Hematopoietic Stem Cells Expressing the Myeloid Lysozyme Gene Retain Long-Term, Multilineage Repopulation Potential

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Summary

Single cell PCR studies showed that hematopoietic stem cells (HSCs) express a variety of lineage-affiliated genes. However, it remains unclear whether these cells exhibiting “lineage priming” represent bona fide stem cells or a subpopulation earmarked for differentiation. Here we have used a Cre-Lox approach to follow the fate of cells expressing a lineage-affiliated marker. We crossed lysozyme Cre mice with yellow fluorescent protein (EYFP) reporter mice and found EYFP gene expression not only in myelomonocytic cells but also in a fraction of HSCs as well as B cells and T cells. Transplantation of EYFP+ HSCs into primary and secondary recipients generated mice in which all hematopoietic cells were EYFP+. In contrast, crosses between CD19 Cre and Ick Cre mice with reporter mice showed no EYFP expression in HSCs or intermediate progenitors. Our results demonstrate that lysozyme expression does not mark myeloid commitment and that long-term repopulation potential is maintained in primed HSCs.

Introduction

HSCs are probably the best-studied class of adult vertebrate stem cells, giving rise to more than 10 well-defined lineages through a series of intermediate progenitors. Transplantation experiments performed during the last few years suggest that HSCs can also “trans-differentiate” into a variety of nonhematopoietic cell types (see for example Graf, 2002). In spite of these advances little is known about how HSCs decide what to become, and whether their differentiation is initiated predominantly by extrinsic factors or by stochastic events (Enver and Greaves, 1998; Enver et al., 1998; Metcalf, 1998). There is a consensus, however, that lineage-specific transcription factors establish and maintain cell fate by regulating cell type-specific gene expression programs. For a long time it was thought that the ground state of HSCs corresponds to a “blank slate” and that lineage-specific gene expression programs only become activated as cells become committed. This view was shaken when it was found that single cells from pluripotent hematopoietic cell lines not only express a variety of lineage restricted transcription factors but also classical lineage affiliated marker genes such as β globin and myeloperoxidase (Hu et al., 1997). More recently, these findings were extended to Lin−, c-Kit+, Thy1.1+, Sca-1− bone marrow cells, of which some 20% have long-term repopulation (LTR) potential following transplantation into irradiated recipients (Morrison et al., 1997; Wagers et al., 2002). As in the multipotent cell lines, most single HSCs analyzed by PCR expressed at least one of several myeloid restricted genes tested, such as β globin or myeloperoxidase, and often coexpressed two or more of them (Miyamoto et al., 2002). Together, these observations were interpreted to suggest that HSCs undergo a process of lineage priming and that differentiation is initiated when one gene expression program becomes dominant, closing all other options (Enver and Greaves, 1998; Miyamoto et al., 2002). The implication is that this happens in bona fide HSCs, that is, in cells with LTR potential (Figure 1A). However, approximately 30% of single HSCs expressed no detectable lineage markers (Miyamoto et al., 2002), raising the possibility that only the lineage marker negative cells exhibit LTR potential, while the marker positive cells are earmarked for differentiation (Figure 1B).

These alternatives can be tested by isolating HSCs expressing a given lineage restricted marker and determining whether they have LTR potential. In one study early hematopoietic progenitors expressing the platelet integrin Gpllb/CD41 were found to have lymphoid lineage repopulation potential but no clear LTR activity (Corbel and Salaun, 2002). In another study, HSCs (Lin−, Thy1low, c-Kit+, Sca-1−) expressing low levels of the macrophage integrin Mac-1 were found to be devoid of long-term repopulation potential, although they exhibited multilineage differentiation potential (Morrison et al., 1997). Surprisingly however, Mac-1+ is also expressed by a subset of HSCs from fetal liver that were shown to have self-renewal capacity (Morrison et al., 1995). Markers that are not detectable on the cell surface are more difficult to study since cells cannot easily be sorted and tested. However, it is possible to use lineage-specific promoters to drive reporter genes either directly or indirectly by driving Cre recombinase, whose activity leads to the activation of the reporter (see below). Cells expressing the reporter gene can then be analyzed and isolated using flow cytometry (FACS).

