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Impaired adult myeloid progenitor CMP and GMP cell function in conditional c-*myb*-knockout mice

Yen K. Lieu† and E. Premkumar Reddy

Department of Oncological Sciences; Mount Sinai School of Medicine; New York, NY USA

†Current affiliation: Institute for Cancer Genetics; Columbia University; New York, NY USA

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The differentiation of myeloid progenitors to mature, terminally differentiated cells is a highly regulated process. Here, we showed that conditional disruption of the c-*myb* proto-oncogene in adult mice resulted in dramatic reductions in CMP, GMP and MEP myeloid progenitors, leading to a reduction of neutrophils, basophils, monocytes and platelets in peripheral blood. In addition, c-*myb* plays a critical role at multiple stages of myeloid development, from multipotent CMP and bipotent GMP to unipotent CFU-G and CFU-M progenitor cells. c-*myb* controls the differentiation of these cells and is required for the proper commitment, maturation and normal differentiation of CMPs and GMPs. Specifically, c-*myb* regulates the precise commitment to the megakaryocytic and granulo-monocytic pathways and governs the granulocytic-monocytic lineage choice. c-*myb* is also required for the commitment along the granulocytic pathway for early myeloid progenitor cells and for the maturation of committed precursor cells along this pathway. On the other hand, disruption of the c-*myb* gene favors the commitment to the monocytic lineage, although monocytic development was abnormal with cells appearing more mature with atypical CD41 surface markers. These results demonstrate that c-*myb* plays a pivotal role in the regulation of multiple stages in adult myelogenesis.

Introduction

Myeloid progenitor cells are derived from pluripotent hematopoietic stem cells in the bone marrow (BM). Hematopoietic stem cells undergo progressive commitment to generate multipotent common myeloid progenitor (CMP) cells, which, in turn, can differentiate into either megakaryocyte-erythrocyte progenitor (MEP) or granulocyte-monocyte progenitor (GMP) cells.[1] GMPs give rise to unipotent precusor cells that terminally differentiate into granulocytes or monocytes. The commitment and differentiation of myeloid progenitors to mature cells are highly regulated processes. Transcription factors play a key role in regulating these processes. Dysregulation or mutation of some of these transcription factors is often associated with myeloid leukemias.[2]

The c-*myb* proto-oncogene is the founding member of the *myb* family of transcription factors. It is expressed highest in hematopoietic tissues, but its expression is noted in non-hematopoietic tissues as well.[3] Studies using transformed and leukemic cell lines as well as human bone marrow cells treated with antisense oligonucleotides implicate a role for c-Myb in hematopoiesis.[4,5] c-Myb appears to be required for granulopoiesis but not monopoiesis,[5] and in vitro studies indicate a role for c-*myb* in mediating the monocyte/granulocyte lineage decision.[5] However, these studies have some shortcomings. For instance, the transcriptional program appears to be altered in some of the transformed and leukemic cell lines.[6] In addition, in the antisense studies using normal human bone marrow cells, colony-forming unit (CFU) survival and growth arrest of only bipotent CFU-GM (Granulocyte, Monocyte) and unipotent CFU-G and CFU-M progenitors were determined, but CFU survival of multipotent progenitor CFU-Mix/GEMM (granulocyte, erythrocyte, monocyte, megakaryocyte) was not assayed.[7] Furthermore, possible nonspecific effects associated with the antisense technology raise some concerns.[8] Lastly, it is not completely certain that these in vitro studies reflect myelopoiesis in the whole organism. For instance, the Egr1-knockout mice do not exhibit any myeloid deficits despite a large body of literature implicating the gene in myeloid development.[9]

The embryonic lethality of homozygous c-*myb*-null mice at E15.5 emphasizes the critical requirement of c-*myb* in fetal hematopoiesis, although the precise defect is unclear.[10] Several different c-*myb*-deficient mouse models, which were generated using ENU (N-ethyl-N-nitrosurea) mutagenesis, showed little perturbation in adult myeloid development.[3] Except for the M303V mutant that had a point mutation in the transactivation domain and was devoid of eosinophils, myeloid progenitor cells and the number of neutrophils and monocytes were relatively normal in the other ENU-induced hypomorphic animals.[11,12] However, on the other hand, the adult knockdown mice, where c-Myb expression was reduced to approximately 5–10% of control animals, had elevated peripheral blood platelets and monocytes but

*Correspondence to: Yen K. Lieu and E. Premkumar Reddy; Email: ykl2104@columbia.edu and premkumar.reddy@mssm.edu
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Table 1. Peripheral blood cell counts of pIpC-administered *myb*$^{f/f}$/MxCre and *myb*$^{-/f}$/MxCre mice

Peripheral blood cell counts	Control mybf/f	KO mybf/f /Cre, myb-/f /Cre	Student's t-test
WBC (10^3/μl)	8.91 ± 0.20	4.55 ± 0.25*	0.002
Neutrophils (10^3/μl)	3.94 ± 0.07	1.76 ± 0.09*	< 0.001
Eosinophils (10^3/μl)	0.24 ± 0.02	0.15 ± 0.01	0.129
Basophils (10^3/μl)	0.05 ± 0.00	0.03 ± 0.00*	0.040
Monocytes (10^3/μl)	0.80 ± 0.03	0.50 ± 0.03*	0.011
RBC (10^6/μl)	9.07 ± 0.05	7.78 ± 0.24	0.096
Hemoglobin (g/dl)	10.26 ± 0.06	8.53 ± 0.28	0.091
Hematocrit (%)	38.04 ± 0.26	33.08 ± 0.95	0.134
MCV (fL)	41.96 ± 0.19	42.64 ± 0.14	0.088
MCH (pg)	11.34 ± 0.06	10.92 ± 0.05	0.096
Platelets (10^3/μl)	767 ± 27	249 ± 25*	< 0.001
MPV (fL)	4.61 ± 0.04	5.72 ± 0.07*	0.002
Lymphocytes (10^3/μl)	3.88 ± 0.18	2.12 ± 0.15*	0.028

Peripheral blood cells were measured on the Hemavet 950 FS hematology analyzer. Numbers are presented as mean ± SEM n = 7 for control *myb*$^{f/f}$ mice; and n = 7 for the pIpC-induced *c-myb* knockout (5 *myb*$^{f/f}$ / MxCre and 2 *myb*$^{-/f}$ /MxCre) mice (KO). * Significance differences $p < 0.05$. WBC, whole blood cell; RBC, red blood cell; MCV, mean cell volume; MCH, mean corpuscular hemoglobin; MPV, mean platelet volume.

reduced blood neutrophils.[13] In addition, these adult knockdown animals had an abnormal hematopoietic cell population with a self-renewal capability, which could confound any study on myeloid development.[13] Furthermore, it is not certain that the phenotype of the adult knockdown mice reflects normal adult myeloid development, as a similar phenotype had been observed during fetal development.[11] It is therefore possible that defects in fetal hematopoiesis, seen in the c-*myb*-knockdown mice, could lead to abnormalities in adult hematopoiesis.

