphoid but not myeloid cells (10), a recent study suggests myeloid potential in this cell fraction (21). Among B220⁺ cells, we originally identified the Fr. A pre-pro-B cell stage based on a distinctive low level of CD24/HSA, constituting \sim 1% of bone marrow (13). However, the homogeneity and functional lineage restriction of cells in this “Fr. A” have seen reassessment over time. Thus, it became clear that the Fr. A “pre-pro-B” cell fraction as initially described contained non-B lineage cells (5, 7), including CD4⁺ (and Ly-6C⁺) dendritic cell precursors capable of giving rise to plasmacytoid dendritic cells (22, 23). More recently, using expression of the lymphoid-restricted gene TdT, some have suggested that most early B lineage precursors do not fall within the CD24^{low} fraction of B220⁺CD19⁻ cells (15).

To resolve this ambiguity over the identification of the earliest B lineage precursor(s), we have applied 12-color flow cytometry to purify homogenous precursor populations and then characterize their developmental potential. Importantly, our analysis incorporates multiple approaches for identifying early lymphoid stages, such as expression of TdT (15) and RAG-1/2 (17), use of reporter transgenic mice (17), lineage-negative gating (10, 24), and separation based on key cell surface markers such as Ly6c (15), CD117/cKit, and CD127/IL-7R (10). Using this type of analysis, we can easily correlate our results with analyses done by others (10, 15–17, 25). The goal of our work is to connect the B220⁻CLP stage (10) to the CD19⁺ pro-B stage through a clearly defined B220⁺ pre-pro-B stage (Fr. A).

Our analysis revealed that B lineage specification initiates unexpectedly early, at the MLP/CLP stage in bone marrow, and that there is greater persistence of lineage plasticity in B cell development than previously thought, such that myeloid potential is not lost until B220 expression (Fr. A) and B/T lineage plasticity persists until the CD19⁺ pro-B stage (Fr. B).

RESULTS

Early CD93/AA4.1⁺ B lineage progenitor cell fractions revealed by 12-color flow cytometry

To identify hematopoietic cells transiting from MLPs to the CD19⁺ pro-B stage, we adopted the approach pioneered by Muller-Sieburg et al. (24) and Spangrude et al. (25) using a mixture of lineage-specific staining reagents to deplete erythroid, myeloid, and T lineage cells from bone marrow. To simultaneously analyze the potential B lineage precursors, we added reagents specific for proteins that define stages of B cell

development after CD19 expression, including B220, CD43, CD24, CD93, and IgM (13). We also included reagents recognizing CD117/cKIT and CD127/IL-7R α , which have been used to identify the MLP and CLP fractions (10). Fig. 1 shows analysis with these reagents, eliminating cell doublets/aggregates by forward light scatter height/area gating; focusing on intermediate-size cells by forward/side light scatter gating; eliminating dead cells, highly autofluorescent cells, and T cells by CD3/PI gating; and finally eliminating cells expressing other non-B “lineage marker” proteins by gating with reagents specific for monocyte/macrophage/granulocyte/dendritic cells (CD11b, GR1, and Ly6c) and erythroid cells (Ter119).

Further gating based on a display of CD19 versus CD24/HSA allowed elimination of CD19⁺ (pro-B and later) B lineage cells, focusing on CD19⁻ cells, most of which expressed low levels of CD24. Based on previous findings that CD93/AA4.1 is expressed from hematopoietic stem cells through the immature B cell stage (26–28), we selected CD93^{high} cells, most of which expressed intermediate levels of CD43, similar to that found on CD93^{hi}CD43^{med}CD19⁺ pro-B cells (5). This cell fraction contained B220⁻ and B220⁺ subsets. Analyzing both subsets for expression of CD117 versus CD127 identified the following three cell populations: (a) B220⁻ cells, some with high levels of CD117 and lacking CD127, others with intermediate levels of CD117 and bearing CD127; and (b) B220⁺ cell, most bearing CD127 and many with intermediate levels of CD117. Based on the similarity of the B220⁻ fractions to very early multilineage precursors and common lymphoid progenitors described previously, we provisionally referred to these cells as MLPs and CLPs (10). We refer to the B220⁺ fraction as pre-pro-B “Fr. A” based on their expression of B220 and low-level expression of CD24. We excluded the infrequent and variable numbers of B220⁺ cells having undetectable levels of CD117 because such cells were not found in the B220⁻ (CLP) fraction and showed only lower level expression of a RAG-2-GFP reporter compared with CLP, Fr. A, and pro-B stage cells (not depicted).

We then used a “back-gating” analysis, examining the distribution of cell surface proteins used in delineating these subsets to assess whether we might have excluded significant portions of cells belonging to the CD117^{hi}CD127⁻ and CD117^{med}CD127⁺ cell populations, considered to identify the MLP and CLP stages, respectively (9, 10). Although all CD117^{med}CD127⁺ were included in the CD93^{hi}CD43^{med} gate, this analysis revealed that only a portion of the CD117^{hi}CD127⁻ cells were CD93^{hi}CD43^{med}, with many more exhibiting a CD93^{med}CD43^{hi} phenotype. Most cells in the CD93^{med}CD43^{hi} population belong to the LIN⁻Sca1⁺cKit⁺ (LSK) fraction of very early hematopoietic cell precursors, including stem cells (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20052444/DC1>). Thus, it seems likely that the earliest stages of hematopoietic cell differentiation, such as LSK, have lower levels of CD93 (and higher levels of CD43) than the MLP, CLP, and Fr. A stage cells that are the focus of this work. As Fig. S2 shows, most LIN⁻CD19⁻ cells