A classical lineage-affiliated marker is lysozyme M (hereafter called “lysozyme”), which is highly expressed in myelomonocytic cells. We have generated a mouse line in which the egfp gene was inserted into the first exon of the lysozyme gene by homologous recombination, and which exhibits strong labeling in granulocytes and macrophages but not in cells from other lineages (Faust et al., 2000). Ablation of the lysozyme gene in these mice leads to an increased inflammatory response...
to bacterial infections due to an impaired function of granulocytes and macrophages (Ganz et al., 2003). Other than that the mice can be maintained as homozygote knockouts and show no overt defects in the hematopoietic system (unpublished data). In another knockin line, the cre recombinase gene was inserted into the first exon of the lysozyme gene. When crossed with a reporter mouse containing a lox-P site-flanked cassette, excisions were detected by Southern blotting in myelomonocytic but essentially not in B or T cells (Clausen et al., 1999). However, more recent studies showed that this gene is also expressed in intermediate progenitors (Miyamoto et al., 2002). Here we asked whether lysozyme is expressed in HSCs, using both the lysozyme EGFP and lysozyme Cre knockin mice. The latter were analyzed following crossing with ROSA26 EYFP reporter mice (Soriano, 1999; Srinivas et al., 2001). In the resulting lysozyme “ancestry” mice individual cells can be detected that are either expressing lysozyme or that have expressed the gene earlier during development. Using this in vivo lineage tracing technique (Jacob and Baltimore, 1999), we show that lysozyme is indeed expressed at low levels in a subset of HSCs and that these cells exhibit long-term repopulation potential. In contrast, analyses of lineage ancestry mice in which Cre expression is driven by either the gene encoding the B cell restricted marker CD19 or the T cell restricted marker lck failed to detect expression of the markers in HSCs. Our results indicate that expression in HSCs of the myeloid lineage affiliated gene lysozyme gene does not abolish their “stemness” and suggest that lineage priming is selective.

Results

Lysozyme Ancestry Mice Show Unexpected EYFP Labeling of Lymphoid Cells

To generate animals in which the ancestry of lysozyme expressing cells can be traced, lysozyme Cre knockin mice were crossed with ROSA26 EYFP reporter mice. The resulting cross was compared to a ROSA26 EYFP reporter mouse as a negative control and to a germ line deleted ROSA26 EYFP mouse as a positive control. The latter allows determining the percentage of cells that express the reporter gene driven by the endogenous ROSA26 promoter for each lineage. As shown in Figure 2A (a), negative control cells showed no EYFP labeling, whereas 55% of blood leukocytes of the lysozyme ancestry mouse were labeled (b) compared to 98% of the cells in the positive control (c). The latter mouse showed essentially 100% labeling of myeloid, B, and T cells (data not shown). We then analyzed myeloid cells, B cells,
Figure 2. FACS Analyses of Lysozyme Ancestry Mice
(A) Profiles of blood leukocytes from lysozyme ancestry mice as well as from negative and positive control mice. (a) Lysozyme ancestry mouse, showing 54.6% EYFP^+ cells; (b) ROSA26 EYFP reporter mouse, showing no labeled cells; (c) ROSA26 EYFP mouse with a germ line excision of the stop cassette, showing 98.3% EYFP^+ cells.
(B) FACS profiles of blood leukocytes from a lysozyme ancestry mouse, showing distribution of EYFP^+ cells between myeloid, B, and T cell populations. The upper row of diagrams represent profiles obtained after staining with various antibodies; the lower row shows the corresponding profiles after gating on EYFP^+ cells. Numbers in brackets indicate percentages of EYFP^+ cells relative to all cells stained with a given antibody combination, within the same quadrant.
(C) Histograms of EYFP^+ cell distributions in various tissues of lysozyme ancestry mouse #15. Light gray bars, Mac-1^+ cells; black bars, B220^+ cells; hatched bars, CD4^+ cells, cross hatched bars, CD8^+ cells, stippled bars, CD4^+CD8^+ cells.
(D) Histograms of EYFP^+ cell distributions in various blood leukocyte lineages of different lysozyme ancestry mice. Mice #1 to #5 were six months old; mice #6 to #8 two months. Gray bars, Mac-1^+ cells; black bars, B220^+ cells; white bars, CD4^+ plus CD8^+ cells. No significant differences were seen between the proportion of EYFP labeled CD4^+ and CD8^+ cells (not shown). Values shown represent average of three independent measurements. T-shaped bars, standard deviation.
Figure 3. Comparison of EGFP/EYFP Labeling Indexes in HSCs and Intermediate Progenitors

(A) Lysozyme indicator mouse (Lysozyme EGFP ki/ki). (B) Lysozyme ancestry mouse (Lysozyme Cre ki/ki x ROSA EYFP ki/ki). See Experimental Procedures for definitions of HSCs and intermediate progenitors. Gran/Mac, fraction of Gr-1+/Mac-1+ cells.