Amplification of c-*MYB* expression has been observed in myeloid leukemias.[14] Recent evidence showing genetic alterations in *MYB* by duplication or translocation in a subset of childhood T-cell acute leukemias provides a direct link for *MYB* in human cancer.[15,16] In human myeloid leukemias, *MYB* duplication in two myeloid leukemic cell lines, HL60 and Meg01, has been observed.[17] In addition, genomic gain of *MYB* locus is seen in tissue samples from MYST3/MOZ-linked acute myeloid leukemic patients.[18] Thus, in order to delineate the role of c-MYB in myeloid leukemias, a clear and better understanding of c-MYB's role in normal, adult myeloid development is required.

Here, we used the inducible *myb*$^{f/f}$/MxCre system to conditionally delete c-*myb* gene in various adult myeloid progenitor cells to obtain a definitive and a better understanding of the role of c-*myb* in these populations of the murine BM cells. We demonstrate that c-*myb* is an important regulator of adult myelogenesis.

Results

Loss of c-Myb activity reduces all peripheral blood cells, including neutrophils, monocytes and platelets. To determine the role of c-*myb* in myelogenesis, we first examined the peripheral blood profiles of *myb*$^{f/f}$/MxCre mice and their littermate controls after the administration of synthetic double-stranded RNA polyinosinic polycytidylic acid (pIpC) to induce the in vivo deletion of c-*myb* floxed gene. Total white blood cells (WBC) in the pIpC-induced *myb*$^{f/f}$/MxCre mice were decreased as compared with control mice (**Fig. S1A**). In addition, blood analysis indicated that there was a statistically significant reduction in the number of neutrophils and platelets, while there was a modest and non-significant reduction in the number of monocytes, eosinophils, basophils, red blood cells and lymphocytes (**Fig. S1A**). This modest decline in some peripheral blood populations may reflect the incomplete deletion efficiency of the c-*myb* floxed allele in blood cells as shown by DNA analysis (**Fig. S1B**).

To increase the deletion efficiency of the c-*myb* floxed allele, we performed our studies using *myb*$^{-/f}$/MxCre mice, where one of the c-*myb* allele was null and the other floxed. Inclusion of the two pIpC-induced *myb*$^{-/f}$/MxCre mice and their littermate controls resulted in a statistically significant decrease in neutrophils, platelets, basophils, monocytes and lymphocytes (**Table 1**). In contrast, the blood profiles of *myb*$^{f/f}$ and *myb*$^{-/f}$ are similar (data not shown). The results from peripheral blood profiles of pIpC-induced *myb*$^{f/f}$/MxCre mice are in agreement with a reduction of neutrophils and monocytes in the BM[19] and spleen (data not shown) of these mice.

Interestingly, the number of platelets in peripheral blood was significantly reduced when the c-*myb* gene was disrupted (**Fig. S1A and Table S1**). In addition, not only was there a reduction in the number of blood platelets, the size of these mutant platelets as shown by the mean platelet volume (MPV) was larger than that of the littermate controls (**Fig. S1A and Table S1**), suggesting the possibility of other alterations in megakaryocyte development when the c-*myb* gene is disrupted. Hence, our data from peripheral blood analysis are consistent with our findings in the BM, suggesting that c-*myb* is required for the development of adult myeloid lineages[19] (**Fig. S1**; **Table 1**).

In vivo disruption of c-*myb* activity results in a dramatic decrease in the myeloid progenitor CMPs, GMPs and MEPs. To begin to understand the nature of the myeloid deficiency in the bone marrow and peripheral blood cell counts as a result of loss of c-*myb* expression, we examined the number of various myeloid progenitor cells in pIpC-treated *myb*$^{f/f}$/MxCre mice and their littermate controls. We performed flow cytometric staining to identify the three myeloid progenitors: common myeloid progenitor (CMP), granulocyte-monocyte progenitor (GMP) and megakaryocytic-erythroid progenitor (MEP) cells.[1] **Figure 1A** shows, by RT-PCR, high expression of c-*myb* in various BM-derived myeloid progenitor cells CMPs, GMPs and MEPs. The percentages of CMPs, GMPs and MEPs in the pIpC-treated *myb*$^{f/f}$/MxCre mice were quite similar to control animals (**Fig. 1B**). However, due to the huge cellular loss in the lineage$^-$c-Kit$^+$Sca-1$^-$ (LKS-) myeloid compartment (~53%) and in the BM (~59%) of the pIpC-treated *myb*$^{f/f}$/MxCre mice[19] (**Fig. 1B**), the converted percentages to absolute cellular numbers of CMPs, GMPs and MEPs were actually decreased by more than 80% (**Fig. 1C**). This number (80%) was derived by multiplying the

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percentage of remaining total bone marrow cells (41%) with the percentage of LKS- cells (47%), which represents the percentage of remaining cells (20%). This represents a loss of 80%. No abnormality was detected in the pIpC-treated $myb^{f/+}$/MxCre mice (data not shown). These results suggest that c-*myb* is required for the development of adult myeloid progenitor CMP, GMP and MEP cells.

The requirement of c-*myb* in CMPs, GMPs and CFU-G and CFU-M precursor cells is intrinsic. Though loss of c-*myb* expression resulted in the decrease of various myeloid progenitors and mature myeloid cells in the BM and peripheral blood of the pIpC-induced $myb^{f/f}$/MxCre mice as compared with littermate controls, it was not clear whether this was an intrinsic effect on myelogenesis or an indirect effect due to upstream defects in the hematopoietic stem cells, as we had previously described.[19] To determine whether c-*myb* plays a direct role in myeloid progenitor cells, we performed hematopoietic colony-forming assays using FACS-sorted CMPs and GMPs that had been treated with interferon (IFN) to delete the c-*myb* floxed gene; some deletion could be seen starting at 12 h after IFN addition and peaked around 24 h (data not shown). After treatment for 16–18 h, cells were plated on semi-solid cytokine-containing medium that allows for the growth and differentiation of multipotent, bipotent and unipotent myeloid progenitor cells.