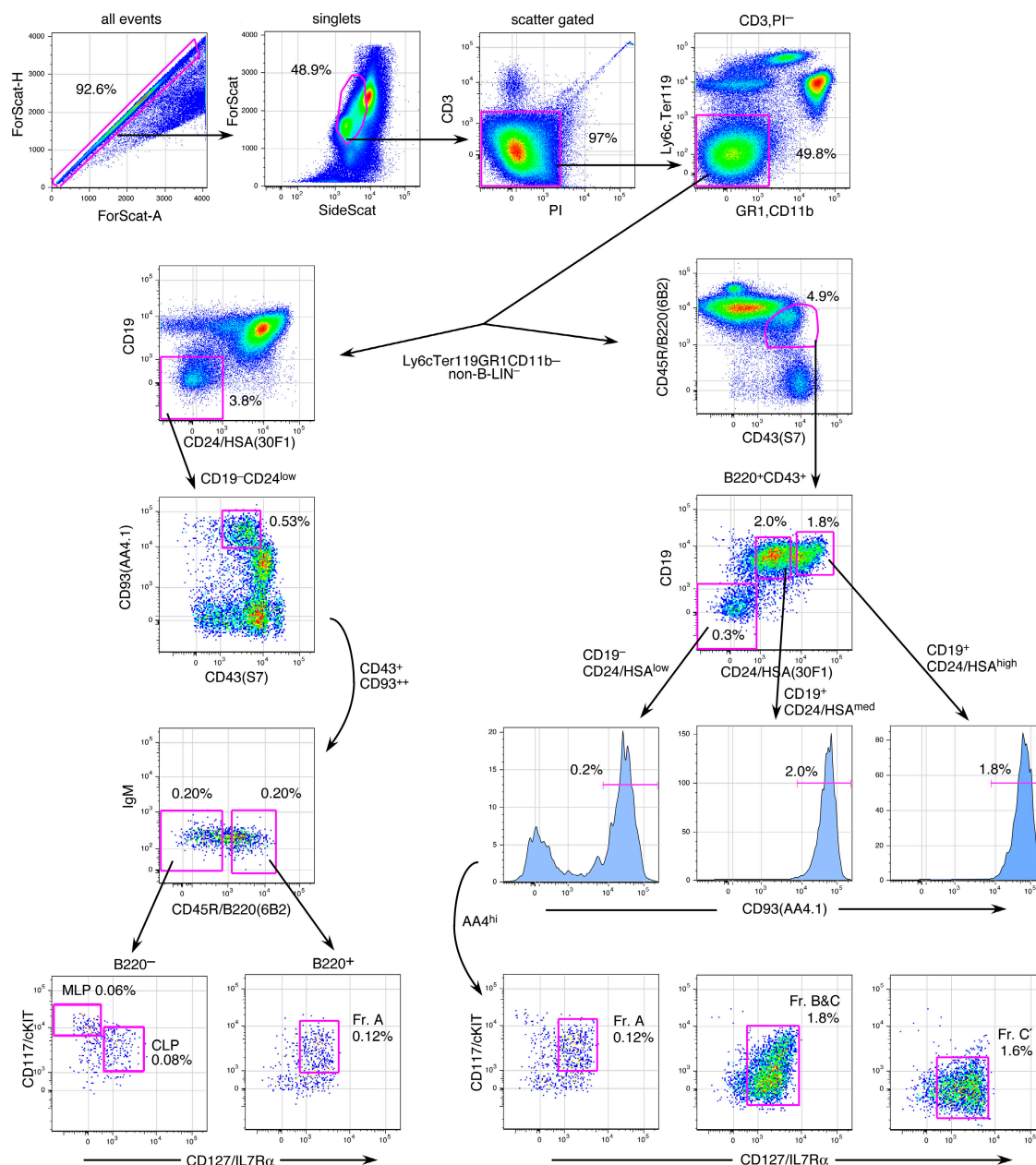


Figure 1. Identification of MLP, CLP, pre-pro-B (Fr. A), pro-B (Fr. B and C), and early pre-B (Fr. C') stages using a combination of fluorescent reagents and 12-color flow cytometry. The staining reagents are described in Materials and methods. Two gating approaches

are used to discriminate these stages: one for the three earliest, shown on the left, and another for three later stages, shown on the right. 5×10^5 bone marrow cells were analyzed. Data shown are representative of more than 10 independent analyses using 6–12-wk-old BALB/c female mice.

expressing very high levels of a RAG-2 GFP reporter (see next section) fall within the $CD117^{med}CD127^+$ gate and these cells are predominantly $CD43^{med}$ and $CD24^{low}$ (i.e., are CLP and Fr. A as we define them). We conclude that using the gates in Fig. 1 encompasses essentially all early B lineage precursors.

In a second gating approach with the same stained sample, we focused on cell surface proteins whose expression identifies Fr. A through Fr. C', focusing on $B220^+CD43^{med}$ cells, subdividing them on the basis of CD24 and CD19. Among

these cells, most of those lacking CD19 have a distinctively low to undetectable level of CD24 (Fig. 1, right side). Furthermore, in contrast to the relatively unimodal high-level CD93 expression found with $CD19^+$ stage cells, there is considerable heterogeneity in the $CD24^{low}$ fraction. Again, based on previous work showing that $CD93^-$ cells with this phenotype do not belong to the B lineage (5), we excluded $CD93^-$ cells and identified a set of $CD93^+CD117^{med}CD127^+$ cells, corresponding to the pre-pro-B population (Fr. A) identified in the first gating analysis described above. We also distinguish

Table I. Cell surface protein expression used to define early B cell precursors in bone marrow

Cell surface protein	MLP	CLP	Fr. A	Fr. B&C
(all LIN ⁻)				
CD93/AA4.1	high	high	high	high
CD117/cKit	high	medium	medium	low/-
CD127/IL-7R α	-	+	+	+
CD24/HSA(30F1)	low	low	low	+
CD45R/B220(6B2)	-	-	+	+
CD19	-	-	-	+
CD43(S7)	medium	medium	medium	medium

two subsets of CD43⁺CD19⁺ stage cells, with intermediate and high levels of CD24, respectively. Both of these fractions, corresponding to stages termed pro-B (Fr. B and Fr. C) and early pre-B (Fr. C'; reference 13), express homogenous high levels of CD93 and are CD127⁺, but have variable (in B and C) to undetectable (in C') levels of CD117. Table I summarizes the surface markers used in identifying the CD19⁻ fractions and CD19⁺ pro-B fractions examined in this work.

Cell transfer analysis supports designation of CD19⁻ cell fractions as MLP, CLP, and Fr. A

Next, we tested the capacity of cells in these cell fractions to engraft B, T, NK, and myeloid cells using a standard Ly5-congenic competition assay (Fig. 2). Thus, we injected Ly5.2 sorted cells mixed with a constant amount of Ly5.1 unfractionated bone marrow i.v. into lethally irradiated Ly5.1 recipient mice and examined the Ly5.1/Ly5.2 chimerism in different hematopoietic cell lineages 3 wk after transfer. This approach shows how a hematopoietic progenitor's fate is read out in the context of the whole organism environment.

Fig. 2 A shows the percent of each specific cell type contributed by the purified precursor population in various tissues, demonstrating robust multilineage reconstitution by the CD117^{hi}CD127⁻ (MLP) fraction, where we obtained large numbers of Ly5.2⁺ myeloid cells and macrophages in bone marrow, T lineage cells in thymus and spleen, and B cells in spleen. We also detect excellent engraftment of DX5⁺ NK cells. T cell reconstituting capacity at 3 wk after transfer was most clearly revealed by analysis of thymus engraftment, the primary site of T cell development. Therefore, Fig. 2 B presents the absolute numbers of myeloid, T, and B cells generated in bone marrow, thymus, and spleen, setting the values obtained with the MLP fraction to 1.0 and expressing the others relative to it. The greater numbers engrafted by MLP stage cells likely represent their capacity to proliferate significantly as precursors, before differentiating into the various hematopoietic cell types analyzed here. Although fewer cells were produced with transfers of CD117^{med}CD127⁺B220⁻ (CLP) fraction cells, we nonetheless observed a clear lymphoid-restricted repopulation of T, B, and NK cells (and few myeloid cells). Transfer of CD117^{med}CD127⁺B220⁺ (Fr. A) cells engrafted far fewer T cells (evident from analysis of thymus; Fig. 2 B) and few NK cells, and still generated a significant

population of B cells, suggesting a clear delineation of Fr. A pre-pro-B cells. As expected, the CD19⁺ Fr. B subset produced the clearest B cell-restricted repopulation. In

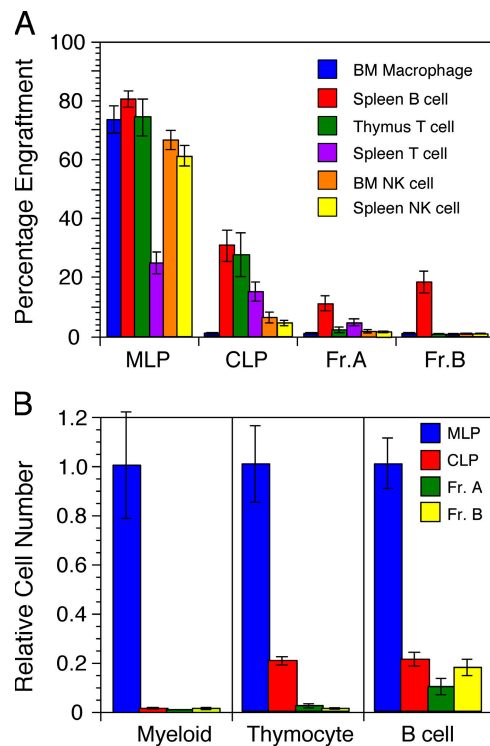


Figure 2. B, T, NK, and myeloid engraftment from MLP stage cells, in contrast with predominant lymphoid repopulation using CLPs, and predominant B cell repopulation using Fr. A. Cell populations defined as in Fig. 1 were isolated by cell sorting from B6.Ly5.2 mouse bone marrow and injected i.v. together with unfractionated bone marrow from B6 wild-type (Ly5.1) mice into lethally irradiated B6 mice. After 3 wk, recipient animals were killed and indicated tissues were analyzed by flow cytometry for the presence of Ly5.1/Ly5.2 cells in B (B220⁺CD19⁺IgM⁺), T (CD4/C8⁺CD3⁺), NK (NK1.1/DX5⁺), and myeloid (CD11b/GR1⁺) cell populations. (A) Percent engraftment reported is frequency of Ly5.2⁺ cells divided by total cell frequency for the indicated population. (B) Absolute numbers of Ly5.2⁺ cells of the indicated cell type were determined and then compared with the number obtained with MLP stage cells (defined as 1.0 for each cell type). Error bars show standard error for analyses of 6–10 individual recipients from four separate experiments.