the MEP fraction. Lysozyme expression was upregulated in the myeloid pathway, as indicated by the presence of 39% EYFP+ cells in the CMP fraction, 64% in the GMP fraction, and 96% in the myelomonocytic fraction. In contrast, expression was downregulated in the lymphoid pathway, being essentially undetectable in B and T cell progenitors. FACS analysis of the corresponding fractions from a lysozyme ancestry mouse (Figure 3B) revealed similarities but also striking differences: In this mouse, about 9% of HSCs were EYFP+, 11% of CMPs and GMPs each and 74% of the myelomonocytic fraction. In addition, in contrast to the lysozyme indicator mouse, the proportion of EYFP+ cells did not decrease in the various lymphoid progenitors or in MEPs, and remained comparable to that seen in HSCs. This reflects the fact that Cre-mediated deletion of the stop cassette in HSCs, activating the EYFP reporter, leads to the labeling of all progeny derived from these cells. Compared to the lysozyme indicator mouse, the ancestry mouse had unexpected low labeling indexes of GMPs, MEPs, and myelomonocytic cells. A possible explanation is that the synthesis rates of active Cre (a tetramer) and EGFP differ, causing a delay between the production of the recombinase and its ability to cause excisions. As a consequence, short-lived GMPs and granulocytes show relatively low percentages of EYFP+ cells, although they already express lysozyme. This interpretation is also supported by the relatively low fluorescence intensity of the Gran/Mac cell fraction in Figure 3B, in which recently formed myeloid cells can be seen in the process of upregulating ROSA2-EYFP. We do not think that the differences in the percentage of EYFP+ cells between HSCs on the one hand and MEPs, CLPs, pro T and pre T cells on the other, are significant since they could not be reproduced in a second mouse. Together, our observations indicate that lysozyme is expressed in HSCs and suggest that the EYFP labeled B and T lineage cells seen in the lysozyme ancestry mice arise from a Cre-mediated activation of the EYFP reporter gene in HSCs. Expression of the lysozyme M gene is not aberrantly induced by the insertion of the cre or egfp knockin genes since we have detected Lysozyme...
by RT-PCR in HSCs (Lin<sup>-</sup>, c-Kit<sup>+</sup>, Sca-1<sup>+</sup>, CD34<sup>-</sup>) of a lysozyme EGFP <i>ki</i>/<i>−</i> mouse (data not shown) and of a wild-type mouse by cDNA array analyses (Akashi et al., 2002).

**EYFP Positive Bone Marrow Cells from Lysozyme Ancestry Mice Have Long-Term Repopulation Potential**

To test whether lysozyme expressing HSCs exhibit both lymphoid and long-term repopulation potential, EYFP<sup>+</sup> bone marrow cells from lysozyme ancestry mice were transplanted into irradiated recipients. The advantage of this strategy, as opposed to using EGFP<sup>+</sup> cells from the lysozyme indicator mice, is that EYFP<sup>+</sup> cells of the ancestry mice form a distinct population that can easily be sorted (Figure 3B). However, more importantly, they can be unambiguously traced in the transplanted animals due to the irreversible Cre-mediated activation of the reporter gene. Two experiments were performed, with similar outcomes.

In Experiment 1, bone marrow cells from two lysozyme ancestry mice averaging 15% EYFP<sup>+</sup> B cells were stained with a cocktail of antibodies against lineage markers (all conjugated to phycoerythrin) as well as against c-Kit (conjugated to allophycocyanin). Lin<sup>+</sup>, c-Kit<sup>−</sup> cells (containing HSCs as well as various intermediate progenitors) were then sorted into EYFP positive and negative fractions, yielding essentially pure populations (Figure 4A). For each fraction, 10<sup>4</sup> cells were then injected into two sublethally irradiated SCID mice and blood leukocytes analyzed over a period of 20 weeks. As can be seen in Figure 4B, cells from both fractions were capable of reconstituting both B and T cells at roughly comparable efficiencies. We next determined the EYFP labeling index in the lymphoid cell populations of mice transplanted with EYFP<sup>+</sup> fraction. As illustrated in Figures 5A and 5B, the percentage of EYFP<sup>+</sup> B and T lymphocytes reached almost 100% after 8 weeks. Two mice were sacrificed 7 months after transplantation and their cells analyzed by FACS. In animal #E1-1, more than 95% of B and T lineage cells in the blood, bone marrow, spleen, and thymus were EYFP<sup>+</sup>, while mouse #E1-2 showed slightly lower values. We also analyzed the stem cell fraction of mouse #E1-1. As shown in Figure 5C, 99.3% of Lin<sup>−</sup>, Sca-1<sup>−</sup>, c-Kit<sup>+</sup> cells were found to be EYFP labeled. Results obtained with mice transplanted with the negative fraction will be discussed below.