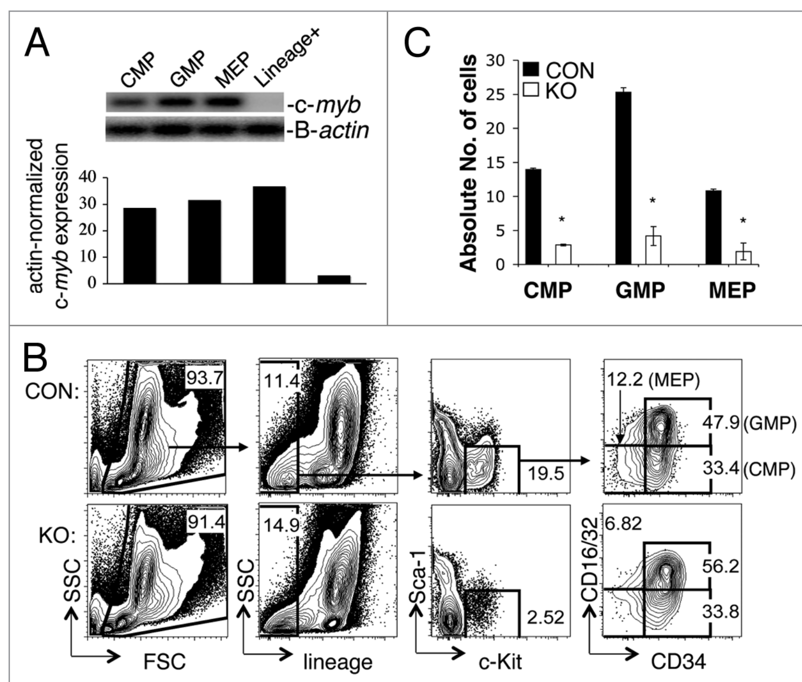

Figure 1. c-*myb* is required for the formation of myeloid progenitor CMP, GMP and MEP cells in the bone marrow of pIpC-induced $myb^{f/f}$/MxCre mice. (A) RT-PCR showing the level of c-*myb* expression in various myeloid progenitor cells CMP, GMP and MEP from BM of wild-type mouse. (B) Representative two-color flow cytometric analysis of myeloid progenitor cells CMP, GMP and MEP from 35 pIpC-administered $myb^{f/f}$/MxCre mice (KO) and 35 pIpC-treated control littermates (CON). (C) Bar graph depicts the absolute number of progenitor cells CMP, GMP and MEP ($\times 10^4$) in pIpC-administered animals. *, $p < 0.001$. Numbers are presented as mean ± SEM n = 35 mice for each genotype. Lin+, lineage-positive BM cells.

Hematopoietic colonies from IFN-treated $myb^{f/f}$/MxCre CMPs or GMPs were significantly reduced by 82% and 75%, respectively, with corresponding reductions in all types of colonies as compared with controls (**Fig. 2A and B**), indicating an important role for c-*myb* in the growth and/or survival of these cells on semi-solid cytokine-containing medium. Specifically, CFU-Mix and CFU-GM were equally reduced in FACS-purified CMPs, while CFU-GM, CFU-M and CFU-G were proportionally diminished in sorted GMPs (**Fig. 2A and B**). In addition, disruption of the c-*myb* gene in purified lineage-negative c-Kit+Sca-1- (LKS-) resulted in a substantial reduction of hematopoietic colonies as seen with CMPs and GMPs (data not shown). Furthermore, addition of G-CSF, M-CSF or GM-CSF to the SCF/IL-3/IL-6 methylcellulose medium did not rescue colony growth (data not shown). In addition, no significant difference in hematopoietic colonies was detected between IFN-treated $myb^{f/+}$/MxCre and IFN-treated $myb^{f/f}$ CMPs or GMPs (data not shown). Hence, these studies support an intrinsic role for c-*myb* at multiple stages in myelogenesis, from multipotent CMP and bipotent GMP progenitors to unipotent CFU-G and CFU-M precursor cells.

c-*myb* regulates the functionality of CMPs. To understand the functional role of c-*myb* in myelogenesis, we sorted CMP and GMP progenitors, treated these cells with type I interferon to delete c-*myb* floxed alleles in vitro and subjected these cells to FACS analysis after 24 h and 48 h of interferon treatment. To examine the possible roles of c-*myb* in proliferation, survival and differentiation, we performed BrdU labeling, caspase and surface lineage marker staining on these cells, respectively. Our initial study on purified LKS- showed little or no difference at 24 h following interferon treatment for the above three functional assays, perhaps reflecting the fact that deletion of c-*myb* floxed allele started at ~12 h after IFN addition and peaked at ~24 h (data not shown). Hence, we performed all our experiments at the 48 h time point.

By surface staining, reduction of c-*myb* expression by 81%, as assessed by RT-PCR, in CMPs leads to altered expression of surface antigens (**Fig. 3A** and data not shown). Cell surface expression of CD11b, CD41 (a marker of megakaryocytic differentiation) and CD115 (a mature monocytic differentiation receptor) on IFNα-treated c-*myb* deleted $myb^{f/f}$/MxCre CMPs was upregulated, whereas Gr-1 antigen levels was downregulated. The expression of c-Kit remained unchanged as compared with IFN-treated $myb^{f/f}$ CMPs (**Fig. 3A**). We did not detect any differences in the levels of the surface antigens examined in untreated $myb^{f/f}$/MxCre and untreated $myb^{f/f}$ CMPs (data not shown). To understand these phenotypic changes in further detail, we performed two-color flow cytometric analysis using antibodies directed against the CD11b and CD41 or Gr-1 surface markers (**Fig. 3B**). On the other hand, CD11b is expressed during development in both the monocytic and granulocytic pathways.[20] Gr-1 is expressed on granulocytes, with increasing levels of expression correlating with maturity;

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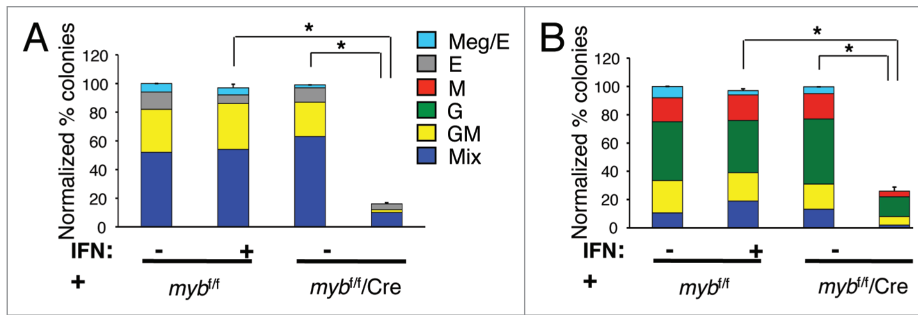