summary, cell transfer data support the designation of these stages as MLP, CLP, and Fr. A.

Bipotential B/myeloid assay reveals myeloid potential in CLPs

Next, we asked whether all CLP stage cells are lymphoid committed. Although the cell transfer analysis appeared to indicate such restriction, this assay suffers from at least two deficits: (a) lineage plasticity may be masked by failure of cells to migrate into inducing microenvironments for all potential alternate cell lineages; and (b) the seeding efficiency may vary among different cell subsets, making it difficult to estimate the frequency and homogeneity of subsets under analysis. Therefore, we assessed lineage plasticity of cells in these early stages by a series of clonal (or near-clonal) *in vitro* assays. First, we investigated B/myeloid potential using a bipotential assay (29), depositing individual cells (1 cell/well) into a 96-well plate containing a preestablished S17 stromal cell monolayer and medium supplemented with SCF, Flt3 ligand, and IL-7 (Fig. 3 A). This combination of cytokines and stromal cells supports the growth and differentiation of most early hematopoietic cells (30) and, under these conditions, both myeloid and B lymphoid cells can develop clonally with high efficiency. Most clones developing from MLPs were myeloid (CD11b⁺/CD19⁻) with occasional B lineage colonies. CLP stage cells generated more B lineage colonies, but we also observed a significant number of myeloid colonies. In contrast, plates sorted with the B220⁺ Fr. A and the CD19⁺ pro-B Fr. B and C contained very few myeloid colonies. Thus, although CLPs generated numerous myeloid colonies, few were seen in plates containing sorted Fr. A cells.

One possible explanation for our detection of myeloid potential from CLPs is heterogeneity within the cell fraction we identified, with only a portion being “true” CLPs. To address this, we examined the expression of a RAG-2 fluorescent reporter BAC transgene (31) in these early B lineage fractions. Expression of recombinase activating genes has been considered one of the hallmarks of lymphoid specification (17). As Fig. 4 A shows, we found that all RAG⁺ cells are CD93⁺. Furthermore, using the high-level CD93 gate we use (see Fig. 1), essentially all MLP cells express low but detectable levels of GFP and some express higher levels (Fig. 4 B). Cells in the CLP fraction show a 10-fold higher level than MLP, and those in Fr. A express a yet higher level. Although most cells in the CLP fraction show very high levels of RAG-2-GFP, there are some cells with a lower level, similar to MLP, so we fractionated cells based on levels of RAG-2-GFP to determine whether enriching for the RAG-2/GFP highest-expressing fraction would eliminate the myeloid colonies for MLP or CLP cultures. This was not the case, as we found many myeloid colonies with MLP fraction cells expressing the highest levels of RAG-2/GFP and also obtained significant numbers of myeloid colonies with RAG-2/GFP-gated CLPs (Fig. 3 B). Thus, the myeloid capacity in CLP stage cells detected by the bipotential culture assay does not reflect a heterogeneity in the cell population that can be

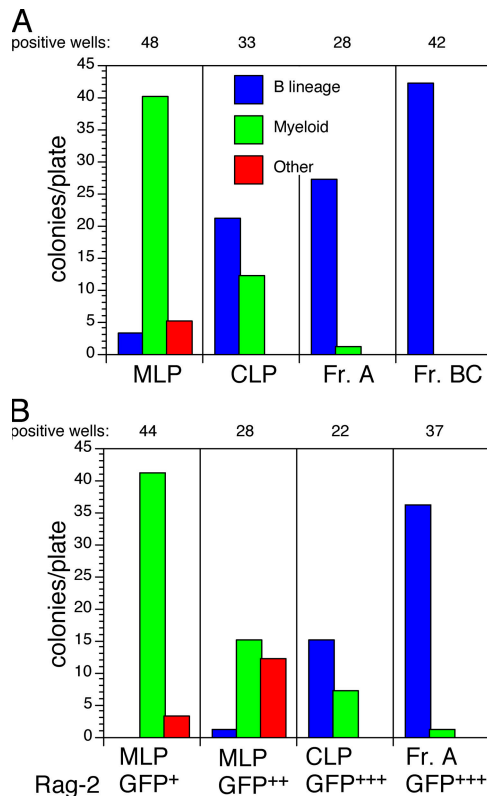


Figure 3. Bipotential stromal cell assay reveals unexpected myeloid capacity in CLP stage cells that is lost upon progression to the B220⁺ (Fr. A) stage. (A) Individual cells from the indicated population were deposited into wells of a 96-well flat-bottom plate containing pre-established S17 stromal cells in complete RPMI 1640 medium, supplemented with SCF, Flt3L, and IL-7. After 7–10 d, plates were examined for cell colonies and these were harvested and analyzed by flow cytometry. Colonies were classified as B (CD11b⁺CD19⁺), myeloid (CD11b⁺CD19⁻), or other (CD11b⁻CD19⁻). No mixed colonies were observed. Representative data from more than four separate experiments is shown. (B) A similar analysis was performed with cells sorted from the bone marrow of RAG-2-GFP reporter mice, selecting the GFP intermediate (+) or brightest (++) MLP stage cells, or the GFP-high (+++) CLP or Fr. A stage cells. Gated regions are marked on the GFP histograms in Fig. 4. Representative data from more than four separate experiments is shown.

fractionated based on RAG expression. Rather, appearance of surface B220 expression on CD117^{med}CD127⁺ cells, recognized as Fr. A, represents a clearer indicator of the loss of myeloid potential as B cell development progresses.

T lineage potential in Fr. A stage cells

Having observed that CLP has myeloid potential, the question arises whether a more clearly B/T-restricted “CLP” stage could be identified. To examine T lineage potential, we undertook fetal thymic organ culture (FTOC) using the high oxygen submersion modification developed by Dou et al. (32) that facilitates clonal generation of T cells. We compared the capacity of the CLP, Fr. A, and pro-B stage cells (3 cells/well) to produce T lineage cells in a lymphoid lobes isolated