In Experiment 2, Lin<sup>−</sup>, c-Kit<sup>−</sup>, Sca-1<sup>−</sup> cells were sorted from bone marrow of a pool of three lysozyme ancestry mice (averaging 11% EYFP<sup>+</sup> B220<sup>−</sup> cells) into an EYFP<sup>+</sup> and an EYFP<sup>−</sup> fraction. Two animals each were injected with EYFP positive and negative fraction HSCs and analyzed 6 months after transplantation. Here again, mice transplanted with the EYFP positive fraction not only exhibited nearly 100% EYFP positive myeloid cells but also lymphoid cells in the blood, bone marrow, and spleen. Bone marrow from one of these animals was used to perform a secondary transplantation into three irradiated SCID mice. These recipients, analyzed 6 weeks later, showed between 98.2% and 100% labeling in their peripheral blood B and T cell population (B220, CD4, CD8; data not shown). We conclude that EYFP labeled HSCs from lysozyme ancestry mice have long-term repopulation potential.

Mice Transplanted with the EYFP Negative Cell Fraction Show Restoration of EYFP Labeling in HSCs and B Cells

The observations described above suggest that EYFP<sup>+</sup> and EYFP<sup>−</sup> progenitors are indistinguishable in their capacity for multilineage differentiation and self-renewal. This implies that, given enough time, EYFP negative HSCs from lysozyme ancestry mice should begin to express lysozyme (and thus EYFP) after transplantation. Alternatively, it is still also formally possible that this is not the case, and that the lysozyme expressers represent a distinct and stable subpopulation. For example, an earlier mesodermal precursor expressing lysozyme might be responsible for the labeling of HSCs (if it is assumed that Cre expression levels in HSCs are too low to cause excision of the stop cassette). In the first alternative, transplanted HSCs should eventually become EYFP positive; in the second, they should remain EYFP negative, even after prolonged periods of time. Therefore, to distinguish between these alternatives we analyzed mice transplanted with EYFP<sup>−</sup> cells. One mouse from Experiment 1 was sacrificed 2 months after transplantation (analysis of the second mouse failed because of a technical problem). As expected, its myeloid cells were highly labeled (80% EYFP<sup>−</sup>), suggesting that most or all cells were donor derived. Significant proportions of EYFP<sup>−</sup> cells were also found in the B cell and T cell compartments (0.9% and 2.5% B220<sup>−</sup> cells in bone marrow and spleen; 2.0% and 1.1% CD4<sup>−</sup> and CD8<sup>−</sup> cells, in spleen). Two mice were sacrificed from Experiment 2 (they had been transplanted 6 months earlier with 10<sup>4</sup> HSCs that were 100% EYFP<sup>−</sup>) and analyzed by FACS. While one animal was essentially fully reconstituted with donor cells (mouse #E2-2, average 77% EYFP<sup>−</sup> Mac-1<sup>−</sup> cells in three tissues), the other was only partially reconstituted (mouse #E2-4, average of 48% EYFP<sup>−</sup> Mac-1<sup>−</sup> cells). As shown in Figure 6A for mouse #E2-2, the spleen EYFP labeling indexes for B220, CD4, and CD8 cells were 10.8%, 3.6%, and 5.0%, respectively, while the labeling index for bone marrow HSCs was 6.8% (Figure 6B). Slightly lower values were obtained for the second mouse (data not shown).

The above observations are compatible with the notion that both EYFP<sup>−</sup> and EYFP<sup>+</sup> HSCs have long-term repopulation potential and that they express lysozyme in an oscillating manner. This predicts that in lysozyme ancestry mice, while the population of EYFP<sup>+</sup> cells is maintained, there should be a continuous recruitment of novel EYFP<sup>−</sup> cells from the negative fraction. Hence, assuming that there is no difference in the repopulation potential of cells from the two fractions, this should result in the gradual increase in the percentage of EYFP labeled cells over time, starting from the original baseline. However, a comparison between 2- and 6-month-old lysozyme ancestry mice showed no significant differences (Figure 2D), either because the age difference was too small or because HSCs were largely quiescent. Therefore, to test whether an age-dependent increase can be seen under conditions of transplantation (where HSCs are induced to proliferate), we injected 2 x 10<sup>4</sup> bone marrow cells from a single lysozyme ancestry mouse (in which 5.5% of the B cells were EYFP labeled) into each of five lethally irradiated C57BL/6 mice (Experiment 3). As can be seen from the analysis of the mice
14 months later (Figure 6C), all animals exhibited an up to 6-fold higher B cell labeling index compared to that of the original donor mouse. The data also show similar animal-to-animal variations as the original ancestry mice (Figure 2D). The observed increase was not due to an upregulation of lysozyme expression within the B cell lineage, since the mouse with the highest proportion of labeled B cells (31.5%, animal #1 in Figure 6C) showed a similar HSC labeling index (36.6%, Figure 6D). The T cell labeling index also increased in all five mice, although CD4+ cells generally exhibited a higher proportion of labeling than CD8+ cells.