Figure 2. c-*myb* is required intrinsically for the growth and differentiation of myeloid progenitor CMP and GMP, and precursor CFU-G and CFU-M cells. Hematopoietic colony assays performed using untreated or 16 h interferon (IFN) treated purified (A) CMPs (n = 9) and (B) GMPs (n = 8) from $myb^{f/f}$/MxCre mice and littermate controls. The number of colonies from the three groups was normalized to the untreated control, which was set to 100%. Legends for (B) are shown in (A). Granulocytic (G), erythroid (E), monocytic (M), megakaryocytic (Meg) and various (Mix) myeloid CFU colonies are shown. Data are expressed as mean ± SEM *, $p < 0.001$.

immature monocytes also express Gr-1, but at intermediate levels and only transiently during development.[21,22] The two-color FACS plots of CD11b and CD41 demonstrate that disruption of the c-*myb* gene led to increases in single positive CD11b and CD41 cells as well as to double positive $CD11b^+CD41^+$ cells (**Fig. 3B**), suggesting that loss of c-Myb activity induces differentiation toward the granulo-monocytic and megakaryocytic pathways, and also leads to aberrant differentiation as exhibited by presence of double positive $CD11b^+CD41^+$ cells.

To examine the granulo-monocytic pathway in greater detail, we performed two-color FACS analysis of CD11b and Gr-1. These results indicate that loss of c-Myb activity leads to a shift toward single positive CD11b and a decrease in single positive Gr-1 cells, possibly suggesting that absence of c-*myb* expression may favor monocytic differentiation (**Fig. 3B**). As monocytes also express Gr-1 during development, we scrutinized the $CD11b^+Gr\text{-}1^+$ compartment of IFNα-induced $myb^{f/f}$/MxCre and control CMPs by surface expression of CD115, CD11b and Gr-1 (**Fig. 3C**). Overlay histograms show that loss of c-*myb* expression led to an increase in surface expression of CD11b and CD115 (**Fig. 3C**). In addition, an increase in cells expressing intermediate levels of Gr-1, with a slight decrease in cells expressing high levels of Gr-1 surface antigen was observed as a function of c-*myb* deletion (**Fig. 3C**), indicating that the granulocytic developmental pathway is blocked, while the monocytic differentiation pathway is favored. Consistent with the observation that loss of c-*myb* induced differentiation, the proliferative capacity, which is inversely correlated with maturity,[21,23] was decreased in the $CD11b^+Gr\text{-}1^+$ disrupted c-*myb* cells as compared with control cells as measured by BrdU incorporation (**Fig. 3C**). Furthermore, compared with control cells, the mutant cells in the $CD11b^+Gr\text{-}1^-$ compartment also expressed elevated surface CD115 and CD11b antigens and exhibited decreased uptake of BrdU (**Fig. 3D**). These studies indicate that loss of c-*myb* activity led to an increase in the frequency of $CD11b^+Gr\text{-}1^-$ cells that are committed to the monocytic development. A closer examination of the $CD11b^+Gr\text{-}1^-$ and the $CD11b^+Gr\text{-}1^+$ populations reveals another difference in these mutant cells as compared with control cells (**Fig. 3C and D**). Specifically, there was an increase in surface expression of the CD41 marker on both of these populations in the c-*myb* disrupted cells as compared with control cells (**Fig. 3C and D**), suggesting possibly further aberrant development in both the monocytic and granulocytic pathways. Together, our data indicate that c-*myb* regulates the commitment to the megakaryocytic and granulo-monocytic pathways. Also, c-*myb* is required for the differentiation of granulocytes and for the normal development of monocytes.

There were no significant changes in polycaspase staining among the four CMP experimental and control groups (data not shown), indicating that apoptosis via caspases at the 48 h time point is not activated when the c-*myb* gene is disrupted. However, the c-*myb* deleted CMPs had a modest, but significant, reduction in the proliferative capacity as compared with that of untreated $myb^{f/f}$/MxCre and IFN-treated and untreated $myb^{f/f}$ CMP controls (**Fig. S2A**). A detailed analysis to understand the proliferative defect in the c-*myb*-depleted CMPs showed that the decrease in the proliferative CMPs was accompanied by an increase in cells expressing the monocytic-granulocytic differentiation marker CD11b, further suggesting that c-*myb* is a regulator of differentiation (**Fig. 3E**).

c-*myb* is required for the functionality of GMPs. To assess lineage commitment and differentiation potential of GMPs when c-Myb activity is lost, we performed flow cytometric analyses using interferon-treated, purified GMPs to determine the level of surface lineage antigen expression as well as that of c-Kit (**Fig. 4**). Reduction of c-*myb* expression by 76%, as assessed by RT-PCR, in GMPs leads to upregulation of CD11b, Gr-1, CD115 and, to a lesser extent, CD41, with little alteration in c-Kit expression (**Fig. 4A** and data not shown). We did not detect a difference in the levels of any of the markers examined between untreated $myb^{f/f}$/MxCre and untreated $myb^{f/f}$ GMPs (data not shown). To further assess these phenotypic changes, we performed two-color flow cytometric analysis of CD11b and CD41 or Gr-1 surface markers (**Fig. 4B**). The FACS plot of CD11b and CD41 reveals that approximately 67% of interferon-treated $myb^{f/f}$ GMPs exhibited increased levels of the CD11b surface antigen, while only outliers expressed CD41 (**Fig. 4B**), indicating that differentiation could only occur in the direction of the granulo-monocytic pathway. This observation is consistent with the fact that GMPs are bipotential and more committed than the mutipotent CMPs in certain lineage fate (**Figs. 3B and 4B**). On the other hand, disruption of the c-*myb* gene in GMPs led to additional increases in CD11b expression and a slight increase in CD41 surface antigen (**Fig. 4B**), indicating that loss of c-Myb activity induces differentiation toward the granulo-monocytic pathway.