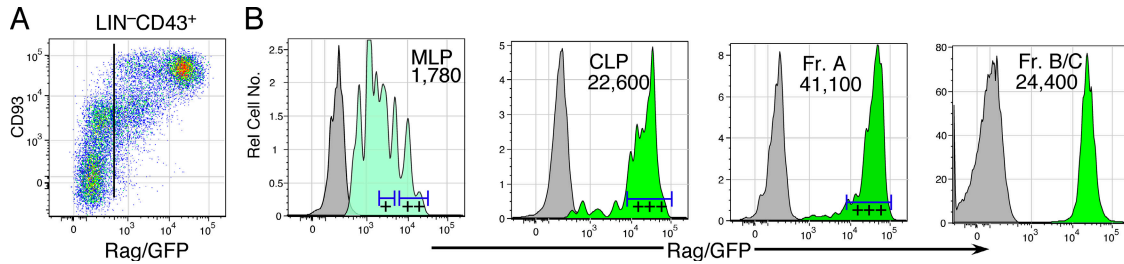


Figure 4. (A) Detection of RAG-2 gene transcriptional activation very early in hematopoietic development. LIN⁻ cells, gated to eliminate CD43⁻ pre-B and later stage B lineage cells, were analyzed for expression of RAG-2-GFP and CD93. Vertical bar indicates the demarcation between positive and negative GFP levels. (B) Comparison of levels of RAG-2-GFP in early stages of B lineage development in mouse bone marrow. The level

of green fluorescence detected in RAG-2-GFP transgenic littermates is shown for each cell subset analyzed. Numbers on the plots show the mean fluorescence intensity for each population. Sort gates for the cells analyzed in B/Myeloid bipotential assays (Fig. 3 B) are indicated. Representative data of three separate analyses is shown.

from RAG-2/common γ chain double-deficient embryos that lack endogenous lymphoid/NK lineage development (Fig. 5 A). It was striking that equal numbers of wells containing early T lineage cells ($\sim 60\%$) arose from both CLP and Fr. A. And, as would be expected, none were generated using CD19⁺ pro-B cells. These early T lineage cells, generated under conditions of high levels of IL-7, typically showed a very immature phenotype, expressing CD90 and CD25, but lacking CD3, CD4, or CD8. They were not stained by reagents specific for B lineage (CD19), myeloid lineage (CD11b and GR1), or NK lineage (NK1.1 and DX5) cells.

Although our FTOC data indicated a significant capacity for T cell production from Fr. A, comparable to the CLP fraction, limitations on the number of thymic lobes required a 3 cell/well assay to generate significant numbers of engrafted lobes. Therefore, we undertook a single cell assay using the recently described Delta-like 1 (DL1)-OP9 system (33). Engagement of Notch-1 by DL1 delivers a critical signal specifying the T cell fate in developing hematopoietic cells (34, 35), and Schmitt et al. (33) found that DL1-transduced OP9 stromal cells could induce T cell development from early precursors *in vitro*. The OP9 cell line also supports efficient development of B lineage cells under these culture conditions and thus a T/B bipotential assay is possible using this approach. We found that the assay works well at the single cell/well level and compared the capacity of CLP and Fr. A to produce T and B lineage cells. As shown in Fig. 5 B, similar to the FTOC assay, we observe efficient T lineage generation from both CLP and Fr. A, but with a bias toward B lineage development apparent in Fr. A (Fig. 5 B). Interestingly, in our analysis, T cell generation occurs at the expense of B cell generation, with similar numbers of total clones in both control and DL1 cultures. Thus, under conditions favorable for generating B cells, Fr. A can generate T cells if a Notch-1 signal is provided.

Ig heavy chain rearrangement initiates very early in B lineage development

While our RAG-2 reporter analysis (Fig. 4 B) indicated that RAG transcription is already activated in nearly 100% of

MLP stage cells, it was unclear when D_HJ_H rearrangement initiated because chromatin configuration at the heavy chain locus will play a key role in determining heavy chain locus accessibility. Therefore, we then focused on this issue. To determine the frequency of cells with Ig heavy chain locus recombination we used a single cell DNA PCR assay (7, 36, 37). This approach uses two rounds of amplification with nested primers, one set that amplifies a germline band and another set that detects many of the possible D_HJ_H rearrangements, allowing us to assess the extent of heavy chain rearrangement at the single cell level. Importantly, this assay was very efficient, recovering signals from 80% of the cells analyzed (Table II). The earliest fraction, MLP, showed germline bands with little detectable D_HJ_H rearrangement (<2%). Yet, we detected D_HJ_H rearrangement in 48% of signal-positive CLP stage cells, and rearrangement increased to 88% of Fr. A cells (Table II, see values in parenthesis). Thus, even cells with extensive DJ rearrangement retain the capacity to develop into T cells. In comparison, CD19⁺ pro-B stage cells had extensive D_HJ_H bands with essentially no germline signal, indicating that most cells had D_HJ_H rearrangements on both chromosomes, consistent with a previous analysis using a germline-loss bulk PCR assay (13).

Quantitative analysis of expression of B, T, and myeloid-associated genes by RT-PCR

To more completely understand the progressive restriction of lineage potential in each cell fraction at these developmental stages, we next performed quantitative analysis of gene expression using a TaqMan PCR assay for a set of genes previously used by us and others to characterize early stages in B cell development, in contrast with that of myeloid and T lineage cells (Fig. 6). A very clear finding was the presence of mRNA for genes involved in Ig recombination earlier than conventionally expected. TdT, RAG-1, and RAG-2 were all found at significant levels in CLP stage cells, well before surrogate light chain message (Fig. 6 A). The activation of these key components of the recombinase system explains the observation of extensive D-J rearrangement in CLP stage cells shown above. TdT, previously suggested as a marker for

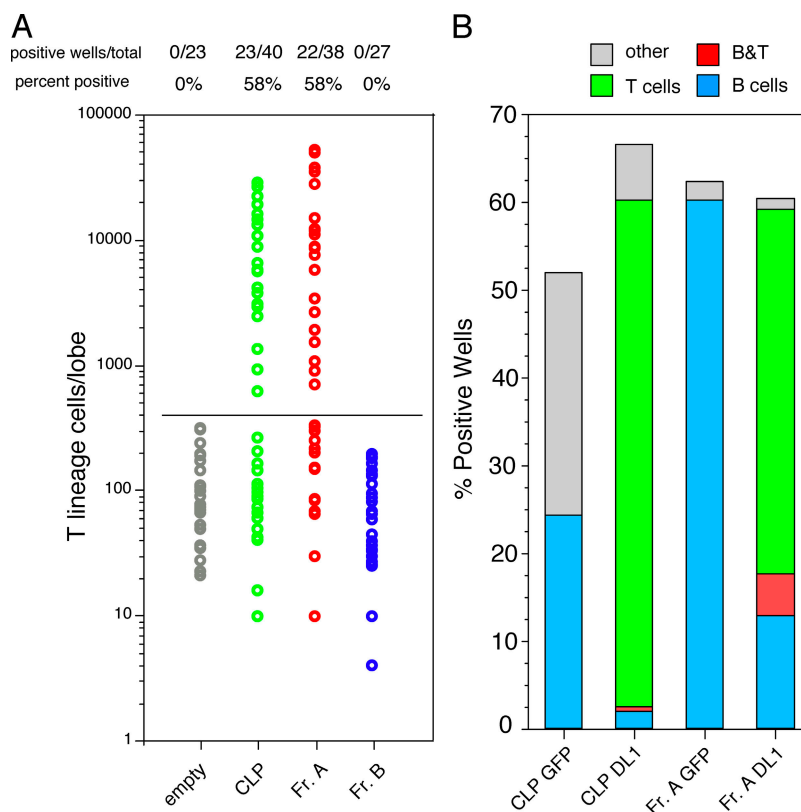


Figure 5. Comparable T cell potential in CLP and Fr. A stage cells.

(A) Three cells of the indicated population were deposited into wells containing a fetal thymic lobe. Plates were then maintained under high oxygen submersion FOC conditions in the presence of SCF and IL-7. Wells were analyzed after 2 wk and evaluated for the presence of CD90/Thy1.2⁺CD25⁺ early T lineage cells or CD19⁺ B lineage cells. B lineage

cells were not efficiently generated under the conditions used. Data from five separate experiments is shown. (B) Individual CLP and Fr. A cells were deposited by electronic cell sorting into microplate wells containing DL1-OP9 or GFP-OP9 (control) stromal cells and the type of cells generated was assayed 7–10 d later. B and T lineage cells were identified as described above. "Other" stands for not falling into either gate.