Together, these experiments show that half a year after transplantation of EYFP− progenitors from lysozyme ancestry mice a subset of HSCs had again become EYFP labeled. Likewise, in mice transplanted with a mixture of EYFP− and EYFP+ bone marrow cells the proportion of EYFP labeled B cells and HSCs increased dramatically over the period of a year.

Analysis of Lymphoid Ancestry Mice Shows Absence of CD19 and Ick Priming in HSCs
To determine whether HSCs not only “preview” myeloid genes but also lymphoid affiliated genes, we created B and T cell lineage ancestry mice, using lines where Cre is driven by the endogenous CD19 promoter (Rickert et al., 1997) or the Ick promoter (Lee et al., 2001), respectively. In a set of pilot experiments we analyzed the blood leukocytes of a total of 8 CD19 and 34 Ick ancestry mice. While all of the CD19 mice showed the expected pattern (specific EYFP labeling of B cells), the situation with the Ick ancestry mice was more complex. Thus, of 34 mice examined, 29 showed a specific EYFP labeling of the T cell fraction while 5 showed strong labeling in all lineages. Chris Wilson’s lab, which created the mice, has made similar observations (see http://depts.washington.edu/immunweb/faculty/wilsonlab-reagent.html). Two CD19 ancestry mice and two Ick ancestry mice from the narrowly expressing group were chosen for more detailed analyses. For CD19 Cre ROSA EYFP no significant numbers of EYFP+ cells could be found in Mac-1+ and CD8+ cells from blood (Figure 7A) and in CD4+, CD8+ cells from thymus (data not shown). EYFP labeled cells could neither be detected in HSCs (Figure 7B) nor in a fraction containing CLPs, CMPs, GMPs, and MEPs (0 EYFP+ cells in 60,000 Lin−, c-Kit−, Sca-1− cells from mouse #9 and 1 EYFP+ cell in 131,000 Lin−, c-Kit−, Sca-1− cells from mouse #10). The analysis of the two Ick ancestry mice likewise revealed no significant pro-
completely EYFP negative (Figure 7D), as were CLPs, CMPs, GMPs, and MEPs (1 EYFP⁺ cells in 195,000 Lin⁻, c-Kit⁺, Sca-1⁺ cells from mouse #1 and 4 EYFP⁺ cells in 152,000 Lin⁻, c-Kit⁺, Sca-1⁺ cells from mouse #2). In conclusion, with the Cre-Lox lineage ancestry approach chosen, we could not detect HSC lineage priming for either of the two lymphoid genes examined.

Discussion

Promiscuous Expression of Lysozyme M Extends the Lineage-Priming Concept to Bona Fide Hematopoietic Stem Cells

Our data demonstrate that lysozyme M is expressed in long-term repopulating hematopoietic stem cells, thus ruling out the possibility that promiscuous expression of a lineage affiliated marker gene necessarily commits them to differentiation. Our observations thus support and extend the “lineage priming” model popularized by Enver and colleagues. (This model is based on the observation that multilineage precursors express a combination of lineage restricted genes before a given gene expression program becomes dominant during lineage commitment [Brady et al., 1995; Enver and Greaves, 1998; Hu et al., 1997]). They also support our earlier findings that showed that lysozyme is expressed in a subpopulation of CMPs without reducing the capacity of the expressers to form erythroid/megakaryocytic colonies (Miyamoto et al., 2002). Although it is clear that both lysozyme positive and negative HSCs exhibit long-term repopulation activity and are capable of reconstituting secondary recipients, the present study did not address the question of whether the two populations differ in quantitative terms. However, we think that lysozyme expressing HSCs (or HSCs that expressed it at some point) are at least as competent as lysozyme negative HSCs since they exhibit similar lymphoid repopulation kinetics (Figure 4B and data not shown). In addition, neither population is strongly dominant, as suggested by the results of the transplantation experiment with a mixture of EYFP⁺ and EYFP⁻ bone marrow cells (Figure 6C). It cannot be excluded that lysozyme M has an as yet undetected role in HSCs, in which case the observed expression might not represent “lineage priming.” However, this seems unlikely since HSCs from lysozyme defective mice can be transplanted and yield multilineage progeny in normal proportions.