To further assess the altered differentiation potential of c-*myb*-deleted GMPs, we performed two-color FACS analysis of CD11b and Gr-1 (**Fig. 4B**). Disruption of the c-*myb* gene resulted in

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further increase in single positive CD11b and double positive CD11b+Gr-1+ cells as compared with control GMPs (**Fig. 4B**). A closer examination of the CD11b+Gr-1+ compartment indicates that c-*myb*-deleted cells had increased expression of CD11b, CD115 and Gr-1 compared with control IFNα-treated *myb*$^{f/f}$ cells (**Fig. 4C**). While the increase in Gr-1 marker indicates further development along the granulocytic lineage, the augmented expression of CD11b and CD115 suggests differentiation toward the monocytic pathway. In the single positive CD11b compartment, IFNα-induced *myb*$^{f/f}$/MxCre GMPs had greater surface expression of CD11b, CD115 and CD41 than control cells (**Fig. 4D**), indicating not only increased differentiation toward the monocytic lineage but also some atypical development in the absence of c-Myb activity in GMPs. Furthermore, the decrease in BrdU uptake in both the single positive CD11b and CD11b+Gr-1+ compartments of IFNα-induced *myb*$^{f/f}$/MxCre GMPs is further suggested that c-*myb*-deleted cells experienced increased developmental maturity than control cells (**Fig. 4C and D**). Together, our data indicate that c-*myb* regulates commitment to the granulocytic and monocytic pathways and is required for the proper maturation and development of myeloid cells.

Similar to the CMPs, c-Myb does not appear to play a role in apoptosis via caspases at the GMPs, at least at the 48 h time point and under these experimental conditions, as measured by polycaspase staining (data not shown). There is a slight but significant decrease in BrdU incorporation when c-*myb* gene was deleted in GMPs (**Fig. S2B**). Further analysis revealed that when c-*myb* was lost in GMPs, the percentage of proliferating cells was reduced, while the percentage of cells expressing the differentiation marker CD11b was increased, indicating that c-*myb* is a regulator of differentiation in GMPs (**Fig. 4E**).

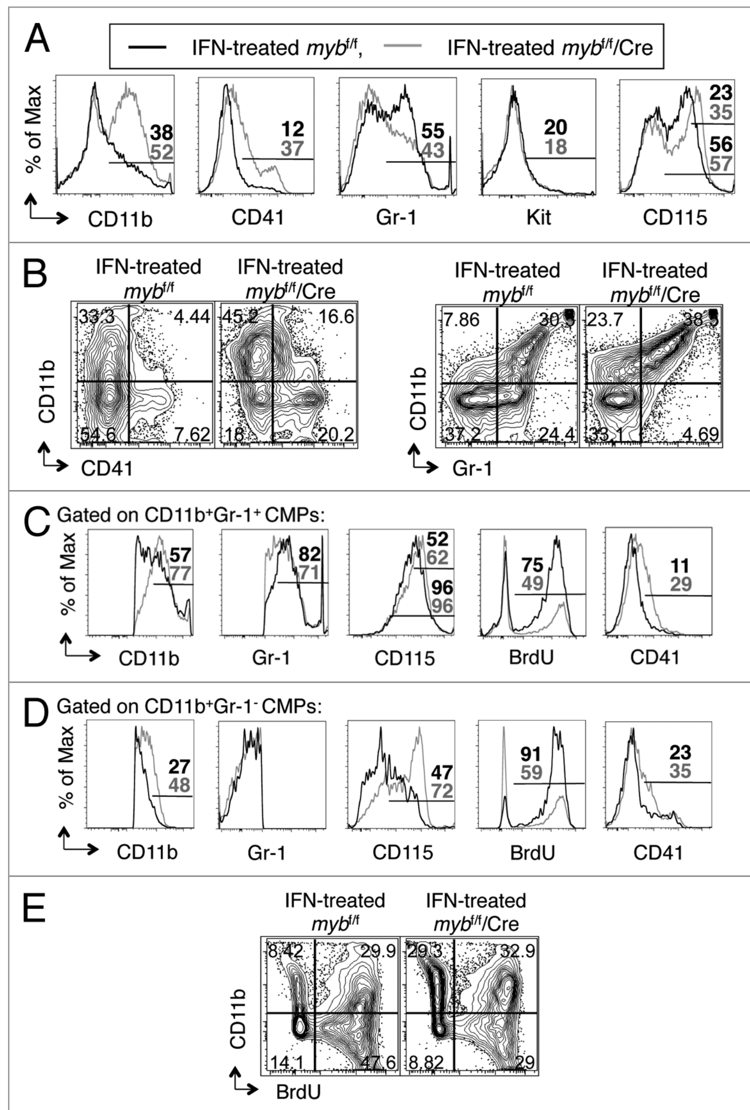

Figure 3. c-*myb* regulates the functionality of CMPs. Following 48 h interferon treatment to induce the disruption of c-*myb*, the purified CMPs were stained for surface expression of various markers. Shown are the representative (A) overlay histograms and (B and E) two-color flow cytometric analysis of various surface antigens on CMPs, purified from myb$^{f/f}$/MxCre mice and littermate controls. Representative overlay histograms of antigens on (C) the CD11b+Gr-1+ and (D) the CD11b+Gr-1- cell compartments. Legends for (C and D) are same as (A). Panels are representative of three independent experiments. The percentages for each surface marker in the indicated bar region of the histograms are specified for IFN-treated *myb*$^{f/f}$ (black) and IFN-treated *myb*$^{f/f}$/MxCre (gray) cells.

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Disruption of c-*myb* in CMP and GMP progenitor cells leads to altered gene expression. To determine the molecular mechanism by which c-*myb* functions in CMPs and GMPs, we performed DNA microarray analysis on purified lineage-c-Kit+Sca-1- (LKS-) BM cells, as these cells could be obtained in sufficient abundance and in high purity. After the addition of interferon to induce the deletion of the c-*myb* floxed gene, some deletion could be seen starting at 12 h and peak at 24–48 hr (data not shown). We wanted to assess early changes in gene expression after the loss of c-Myb activity. Thus, LKS- cells were treated with interferon for 18 h to induce the disruption of the c-*myb* floxed gene. *gfi-1*, *cxcr4*, *cebpa* and *bcl2* were shown to be downregulated in interferon-treated LKS- cells when the c-*myb* gene was disrupted (**Table S1**). We assessed the expression of these genes in 18 h, interferon-treated *myb*$^{f/f}$/MxCre CMPs and GMPs by RT-PCR (**Fig. 5**). Disruption of c-*myb* expression in CMPs resulted in significant downregulation of *gfi-1*, *cxcr4*, *cebpa* and *bcl2* genes by RT-PCR (**Fig. 5A**). In addition, expression of c-*myc* was also decreased when c-*myb* was deleted in CMPs (**Fig. 5A**). On the contrary, in GMP cells, disruption of c-*myb* gene led to statistically significant downregulation of only *cxcr4* and *bcl2* genes (**Fig. 5B**).