B lineage– (15, 38) or lymphoid-restricted precursors (16), is expressed at significant levels even before the CLP stage. MLP stage cells show ~10–15% of the peak levels detected in CLP and Fr. A (Fig. 6, A and B). As we noted earlier (5), Ig- β mRNA (B29) is expressed early in the B lineage pathway, before detectable Ig- α mRNA (MB1), and we confirm this in our quantitative analysis.

Several transcription factors act to regulate the B lineage gene program (2) and several key players are shown in Fig. 6 C. Interestingly, mRNA for the early acting and critical E2A proteins E12 and E47, diagnostic of B lineage potential (39), are well above background in all fractions tested (Fig. 6 C), suggesting that the MLP fraction identified by high levels of CD93 and characterized by significant RAG-2–GFP expression is already being induced along the B lineage pathway. In contrast, two other key B lineage transcription factors, EBF (40, 41) and Pax-5 (42–45), showed later induction in the progression from CLP (B220[–]) to Fr. A (B220⁺). PU.1, a transcription factor regulating B/myeloid potential by activating growth factor receptors at different levels of expression (46), decreases from MLP to CLP, and then decreases further from Fr. A to Fr. B. This is consistent with a decrease in my-

eloid potential by loss of myeloid growth factor receptors and induction of lymphoid potential by expression of the IL-7R during the course of this progression.

We also surveyed expression of genes representative of alternative lineage fates, including T lineage, GATA3 (47) and Notch-1 (33, 34), and myeloid lineage, Csf1r (48) and C/EBP α (49). These genes showed very significant decreases in the progression from CLP to Fr. A to the CD19⁺ pro-B

Table II. Single cell heavy chain locus germline/D_HJ_H rearrangement analysis

Band(s) detected	Bone marrow fraction			
	MLP	CLP	Fr. A	Fr. B&C
GL	89.6 (98.4)	42.2 (51.6)	9.9 (12.0)	0.5 (0.8)
GL/D _H J _H	1.0 (1.1)	12.0 (14.7)	10.4 (12.6)	0.5 (0.8)
D _H J _H	0.5 (0.6)	27.6 (33.7)	62.0 (75.3)	62.0 (98.4)

Frequency of samples with the indicated band pattern (GL only, GL and D_HJ_H, or D_HJ_H only). 240 wells of each cell type were analyzed, one half of five separate 96-well plates, representing three separate sorting experiments. Value in parenthesis is frequency out of samples where a signal was recovered. GL, germline.

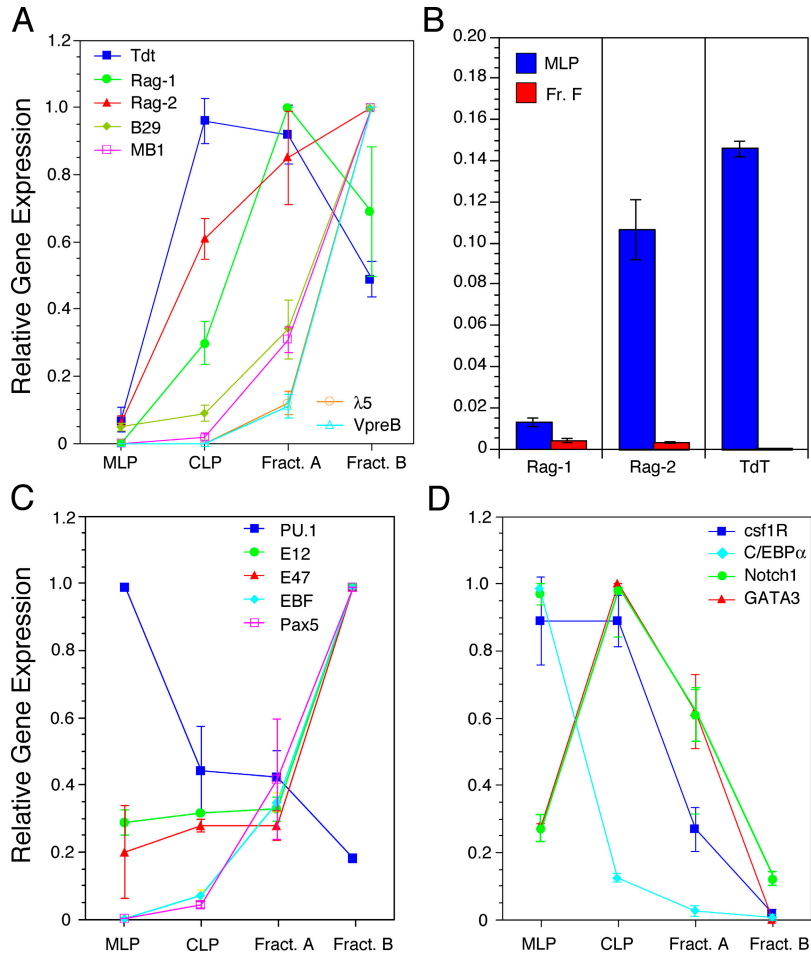


Figure 6. Real-time quantitative RT-PCR analysis of gene expression in early B lineage fractions isolated from mouse bone marrow. Results show average and standard error for three separate sorted samples. For each gene, maximum expression observed in the four fractions tested is set to 1.0. (A) B lineage-associated or -restricted genes are expressed from very early stages of hematopoietic development. (B) Gene expression in MLP stage cells compared with that in mature

recirculating (Fr. F) B cells, showing readily detectable levels of mRNA for TdT and RAG-2 at this very early stage. (C) Patterns of expression of transcription factors indicate an ordered sequence of gene activation early in B cell development. (D) Genes associated with other hematopoietic lineages (Csf1R, C/EBP α , myeloid; Notch-1, GATA3, and T lineage) show diminishing expression as cells progress from CLP to Fr. A to CD19⁺ Fr. B.

stage (Fig. 6 D). In fact, the expression of these genes was reciprocal to EBF and Pax-5, consistent with a progressive restriction to the B lineage fate as cells pass through these three stages. Consistent with the myeloid potential in CLPs, there is little change in Csf1r mRNA levels from MLP to CLP, whereas the level of GATA3 rises sharply in CLPs, possibly indicating the activation of a T lineage program that is then extinguished as cells progress to express B220 and then CD19 in the absence of Notch-1 signaling. Significantly, Notch-1 expression is sharply up-regulated at the CLP stage, declining thereafter, providing a mechanism for T lineage specification via Notch-1 signaling in CLP and Fr. A stage cells.