Possible Explanations of the Observed Animal-to-Animal Variations in the Lysozyme Ancestry Mice

A comparison of individual lysozyme ancestry mice of identical genotype revealed surprisingly large variations in the proportions of EYFP labeled cells in the HSC and B cell compartments (Figure 2D and not shown). One possibility to explain these variations is the heterogeneity in the genetic background of the lysozyme ancestry mice, although similar variations were seen in C57BL/6 mice transplanted with aliquots of the same bone marrow sample from a single lysozyme ancestry mouse. We therefore think it is more likely that stochastic events play a role, assuming that during transplantation the bone marrow is reconstituted by a few dominant HSC clones. As a consequence, animals with high labeling

Figure 5. Analysis of Mice Transplanted with EYFP Positive Hematopoietic Progenitors from a Lysozyme Ancestry Mouse

(A) Profiles of blood leukocytes from mouse #E1-1, 5 months after transplantation with the EYFP positive cell fraction. FACS profiles are shown before and after gating on EYFP⁺ cells. (B) Kinetics of lymphoid repopulation in mice transplanted with the EYFP positive cells, showing EYFP⁺ cells only. Values shown represent the average observed for mice #E1-1 and 2 (variations were below 15%). Not all lymphoid cells were EYFP⁺ after 4 and 6 weeks because SCID mice develop some B220⁺, CD4⁺, and CD8⁺ cells following irradiation (unpublished data). (C) FACS profiles of Lin⁻ bone marrow cells from mouse #E1-1 transplanted with EYFP⁺ progenitors 7 months earlier, showing Sca-1 and c-Kit stained cells before and after gating of EYFP⁺ cells. Numbers in brackets indicate percentage of cells that are EYFP⁺ relative to all cells in the corresponding quadrant.

portions of EYFP positive B220⁺/Mac-1⁺ cells in the blood (Figure 7C), spleen, and bone marrow (data not shown). The EYFP labeled CD4⁺/CD8⁺/Mac-1⁻/B220⁻ cells observed in the blood (Figure 7C) might represent delta γ T cells and/or NK cells. HSCs were again com-
indexes might have been repopulated with a larger proportion of EYFP positive clones. The timing of the onset of lysozyme expression also matters: the earlier EYFP becomes expressed in the transplanted HSCs the higher will be the proportion of EYFP labeled progeny observed after prolonged time intervals. While there was a good correlation between the labeling index of HSCs and B lineage cells, this was less true for T cells, which in transplanted animals typically exhibited lower labeling indexes. Since EYFP$^{+}$ T cells develop normally in the absence of competing EYFP$^{+}$ T cells (Figure 5B) reporter positive T cells are probably at a disadvantage when competing with nonexpressers.

**Implications for Gene Oscillation Models**

The finding that EYFP negative cells become positive after transplantation is compatible with the notion that HSCs express lysozyme in a oscillating manner, as has been observed for transgenes expressed in muscle fibers (Newlands et al., 1998) and in erythroleukemia cells (Feng et al., 1999; Hume, 2000). That no distinct EGFP$^{+}$ population could be seen in HSCs from the lysozyme indicator mice is consistent with the idea that there is a continuum of cells with different levels of lysozyme.

In this view, the observed lysozyme expression profile represents a “snapshot” that becomes arrested in the lysozyme ancestry mice. Here, because EYFP is driven by the strong ROSA26 promoter, expressers constitute a distinct population. As estimated from a comparison of the fluorescence intensity of EGFP in myelomonocytic cells and HSCs from lysozyme indicator mice, HSCs express less than 100 times lysozyme. It is not known whether this low level expression (which has also been described for other lineage marker genes, such as myeloperoxidase and $\beta$ globin (Hu et al., 1997; Miyamoto et al., 2002), is due to a transcription factor dependent
or independent mechanism. If the former would be the case, myelomonocytic genes regulated by the same set of transcription factors, such as PU.1, C/EBP, AML-1 and Myb (Tenen et al., 1997), should oscillate within one cohort while erythroid restricted genes should oscillate within another. If the latter would be true, their expression would be equally distributed between the lysozyme positive and negative fraction. These alternatives are now testable.

Apparent Selectivity of Lineage Priming
Can the observed expression of lysozyme in bona fide HSCs be generalized to other myeloid genes? As mentioned in the introduction, the answer is probably no, since low levels of both GpIIb and Mac-1 seem to mark adult HSCs devoid of long-term repopulation potential (Corbel and Salaun, 2002; Morrison et al., 1997). Also, in striking contrast to the lysozyme gene, lineage priming in HSCs could not be observed for the B cell marker gene CD19 and for the T cell marker gene lck. While none of the published work indicated that CD19 or lck is expressed in HSCs, this also seemed to be the case for lysozyme at the outset of our studies (Clausen et al., 1999). This can be explained by the exquisite sensitivity of the lineage ancestry approach, which allows detecting not only cells that actually express the Cre reporter gene but also all cells that expressed it at an earlier point and that are therefore irreversibly marked. In addition, the marked cells are amplified through expansion of intermediate and monopotent progenitors, generating large numbers of reporter positive cells in all lineages. However, in spite of the assay’s sensitivity, we cannot rule out that HSCs express CD19 and lck at levels too low to excise the stop cassette in the reporter mouse. It is also possible that the lck transgene in the mouse line used does not fully recapitulate the activity of the endogenous promoter/enhancer. In spite of these caveats our observations strongly reinforce the notion that CD19 and lck genes are expressed in a highly lineage restricted manner. Our results also indicate that CD19\(^+=\), B220\(^-=\) cells from adult bone marrow, described to have both B cell and macrophage potential in culture (Montecino-Rodríguez et al., 2001), do not contribute to the myelomonocytic population in vivo. Finally the data suggest that in unperturbed mice, once progenitors have entered the B or T cell differentiation pathway, cells do not switch to acquire new identities.