Noteworthy, other genes of interest that were downregulated in the LKS- microarray when c-*myb* expression was deleted were myeloperoxidase (MPO), neutrophil elastase and myeloblastin (**Table S1**). All three genes, as well as *cxcr4* and *bcl2*, have been reported as c-*myb* target genes.[24-28] Together, these studies indicate that loss of c-*myb* expression in LKS-, CMP and GMP cells leads to altered gene expression, affecting many known genes in the myeloid pathways.

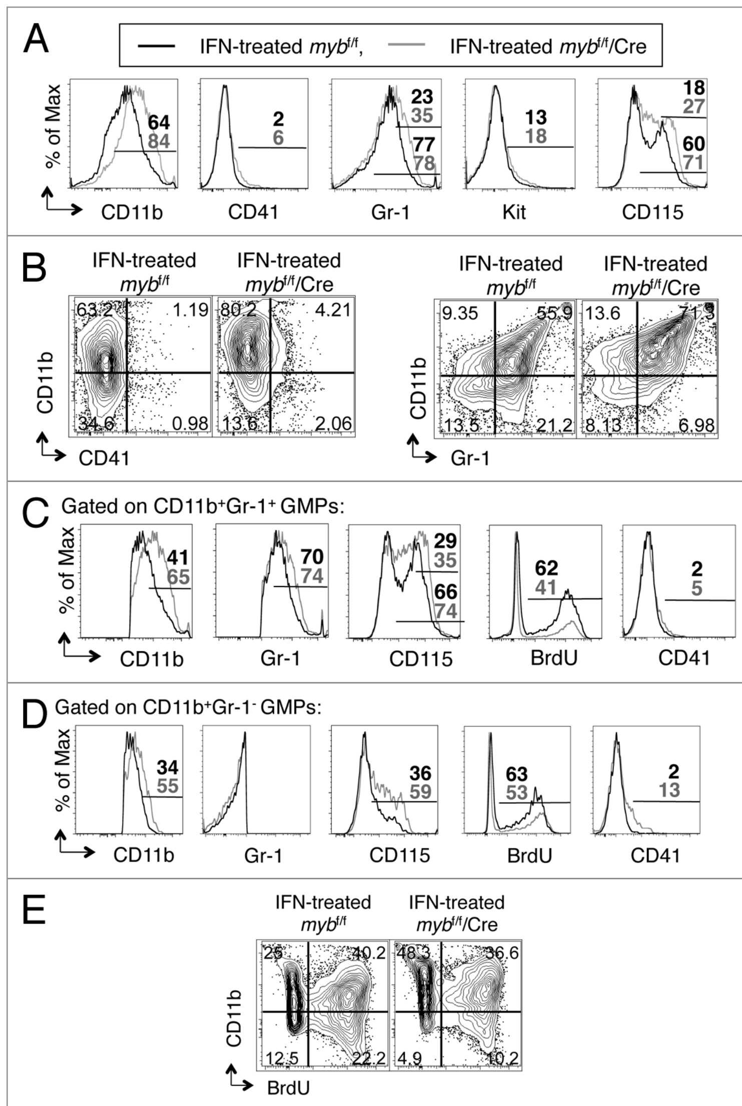

Figure 4. c-*myb* is required for the functionality of GMPs. Following 48 h interferon treatment to induce the disruption of *c-myb*, the purified GMPs were stained for surface expression of various markers. Shown are the representative (A) overlay histograms and (B and E) two-color flow cytometric analysis of various surface antigens on GMPs, purified from myb$^{f/f}$/MxCre mice and littermate controls. Representative overlay histograms of antigens on (C) the CD11b$^+$Gr-1$^+$ and (D) the CD11b$^+$Gr-1$^-$ cell compartments. Legends for (C and D) are same as (A). Panels are representative of three independent experiments. The percentages for each surface marker in the indicated bar region of the histograms are specified for IFN-treated *myb*$^{f/f}$ (black) and IFN-treated *myb*$^{f/f}$/MxCre (gray) cells.

Discussion

Our current study, which used the inducible *myb*$^{f/f}$/MxCre system to conditionally disrupt c-*myb* expression in adult BM cells, supports a critical role for c-*myb* in the regulation of adult myeloid progenitor CMP, GMP, CFU-M and CFU-G cells. Disruption of the c-*myb* gene resulted in dramatic reductions in CMP, GMP and MEP myeloid progenitor cells in adult mice, leading to a reduction of all peripheral blood cells, significantly in neutrophils, basophils, monocytes and platelets. The requirement of c-*myb* in myeloid progenitor CMPs and GMPs, as well as precursor CFU-M and CFU-G, is intrinsic, as demonstrated by hematopoietic colony assays and/or in vitro functional assays. In CMPs and GMPs, c-*myb* acts as a regulator of differentiation. c-*myb* is required for the proper commitment, normal maturation and differentiation of CMP and GMP progenitor cells. Specifically, c-*myb* regulates the commitment to the megakaryocytic and granulo-monocytic pathways and governs the granulocytic-monocytic lineage choice. c-*myb* is required for the commitment to the granulocytic development for early myeloid progenitor cells. The disruption of the c-*myb* gene favors commitment to the monocytic lineage. However, in this model, monocytic development was abnormal, whereby monocytes appeared more mature, with atypical CD41 megakaryocytic surface markers. Hence, the transcriptional factor c-*myb* is an important regulator of adult myelogenesis.

Due to the accumulating knowledge in this field, it is perhaps not surprising that conditional disruption of c-*myb* expression in adult myeloid cells would perturb granulopoiesis. What is surprising about the data presented here was the direct impact of c-*myb* on monopoiesis. The majority of the published reports suggest that c-*myb* may not play a direct role in monopoiesis.[29-34] Here, we used an inducible, conditional knockout strategy to disrupt c-*myb* expression in sorted CMPs and GMPs from adult BM cells and show that this gene has an intrinsic role in monopoiesis. First, we showed that the significant decrease in BM and peripheral blood monocytes[19] (**Table 1** and data not shown), in fact, reflect an inherent and essential role of c-*myb* in monopoieis as shown by the drastic reduction of CFU-GM and CFU-M colonies in the colony-forming assays (**Fig. 2**). In addition, in vitro functional assays support a role for c-*myb* in regulating the proper maturation and normal development of monocytes. In these assays, when the c-*myb* gene was disrupted, CMPs and GMPs expressed increased levels of the CD11b surface marker and the CD115/M-CSF monocytic differentiation receptor as compared with control cells, indicating a biased maturation in the monocytic pathway (**Figs. 3 and 4**). Furthermore, when c-Myb activity was lost, a portion of the CD11b$^+$Gr-1$^-$CD115$^+$ and CD11b$^+$Gr-1$^+$CD115$^+$ cells expressed CD41 surface marker, indicating further aberrant development (**Fig. 3 and 4**). Although the precise defect in fetal hematopoiesis in the systemic c-*myb*-null embryos is unclear, their fetal livers contained few, but normally appearing, monocytes,[10] suggesting that c-*myb* may not have a direct role in the monocytic developmental pathway. Thus, contrary to most suggestions, c-*myb* has a direct role in monocytic development: in the absence of c-Myb activity, monocytic precursor cells undergo atypical differentiation.