Determining global patterns of gene expression by microarray analysis

Finally, we analyzed RNA prepared from these four early B lineage stages from mouse bone marrow to identify sets of

genes that were coordinately regulated as cells progressed down the B cell development pathway. We generated ratios of signal from amplified RNA to a common reference RNA for two samples per fraction. Genes showing a statistically significant difference in at least one stage were identified by ANOVA, and the resulting set (~1,000 genes) was analyzed by KMeans clustering. The results obtained were visualized by a “heat map” display (Fig. 7) where individual gene levels are coded green for low, black for intermediate, and red for high. Using this approach we can identify a set of genes that show low-level expression in MLP and pro-B stages, but higher expression in CLP and Fr. A (cluster A); another that is low in MLP and CLP, but increasing in Fr. A and Fr. B and C; and yet others where high-level expression found early diminishes in later fractions. Importantly, we could identify genes present in each cluster that were consistent with the patterns of expression shown in Fig. 6. Thus, cluster A

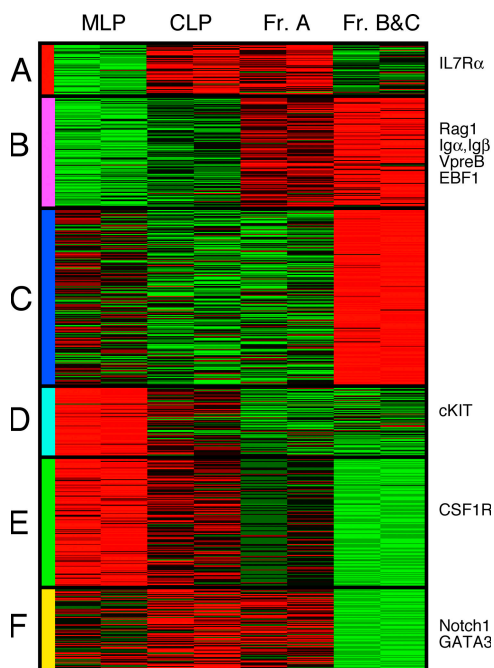


Figure 7. Cluster analysis reveals distinct patterns for 1,000 genes (out of ~40,000 features on the slides used) whose expression varies within the four early B lineage stages analyzed in this work.

Heat map display of expression shows relative level for each individual gene: green, low; black, medium; red, high. Six patterns of expression, including those present at high levels early that are down-regulated (clusters D and E), those up-regulated at the CLP and Fr. A stages (cluster A), and those up-regulated with B cell development (clusters B and C). Cluster B includes many genes considered important in B cell development, such as RAG-1, BLNK, CD19, CD79a, CD79b, EBF, and VpreB.

includes the IL-7R α gene, consistent with the highest staining for this protein on CLP and Fr. A. Cluster B contains well-known B lineage-associated transcription factors (Pou2af1/OCAB, EBF-1, PBX-1, LEF1, IRF4, and SPI-B) and a large number of lymphoid/B lineage-associated genes, including RAG-1, Blnk, CD19, CD79a, Cd79b, and VpreB. Cluster C is a group of genes sharply up-regulated at the pro-B stage and includes ABL-1. Cluster D includes cKit and one of the myeloid colony-stimulating factor receptor genes. Cluster E contains Csf1, the myeloid growth factor receptor gene that we analyzed by quantitative PCR and found was present in CLP and down-regulated in Fr. A (the pattern shown here). Cluster F includes Notch1 and GATA 3, consistent with the T lineage potential of Fr. A that is lost in Fr. B and C. In summary, the patterns of expression that we observe are consistent with our quantitative PCR determination for every gene examined that is contained in this set of differentially expressed genes, and this analysis identifies a large number of additional genes.

DISCUSSION

Here we identify and characterize very early stages in B cell development in mouse bone marrow, focusing on those be-

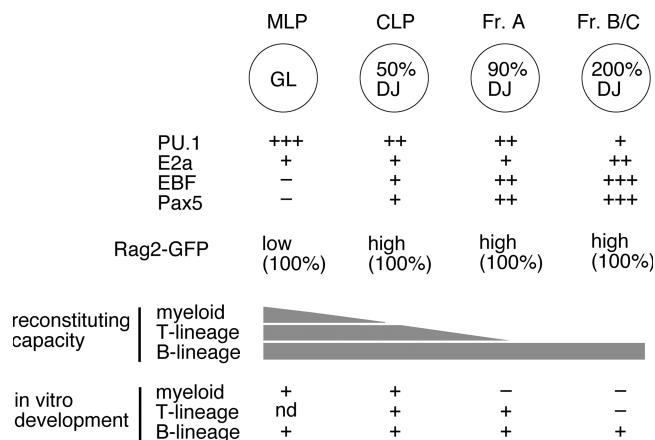


Figure 8. Diagram of early stages in B lineage development, indicating extent of D_HJ_H rearrangement, expression of key transcription factors, expression of a RAG-2-GFP reporter transgene, extent of B/T/myeloid repopulating capacity, and lineage plasticity in vitro.

fore CD19 expression. Fig. 8 summarizes the features of the cell stages we have defined. The earliest stage, MLP, generates all lineages assayed in vivo, including B, T, NK, and myeloid. The next stage, identified by cell surface markers as CLP, gives a lymphoid-restricted repopulation in vivo but has the potential to generate myeloid cells in culture, while half of the cells already contain D_HJ_H rearrangements. The third stage, Fr. A, identified by expression of B220, generates predominantly B cells in vivo but has the potential to produce T cells in culture, while showing an even higher frequency of D_HJ_H rearrangement than CLP. Gene expression profiles are consistent with B lineage activation at the MLP stage, resulting in expression of a large number of B lineage-specific genes at the CLP and Fr. A stages.

T lineage potential in Fr. A was unexpected. Previously we showed that a fraction of B220⁺CD19⁻ cells expressing CD43 and CD93 (“Fr. A2”) were B lineage precursors that failed to generate T cells in i.v. and intrathymic assays (7). Using the present approach, applying greater purification criteria than previously, we still find predominantly B lineage repopulation by a “refined Fr. A” using the standard Ly-5 marked competitive repopulation assay. Although these B220⁺CD19⁻ Fr. A cells constitute only 0.05–0.1% of total bone marrow cells, they constitute an important decision point in B cell development. However, while behaving as B lineage precursors in some assays, their lineage plasticity is revealed in the HOS-FTOC and DL1-OP9 assays. One explanation for this behavior is their expression of Notch-1, which can be activated in the thymic microenvironment or by DL1 interaction in culture, redirecting their lineage fate. The contrasting i.v. repopulation results likely indicate that Fr. A stage cells only inefficiently home to the thymus or else rapidly progress to the irreversibly B lineage-committed CD19⁺ stage compared with earlier stages like CLP or MLP.

Myeloid cell generation from the CLP fraction was also surprising. Our analysis shows that although these cells have

become considerably more lymphoid specified than MLP, expressing higher levels of TdT and RAG message (and functional RAG protein, as indicated by DHJH rearrangement), they nevertheless retain significant myeloid capacity as revealed in the B/myeloid bipotential assay. As with Fr. A, the i.v. repopulation assay shows a more restricted lineage potential, either because these cells predominantly home to microenvironmental niches that disfavor myeloid development or else rapidly progress to Fr. A in vivo. We can only speculate that this in vitro myeloid potential was overlooked previously due to differences in stromal culture conditions that favored lymphoid progression or else to differences in sensitivity of detection of CD45R/B220 that might have included Fr. A in the CLP population, increasing its apparent lymphoid restriction. Therefore, the subdivision of B220⁻ and B220⁺ fractions of CD117^{med}CD127⁺ cells is important because it reveals clear differences in lineage restriction read out in both in vivo and in vitro. That is, in contrast with CLP, most Fr. A cells did not respond to myeloid-inducing signals in stromal cell culture. Thus, Fr. A pre-pro-B cells behave as a strict “common lymphoid progenitor” in terms of their lineage potential.

The finding of B220⁺ CLP is reminiscent of a previous report from Martin et al. (50) describing a lymphoid-restricted “CLP2” cell type. However, in their studies, the B220⁺ CLP-like cells were reported to lack CD117, clearly not the case with Fr. A. Furthermore, the CLP2 cells were described functionally as efficiently homing to the thymus, which we do not observe; Fr. A cells injected i.v. generate far fewer thymocytes compared with classical (i.e., B220⁻) CLP stage cells (Fig. 2 B). The capacity of CLP2 stage cells to home to the thymus led Martin et al. to propose that these cells are a founder population for thymic T cell development. In contrast, we would suggest that Fr. A stage cells are an intermediate between CLP and CD19⁺ pro-B stage cells, in a B lineage-specified, but not yet committed, state. Recent work from Allman et al. (51) has identified an early thymic progenitor that appears distinct from CLP2. Clearly, this issue will require further investigation to clarify the relationships among B220⁺CD19⁻ subsets from bone marrow and thymus in terms of lineage potentials.