Although our work is limited to only two lymphoid genes, it supports and extends earlier findings obtained...
by PCR analysis of single HSCs (Miyamoto et al., 2002), and by cDNA array analysis (Akashi et al., 2002). However, in the latter study a few of the more than 100 lymphoid affiliated genes tested were also detected in the HSC fraction. The apparent absence of lymphoid lineage priming is especially intriguing in view of the fact that HSCs express, besides myeloid lineage markers, a variety of genes that are normally associated with the differentiation of nonhematopoietic cells (Akashi et al., 2002). A similar selective, low level promiscuous gene expression has been described for thymic epithelial cells (Kwek et al., 2002). Ectopic activation of these genes has recently been ascribed to the transcription factor AIRE and shown to play an important role in T cell tolerance (Andersson et al., 2002). What causes selectivity of lineage priming in the hematopoietic system? Are chromatin domains corresponding to a subset of myeloid/nonhematopoietic genes "open for business" while domains associated with lymphoid genes are closed? Studies with chicken multipotent myeloid progenitor cell lines suggest that the lysozyme promoter has an open chromatin configuration, although they do not yet express the gene (Kontaraki et al., 2000). A comparison of the chromatin configurations of the regulatory regions of lysozyme, CD19, and lck genes in HSCs might yield clues as to what initiates the switch from hematopoietic stem cells to lymphoid progenitors.

Experimental Procedures

Mice

The indicator lysozyme mice were developed by inserting the egfp gene into the first exon of the lysozyme M locus through homologous recombination (Faus et al., 2000).

They were used as homozygotes (lysozyme EGFP ki/ki), Lysozyme ancestry mice were obtained by crossing two mouse lines: Lysozyme-Cre mice, in which cre cDNA was inserted via homologous recombination into the first exon of the lysozyme M gene (Clausen et al., 1999), and ROSA26 reporter mice, in which a stop cassette was inserted into the ROSA26 gene by homologous recombination (Soriano, 1999). In the particular reporter line used, removal of the loxP site flanked stop cassette by Cre recombinase activates EFYFP expression (Soriano, 1999; Smivals et al., 2001). Mice homozygous for both lysozyme Cre and ROSA26 EFYFP were used in this study (lysozyme Cre ki/ki x ROSA EFYFP ki/ki). To obtain CD19 ancestry mice, CD19 Cre mice, generated by insertion of the cre gene upstream of the CD19 gene (Rickert et al., 1997), were crossed with the ROSA26 EFYFP reporter line. For the present studies mice heterozygous for CD19 Cre and homozygous for ROSA26 EFYFP were used (CD19 Cre ki/+ x ROSA EFYFP ki/+). To obtain lck ancestry mice, transgenic lck Cre mice (Lee et al., 2001), purchased from Taconic (Germantown, NY), were crossed with the ROSA26 EFYFP reporter mice. Double heterozygous mice (lck Cre +/+ x ROSA EFYFP ki/+ ) were used in this study. The ROSA26 EFYFP mice were obtained after crossing β-actin-Cre mice (Lewandoski and Martin, 1997) with ROSA EFYFP reporter mice, using progeny exhibiting a germ line deletion of the stop cassette. The genetic background of the lysozyme, CD19, and lck ancestry lines is heterogeneous, consisting of mostly C57Bl background mixed with some 129 background. Mice were bred and maintained in the Research Animal Facility at Albert Einstein College of Medicine in accordance with guidelines from the Institute for Animal Studies.