In the c-*myb*-knockdown model, it was concluded that c-*myb* has a role in lineage choice based on the observation that there were an increase in monocytes and a decrease in granulocytes in the fetal liver.[29] However, it's possible that the loss of granulocytes

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in these mice could be attributed to the critical role of c-*myb* in other stages along the granulocytic pathway. In support of this notion, in human bone marrow cells, c-*myb* expression is noted at the myeloblastic, promyelocytic and myelocytic stages.[6,29] Our data provide direct evidence for c-*myb* in governing the granulocytic-monocytic lineage choice in adult myelogenesis and show that there is a direct role for c-*myb* in granulopoiesis. The significant decrease in BM and blood neutrophils and basophils, as well as the impaired colony growth of CFU-G and CFU-GM, demonstrates a critical and an inherent role for c-*myb* in granulocytic development. Loss of c-Myb activity skewed the development away from the granulocytic pathway and toward monocytic differentiation. Immature monocytes also express Gr-1, albeit transiently, at an intermediate levels during development, while granulocytes express Gr-1 at an even higher abundance during maturation.[21,22] Loss of c-Myb activity by early myeloid progenitors resulted in an increase in cells expressing the CD11b surface marker together with the CD115/M-CSF monocytic receptor, but prevented further upregulation of Gr-1 in the IFN-induced *myb*$^{f/f}$/MxCre CMPs (**Fig. 3**), supporting a direct role for c-*myb* in lineage choice.

In spite of the lineage bias toward monocytic development, one reason for the decrease in monocytic colonies and peripheral blood monocytes is that increased differentiation coupled with impaired proliferation caused a loss of the myeloid progenitor and monocytic precursor cells. Alternatively, the aberrant development of these committed monocytic cells could lead to their demise after the 48 h time point. Consistent with this notion of cell death, analysis of DNA isolated from the cells remaining on the IFN-induced *myb*$^{f/f}$/MxCre LKS-, CMP and GMP methylcellulose plates after 10–12 d demonstrated only the presence of the c-*myb* floxed allele by PCR (data not shown). While the significant decrease in BM and blood neutrophils as well as peripheral basophils when c-Myb activity is lost could in part be due to the unfavorable lineage bias, aberrant induced differentiation may also account for the decrease in granulocytes. In support of this notion, while the expression of Gr-1 is already elevated in the control GMPs, the c-*myb*-deleted GMPs expressed even higher Gr-1 on the cell surface (**Fig. 4**). As indicated in **Figure 2B**, most of these cells were further downstream from the bipotent stage, further upregulation of Gr-1 marker in c-*myb*-deleted cells may suggest further progression in granulocytic maturation (**Fig. 4**), possibly indicating that cells which have progressed further along the granulocytic pathway may have proceeded further along that maturation pathway in the absence of c-Myb activity. Another reason for the decrease in granulocytes is that other points along the granulocytic developmental pathway, aside from what was shown here (CFU-Mix, CFU-GM and CFU-G), may depend on c-*myb* as is evident by high levels of c-*myb* expression at the myeloblastic, promyelocytic and myelocytic stages.[6]

It has been assumed that megakaryocytic development may be c-*myb*-independent, because this population in the fetal livers of homozygous c-*myb*-null mice was not affected.[10] Since then, four hypomorphic mouse models seem to suggest a role for c-*myb* in the negative regulation of megakaryocytic development.[11-13,29]

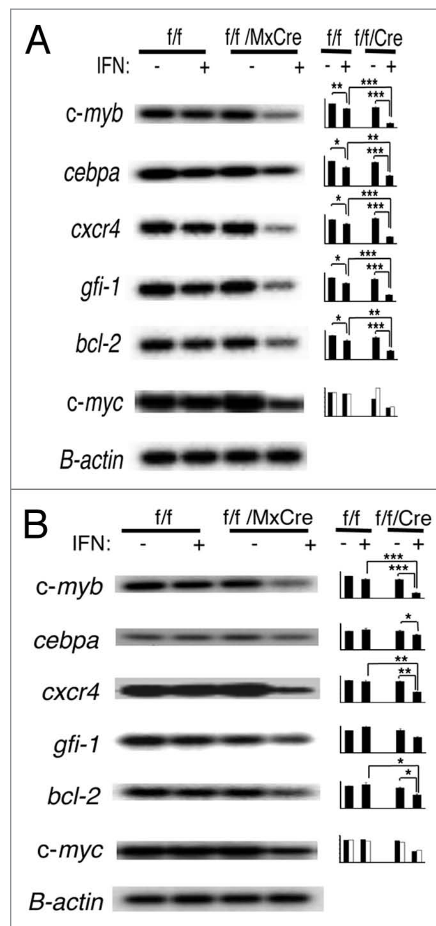

Figure 5. Disruption of c-*myb* results in altered gene expression in CMPs and GMPs. RT-PCR analysis of gene expression of (A) CMPs and (B) GMPs after 18 h of interferon treatment is shown. Results reflect at least three independent experiments. In the case of c-*myc*, results were from two independent sorts. Data on bar graph are β-actin normalized gene expression (y-axis) and are expressed as mean ± SEM *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

All four murine models presented with elevated blood platelets, and in the knockdown and M303V mutants, BM megakaryocytes were increased.[11-13,29] On the contrary, our c-*myb*-disrupted *myb*$^{f/f}$/MxCre mice had a decreasing megakaryocytic trend in the BM (data not shown), and their blood platelets were dramatically decreased (**Fig. S1 and Table S1**); thus, our mouse model seems to be at odds with all the other mouse models to date. Our hematopoietic colony assay, performed on CMPs, suggests a role for c-*myb* in megakarypoietic development. In support of this latter notion, no hematopoietic colonies were derived from CMPs with disrupted c-*myb* alleles, and analysis of DNA isolated from the cells remaining on the IFN-induced *myb*$^{f/f}$/MxCre LKS- or CMP methylcellulose plates after 10–12 d showed only the presence of the c-*myb* floxed allele by PCR (**Fig. 3**, data not shown). Furthermore, the size of the platelets was increased in the pIpC-treated *myb*$^{f/f}$/MxCre mice (**Fig. S1 and Table S1**). Interestingly, the M303V mice were also presented with increased platelet size.[12] Thus, our data suggest that c-*myb* may have a previously unappreciated role in megakaryopoiesis.