A very recent report by Balciunaite et al. (21) described the presence of a B220⁺CD117⁺CD19⁻ hematopoietic progenitor with B, T, and myeloid potential in vitro and lymphoid potential in vivo. Although these authors’ limiting dilution analysis suggests a precursor with some similarity to ours, their population appears more similar to the B220⁻ CLP stage we report here; the expression of B220 in our experience greatly reduces myeloid potential (by 10-fold) found in CLPs. Furthermore, the decrease in csfR1, a gene encoding the receptor for colony stimulating factor 1 (a key myeloid growth factor), in the progression from CLP to Fr. A provides a mechanistic explanation for the difference we observe. In fact, we found that B220 expression was a better marker for the loss of myeloid potential than induction of a RAG-2-GFP reporter, sounding a cautionary note on the

use of RAG reporters for identifying “early lymphoid progenitors” (17).

Both CLP and Fr. A stage cells generated T lineage cells in vitro, revealing T lineage potential, but this does not necessarily mean that either are normal intermediates in a developmental pathway from hematopoietic stem cells to T cells. Although the existence of T cell lines or thymocytes with Ig D_HJ_H rearrangement have been reported (52), there are no T lineage precursors among triple negative (CD3⁻4⁻8⁻) thymocytes with a surface phenotype corresponding to bone marrow CLP (51), and we detect no cells corresponding to Fr. A in the thymus (not depicted). Our results seem most consistent with a type of developmental model where B lineage “specification” precedes B lineage commitment in bone marrow (53–55). In this model, B lineage genes are induced and non-B lineage genes are repressed in progressive stages of hematopoietic development, mediated by a hierarchy of transcription factors, resulting in a B lineage-specified stage, coincident with D_HJ_H rearrangement and the high-level expression of a set of early B lineage genes, such as Igα/β, λ5/VpreB, and RAG-1/2. However, absolute irreversible lineage commitment occurs at a later stage, coincident with high-level expression of functional Pax-5 (42, 43).

In fact, it appears that B lineage specification initiates even before the CLP stage, as MLP cells express some lymphoid/B lineage genes, including TdT, RAG-2, and Ig-β. We also note that most MLP stage cells show activation of a RAG reporter transgene, similar to the previously described CD117^{hi} early lymphoid progenitors fraction that lacks CD127 (17). Nevertheless, we found a robust myeloid lineage engraftment with MLP, suggesting that RAG-2 gene transcriptional activation, along with a significant component of the B lineage program, initiates in a cell fraction that maintains considerable myeloid potential as revealed using the cell engraftment competition assay.

Finally, our microarray analysis illustrates the progressive nature of B cell development. We identify clusters of genes with expression shared between MLP and CLP, between CLP and Fr. A, and between Fr. A and CD19⁺ pro-B stage cells. Continuing examination of the members of these clusters will help to elucidate more fully the gene program resulting in progressive restriction to B lineage development, along with the key microenvironmental interactions that foster this process.

MATERIALS AND METHODS

Animals. 6–12-wk-old BALB/c (ICR) female mice, bred in our animal facility, were used in most experiments. For the i.v. competition cell transfer assay, B6.Ly5.2 female mice were obtained from the National Cancer Institute. RAG-2-GFP BAC transgenic mice were obtained from M. Nussenzweig (Rockefeller University, New York, NY) and bred in our animal facility. RAG-2/common γ chain double-deficient (RAG2^oγC^o) α-lymphoid animals, originally described by Colucci et al. (56), were obtained from D. Wiest at our institute, and timed matings for generating fetal thymic lobes were performed in our animal facility. C57BL/6 mice were obtained from our animal facility production colony. All experiments with mice were conducted under an approved animal protocol.

i.v. competitive repopulation assay. The selected population was sorted from B6.Ly5.2 bone marrow and the yield derived from two bone marrow equivalents was transferred i.v. per recipient, typically $5\text{--}10 \times 10^3$ for CD19⁻ fractions and $5\text{--}10 \times 10^4$ for CD19⁺ cells, together with 10^5 unfractionated Ly5.1 (recipient type) bone marrow. 2-mo-old C57/B6 female recipient mice were lethally irradiated (9 Gy) 1 d before transfer and provided neomycin polymyxin B antibiotic in water. Animals were analyzed 3 wk after transfer, using antibodies specific for Ly5.1 and Ly5.2 in addition to reagents specific for T, B, NK, and myeloid cells (CD3, CD4, and CD8 for T cells; B220, CD19, and IgM for B cells; NK1.1 and DX5 for NK cells; and CD11b and GR-1 for myeloid cells/granulocytes).

Myeloid/B cell bipotential stromal cell cultures. S17 stromal cells were grown in a 37°C humidified, 10% CO₂ gassed incubator in 5% FBS/RPMI 1640 medium (supplemented with $1 \times$ glutamine, 10 mM Hepes, 5×10^{-5} M 2-ME, and 0.5 mg/ml gentamicin). Cells were passaged into 96-well plates in the same medium and allowed to reach confluence 3–5 d before use. Immediately before cloning, medium was replaced with fresh medium containing cytokines (10 ng/ml SCF, 10 ng/ml Flt3L, and 100 U/ml IL-7; R&D Systems). Individual cells of the selected population were sorted using the single cell deposition unit of a FACSVantage/DiVa flow cytometer and then returned to the incubator. 7–10 d later wells were examined for colonies using an inverted microscope. Positive wells were harvested by pipetting, and then cells were stained with antibody to CD19 and CD11b and analyzed by flow cytometry.

T cell potential assays: high-oxygen tension FTOCs and DL1-OP9. The modified FTOC culture under high oxygen tension was performed as described previously (32, 57), except that fetal thymic lobes from RAG2^{γc} embryos were used. Three cells of selected population were sorted per well using the automated cell deposition unit. Cells were analyzed after 14 d by flow cytometry, staining for CD19, CD90/Thy-1, CD25/IL-2R α , DX5, CD11b, and CD11c. Early T lineage cells were recognized as CD90⁺CD25⁺ cells, lacking other markers tested. Alternately, individual cells selected by sorting were deposited into microplate wells containing preestablished stromal cells, either DL1-transduced OP9 or GFP-OP9 (33). Cultures were performed essentially as described previously (33) and analyzed for generation of T or B cells using the staining procedure described above at 7–10 d.

Flow cytometry and monoclonal antibodies. Sorting was performed using a BD Biosciences FACSVantageSE/DiVa, equipped with three laser excitation lines (407, 488, and 600 nm) for 12-color detection. Early B lineage precursors were isolated using the following staining combination: FL-Ter119, FL-anti-Ly6c, PE-anti-IL-7R α (SB/199), PI detected in TR-PE channel, Cy5PE-anti-CD3 (500A-A2), Cy55PE-CD93 (AA4.1), Cy7PE-CD43 (S7), Alexa 594-anti-CD24/HSA (30F1), APC-CD117/cKit (2B8), Cy55APC-anti-CD19 (1D3), Cy7APC-anti-CD11b/Mac-1(M1/70), Cy7APC-GR1, CasBlue-anti-IgM (331.12), and Bi-anti-CD45R/B220 (RA3-6B2). Biotin reagent was revealed by second-step incubation with QDot605-streptavidin. Analysis was performed using either this flow cytometer or a BD Biosciences LSR-II with three lasers (407, 488, and 630 nm) equipped for 10-color detection. All reagents were made in our laboratory, except for QDot605-streptavidin, which was purchased from Quantum Dot Corporation, and DX5 from BD Biosciences. Cells from RAG-2-GFP reporter mice were analyzed by detecting GFP in the FL channel, using the staining combination described above, except that Ter119 and anti-Ly6c antibody were labeled with Cy5PE.