The following oligonucleotides were used for PCR genotyping of the lysozyme-Cre mouse: 5'-CTT GGG GGT CCA GTA TTT CTC-3' (lysozyme 5'); 5'-TTA CAG TGC GCC AGG CTC AC-3' (lysozyme 3'); 5'-CCG GCC TGA AGA TAT AGA AGA-3' (Cre 3'). PCR fragments of 350 bp and 470 bp were indicative of the wild-type lysozyme alleles and cre knockin, respectively. The following oligonucleotides were used for PCR genotyping of the CD19-Cre mouse: 5' -ATT GTT GTG CTG CCA TGA TTC-3' (CD19 5'), 5' -GTG TGA AGC ATT CCA CCG GAA-3' (CD19 3'), and 5'-CCG GGT ATT CAA CCT TGA CCA-3' (Cre 3'). PCR fragments of 560 bp and 300 bp were indicative of wild-type CD19 alleles and the cre knockin, respectively. The Lck-Cre transgenic mice were genotyped with the cre-specific primers 5'-TGA TGA TGT TCG CAA GAA CC-3' and 5'-CCA TGT AGA GAA CCT GG-3', resulting in a 390 bp PCR product. PCR genotyping of the ROSA26 reporter mouse was done with the following three primers: 5' -AGA CTT CGT AGT TAT TGT-3', 5' -GGC AAG AGT TTG TCC TCA ACC-3' and 5' -GGA CCG GGA QAA AGT ATG-3'. The sizes of the wild-type and mutant fragments were 500 bp and 250 bp, respectively. All reactions were performed with 100 ng tail DNA for 30 cycles each at 30 s denaturation at 94°C, 30 s annealing at 60°C, and 30 s elongation at 72°C. The products were analyzed on a 1.5% agarose gel.

FACS Analysis of Hematopoietic Cells

Before analysis of samples by flow cytometry, erythrocytes were first removed by lysis buffer (155 mMol/L NH4Cl, 10 mMol/L KHCO3, and 0.1 mMol/L EDTA) and cells stained with various antibodies.

Phycoerythrin (PE)- or allophycocyanin (APC)-labeled antibodies directed against B220 (RA3-6B2), CD4 (L3T4), CD3 (145-2C11), CD8 (53-6.7), CD19 (D3E), Gr-1 (RB6-8C5), c-Kit (2B8), Mac-1 (M1/70), and Ter119 (Ter-119) were purchased from Pharmingen (San Diego, CA). Between 10,000 and 50,000 events were collected per sample using a FACSCalibur flow cytometer (Becton Dickinson), and data analyzed using Cell Quest (Becton Dickinson) and Flow Jo (San Carlos, CA) software.

Sorting of Stem Cells and Intermediate Progenitors

Analysis of HSCs and intermediate progenitors was essentially performed as described earlier (Akashi et al., 2000). Hematopoietic stem cells (HSCs) were sorted as IL-7RαLin Sca-1hi c-Kithi common myeloid progenitors (CMPs) as IL-7RαLin Sca-1hi c-Kithi, CD34+ , γc RII/IIIhi; granulocyte-macrophage progenitors (GMPs) as IL-7RαLin Sca-1hi c-Kithi, CD34+, γc RII/IIIhi; megakaryocyte-erythroid progenitors (MEPs) as IL-7RαLin Sca-1hi c-Kithi, CD34+,Fc RII/IIIhi; common lymphoid progenitors (CLPs) as IL-7RαLin Sca-1hi c-Kithi, CD34+CD4+CD8+NK1.1hi c-Kithi CD25+CD44hi and CD3-CD4+CD8-NK1.1c-Kithi CD25-CD44+ and CD3-CD4-CD8-NK1.1 c-Kithi CD25+CD44+, respectively. All progenitor populations were sorted using a dual laser of 488 nm (Coherent) and 633 nm (Spectraphysics) with a high-speed cell sorter (MoFlo-MLS, Cytomation, Fort Collins, CO). Cells were reanalyzed using the same machine. To determine the level of autofluorescence in the different populations and to obtain the correct settings, the corresponding populations were sorted in parallel from a wild-type mouse.

Reconstitution of Irradiated Mice

Experiment 1: Two-month-old SCID mice (C.B-17 scid/scid) were sublethally irradiated with 300 rad and 2 mice each injected with either 106 EFYP+ or 106 EFYP-, Lin-, c-Kit+ cells. These cells were obtained from the bone marrow of two lysozyme ancestry mice that exhibited 16.1% and 13.8% EFYP labeling in the B cell compartment, respectively. The levels of donor-derived blood nucleated cells were monitored at different times thereafter. Two mice transplanted with the EFYP+ fraction were sacrificed after 7 months and cells from the blood, spleen, thymus, and long bones analyzed by FACS. Experiment 2: Two-month-old SCID mice were irradiated with 300 rad, and two mice each injected with 106 FACS sorted lin, Sca-1hi, c-Kit+ cells from a pool of cells from three lysozyme ancestry mice averaging 11.1% EFYP+ B cells. The postsort analysis showed that the EFYP negative fraction was 100% pure. Mice were sacrificed 6 months after transplantation for FACS analysis and to perform a secondary transplantation with 104 bone marrow cells into three irradiated SCID mice. The blood of the secondary recipients was analyzed 6 weeks later. Experiment 3: Two-month-old C57BL/6 mice were lethally irradiated 2× with 600 rad prior to intravenous injection of 2 × 105 bone marrow cells. The donor cells came from a single lysozyme ancestry mouse exhibiting 5.5% EFYP+ cells in the B cell
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