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To better understand the role of c-*myb* in myelogenesis, we examined the gene expression of sorted CMPs and GMPs. At the molecular level, the statistically significant downregulation of *gfi-1*, *cebpa* and c-*myc* transcription factors in c-*myb*-disrupted myeloid cells could explain the critical impairment in granulocytes.[35-37] In addition, statistically significant inhibition of *cxcr4* chemokine receptor expression could critically affect myelogenesis.[38] Furthermore, statistically significant downregulation of *Bcl-2* could explain the absence of colony formation and could partially account for the decreases in BM and blood myeloid cells.[39] Our data in totality support a role for c-*myb* at multiple stages in the formation of mature myeloid cells: from multipotent CMP and bipotent GMP to unipotent myeloid precursor, CFU-M and CFU-G. Hence, the transcription factor c-*myb* has a pivotal role as a regulator of differentiation in adult myelogenesis. A better understanding of the role of c-*myb* in normal myelogenesis will hopefully improve further research into the role of c-*myb* in myeloid leukemias for therapeutic intervention.

Materials and Methods

Mice. The $myb^{f/f}$/MxCre mice, which were maintained on a N6 to N10 C57BL/6 genetic background, had been described.[19] The mutant mice were genotyped using a three-primer PCR amplification method: mybG2e 5'-att cca gtg gtt ctt gat agc att atc-3'; mybG11e: 5'-gcc gct aag cca caa tgg aag ggc-3'; mybG19e: 5'-cct tga ctc tga gta aga aag taa ac-3'.

Flow cytometry, sorting and antibodies. Freshly isolated single cell suspensions of BM cells were prepared as previously described.[19] Stained cells were analyzed using the FacsAria (BD Biosciences) following staining with fluorochrome-conjugated antibodies purchased from BD Biosciences or eBiosciences. For cell sorting, lineage-positive cells were partially depleted via StemSep murine progenitor enrichment cocktail (StemCell Technologies). Then enriched progenitor cells were then stained with lineage cocktail antibodies consisting of CD3, B220, TER119, Gr-1 and CD11b as well as stem/progenitor cell markers: c-Kit, Sca-1, CD34 and CD16/32. Labeled cells were sorted and analyzed on a high-speed cell sorter (FacsAria; BD Biosciences). CMPs were defined as $Lin^-c\text{-}Kit^+Sca\text{-}1^-CD34^+CD16/32^-$ and GMPs as $Lin^-c\text{-}Kit^+Sca\text{-}1^-CD34^+CD16/32^+$. Data were analyzed using FlowJo (TreeStar).

Colony assay on methylcellulose. At 16–18 h following ex vivo interferon [IFNα (R&D Systems); 2×10^4 units per ml] treatment, ~500 sorted cells were plated onto dishes containing Methocult M3434 (StemCell Technologies), supplemented with rmGM-CSF (2 ng/ml), rhTPO (10 ng/ml) and rmIL-11 (20 ng/ml). Colonies were scored 10–12 d post-plating. In each experiment, the number of colonies for each of the four groups (untreated $myb^{f/f}$, IFN-treated $myb^{f/f}$, untreated $myb^{f/f}$/MxCre and IFN-treated $myb^{f/f}$/MxCre CMPs/GMPs) was divided by the number of colonies for the untreated $myb^{f/f}$ control; these numbers were then multiplied by 100%. Thus the untreated $myb^{f/f}$ control was set to 100%, enabling statistical comparison among the different independent experiments. The sort purities of the CMPs and GMPs were in the range of 91.1–97.5% and 85.2–93.4%, respectively.

In vivo deletion of the c-*myb* floxed allele and in vitro functional assay. For in vivo disruption of the c-*myb* floxed allele, the $myb^{f/f}$ /MxCre and control mice were given a 250 μl of 2 mg/ml pIpC (polyinosinic-polycytidylic acid, Sigma, P-1530) i.p. injection every other day for a total of seven to nine injections and analyzed 1 or 2 d after the last injection. pIpC was dissolved in sterile PBS by heating at 56°C for 30 min and then stored in frozen aliquot at -20°C. For injections, defrosted pIpC solution was heated at 56°C for 8 min and allowed to cool at room temperature. The sort purities of CMPs and GMPs for the three independent experiments for the functional assays were > 90% and > 95%, respectively.

For in vitro deletion, 2×10^4 units of IFNα (R&D Systems) per ml of SCF/IL3/IL6 cytokine-containing DMEM medium (DMEM, 15% heat-inactivated FBS, 10% Wehi supernatant, 50 units/mL penicillin/streptomycin, 2 mM L-glutamine, 100 mg/mL mSCF, 10 ng/mL mIL-3, 10 ng/mL hIL-6) was used. For cell culture, regardless of the purified cell number, a minimum of 200 μl medium was used with a maximum concentration of 1×10^6 cells/ml.

At the indicated time following IFNα treatment, purified cells were pulsed with BrdU (BD Biosciences) in fresh cytokine medium for 2 h at 37°C in a tissue culture incubator under humidified conditions with 5% CO_2. Then, the indicated fluorochrome inhibitor of caspases for detecting poly-caspase activity (Invitrogen, V35117 FLICA kit) was added, and cells were returned to the incubator for an additional 1 h. After the incubation, purified cells were washed, stained with surface antibodies and then fixed and permeabilized for anti-BrdU staining as specified by the manufacturer (BD Biosciences).

Peripheral blood count analysis. Blood samples collected from the heart were drawn into EDTA tubes and analyzed within 2 h using a Hemavet 950 FS hematology counter, equipped with a mouse-specific software (Drew Scientific).

Microarray and RT-PCR analysis. Agilent gene array analysis of purified $lineage^-c\text{-}Kit^+Sca\text{-}1^-$ (LKS-) cells was performed at the DNA Microarray Core Facility at the Fox Chase Cancer Research Center. RNA isolation, RT-PCR conditions, gene amplification primers and oligonucleotide probes used were previously reported.[19]

Statistical analysis. Data are expressed as means ± SEM. Comparisons were analyzed by using Student's two-pair or unpaired t-test (equal variance). Differences were considered significant when $p < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
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