Single cell heavy chain recombination assay. Analysis was performed using a modification of procedures described previously (7, 36, 37). Cells were sorted directly into 96-well plates (Applied Biosystems) containing 20 μ L/well lysis buffer ($1 \times$ PCR buffer [Applied Biosystems] with 2.5 mM MgCl₂, 9.2 μ g/ml tRNA [Sigma-Aldrich], and 100 μ g/ml gelatin). Plates were stored at -80°C and then just before PCR, plates were thawed, treated with 0.5 mg/ml proteinase K for 1 h at 55°C , and then heated for 10 min at

95°C . After digestion, a two-round nested PCR was used to detect a germline DNA segment (lost upon heavy chain rearrangement) and potential D_HJ_H rearrangements. The PCR program was 95°C for 1 min, 63°C for 1 min, and 72°C for 1.5 min for 30 cycles, with a 10-min end extension at 72°C . For round 1: GL5-1, CCCGGACAGAGCAGGCAGGTGG; D_H5-1, ACAAGCTTCAAAGCACAATGCCTGGCT; and D_H3-1, AGGCTCTG-AGATCCCTAGACAG. For round 2, the following two separate reactions were performed: for germline GL5-2, GAGTTGACTGAGAGGACAG, and GL3-2, CGAAGTACCAGTAGCAC; and for D_HJ_H rearrangements D_H5-2, ACGTCGACTTTT(G/C)TCAAGGGATCTACTACTGT, and D_H3-2, GGGTCTAGACTCTCAGCCGGCTCCCTCAGGG. In the first round, PCR amplification was performed with the contents of each well in a total volume of 50 μ L containing 0.2 μ M dNTPs, 1 μ L BD Advantage cDNA polymerase mix, $1 \times$ BD Advantage PCR buffer, 0.5 mg/ml BSA, and 0.4 μ M of each primer. In the second round, 1 μ L of each first round product was added to a 50- μ L reaction volume using the same conditions described above, except that second round primers (GL-5-2/GL-3-2 or D_H5-2/D_H3-2) were used at 2 μ M. Products were visualized on 1.5% agarose gels stained with ethidium bromide. The validity of these PCR products was verified by sequence analysis.

Quantitative RT-PCR assay. Total RNA was prepared by sorting cells into Solution D lysis/denaturing solution, followed by acid-phenol extraction and isopropanol precipitation, as described previously (5). cDNA was synthesized by adding 1 μ L oligo-(dT)₁₂₋₁₈ primer (0.5 μ g/ μ L; Invitrogen) to 20 μ L total RNA, heating at 70°C for 10 min, cooling on ice for 2 min, adding 8 μ L $5 \times$ first-strand buffer (Invitrogen), 4 μ L 0.1 M DTT (Invitrogen), 4 μ L dNTPs (each dNTP at 10 mM; Promega), 1 μ L random hexamer primers (20 U/ml; GE Healthcare), 2 μ L RNAsin (40 U/ml; Promega), and 2 μ L Superscript II (200 U/ml; Invitrogen), and then incubating at 42°C for 2 h. Gene expression was quantitated by real-time PCR. Analyses were performed in triplicate in 25- μ L volumes using an ABI7500 thermal cycler. For each tube, 12.5 μ L ABI TaqMan 2 \times Mastermix (polymerase and dNTPs), 1.25 μ L probe mix (ABI), 9.25 μ L DEPC-H₂O, and 2 μ L template (typically diluted 1:3 from cDNA synthesis volume) were added. ABI software was used to quantify/calculate Ct values and determine relative gene expression levels, standardizing using β -actin values. All quantitative PCR ABI assay IDs and sequences for custom-designed sets are available upon request.

RNA extraction from sorted cells, RNA amplification, and labeling for microarray. Cells were sorted directly into RNA lysis buffer (6 M guanidine thiocyanate, 0.67% Na *N*-lauroylsarcosine, 33 mM sodium citrate, and 133 mM 2-mercaptoethanol), and total RNA was extracted using the acid phenol method as described previously (5). Integrity and quantity of total RNA samples were analyzed using a 2100 Bioanalyzer (Agilent Technologies). 40 ng total RNA was used for RNA amplification. RNA amplification was performed using the Ovation Aminoallyl RNA Amplification and Labeling System (NuGEN Technologies, Inc.) in accordance with the manufacturer's protocols. 2 μ g amplified cDNA was used for dye-coupling with Alexa Fluor 555 and Alexa Fluor 647 (Invitrogen). Quantification of the fluorescent-labeled probes was performed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). 50 pmol of fluorescent-labeled cDNA (~ 1.5 μ g amplified cDNA) of each probe (experimental sample and reference) was loaded per slide. For use as a reference, total RNA from six cell lines (R1, RS2, J558, EL4, DN3, and P388d) was prepared using TriReagent (Molecular Research Center, Inc.) and 30 μ g (5 μ g from each cell line) was reverse transcribed at 42°C for 2 h in a total volume of 20 μ L that included 1 μ L Superscript II reverse transcriptase (200 U/ μ L; Invitrogen), 1 μ L rRNAsin RNAase inhibitor (40 U/ μ L; Promega), and 2 μ L of a mixture of dNTPs (5 mM each of dGTP, dATP, and dCTP; 2 mM of dTTP; and 3 mM of aminoallyl dUTP). RNA was hydrolyzed by the addition of 10 μ L 1 M NaOH and incubation at 70°C for 10 min. 10 μ L of 1 M HCl was then added to neutralize the sample, and cDNA was precipitated overnight by the addition of 4 μ L of 3 M sodium acetate, pH 4.5, 1 μ L glycogen (20 μ g/ μ L), and 100 μ L ethanol. cDNA was pelleted by centrifugation, washed once with

70% ethanol, and dissolved in 9 μ l coupling buffer, followed by the same dye-coupling procedure described above for amplified cDNA.

Microarray analysis. Two samples were analyzed from each fraction sorted from separate pools of mouse bone marrow cells. RNA prepared from each sample was amplified, used for probe generation, and hybridized with a common reference RNA. Amplified RNA was labeled with both Alexa Flour 555 and Alexa Flour 647 and hybridized with the complementary labeled reference RNA (dye-flip replicates). Whole mouse genome 44K oligo microarray kits (Agilent Technologies) were used for hybridization. The hybridization and SSPE washing and drying procedures were all performed according to the manufacturer's recommendations. The slides were then scanned using an Agilent BA DNA Microarray Scanner. Data from scans were normalized using Agilent feature extraction software and then subject to statistical analysis using GeneSight software (BioDiscovery). Results were determined as ratios of experimental sample to reference, and dye-flip replicates were combined. The resulting data, two ratios for each gene from all four samples, were analyzed by ANOVA, selecting genes differentially expressed reproducibly in at least one stage with a p-value cutoff of <0.005 . This set of $\sim 1,000$ genes was then analyzed by KMeans clustering using a distance measure based on the Pearson correlation and assuming six clusters.

Online supplemental material. Fig. S1 is a flow cytometry analysis showing the relationship between CD43 and CD93 expression with B cell development, comparing very early precursors (LSK) with CLP and Fr. A. Fig. S2 is a flow cytometry analysis showing the expression of a RAG-2-GFP reporter BAC transgene in early stages of B cell development. The microarray data used to generate the cluster analysis shown in Fig. 7 is also available. The online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20052444/DC1>. The microarray data is available from ArrayExpress, European Bioinformatics Institute (<http://www.ebi.ac.uk/arrayexpress>), as accession no. E-MEXP-559.

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