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Enhancement of Hematopoietic Stem Cell Repopulating Capacity and Self-Renewal in the Absence of the Transcription Factor C/EBPα

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Summary

The transcription factor C/EBPα is required for granulopoiesis and frequently disrupted in human acute myeloid leukemia (AML). Here, we show disruption of C/EBPα blocks the transition from the common myeloid to the granulocyte/monocyte progenitor but is not required beyond this stage for terminal granulocyte maturation. C/EBPα-deficient hematopoietic stem cells (HSCs) have increased expression of Bmi-1 and enhanced competitive repopulating activity. Bone marrow in adult C/EBPα-deficient mice was filled with myeloblasts, similar to human AML, supporting the notion that disruption of C/EBPα cooperates with other events in the development of leukemia. Therefore, C/EBPα is not only essential for granulocyte development but, in addition, is a regulator of hematopoietic stem cell activity.

Introduction

Myeloid cells develop from HSCs through intermediate myeloid progenitors, which normally differentiate further into granulocytes and monocytes, and their importance is emphasized by the characteristic accumulation of bone marrow blasts in AML. Several transcription factors are involved in the differentiation from HSC to terminally mature granulocytes and monocytes (Tenen et al., 1997; Tenen, 2003). The transcription factor C/EBPα is a key factor for granulopoiesis (Radomska et al., 1998). C/EBPα is expressed in myeloid cells and regulates many myeloid genes (Iwama et al., 1998; Tenen et al., 1997). In C/EBPα, nonconditional knockouts have a block in granulocyte differentiation in fetal liver and newborns. Immature myeloid cells accumulate in fetal liver and peripheral blood, similar to what is observed in humans with AML (Zhang et al., 1997). C/EBPα mutations are found in a significant number of AML patients with a normal karyotype (Pabst et al., 2001b; Gombart et al., 2002; Preudhomme et al., 2002; Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003). Patients with the t(8;21) AML1-ETO translocation do not harbor C/EBPα mutations, but expression of C/EBPα is downregulated (Pabst et al., 2001a). C/EBPα is involved in the regulation of cell proliferation (McKnight, 2001). These studies suggest that C/EBPα is not only important for granulocyte differentiation but also involved in control of cell proliferation and leukemogenesis. However, these studies did not address the precise stage at which loss of C/EBPα affects myeloid differentiation, nor did they address the role of C/EBPα in adult hematopoiesis and in particular its function in stem cells.

Early hematopoietic precursors can be classified using multicolor FACS according to their cell surface marker expression pattern. Common myeloid progenitors (CMPs) differentiate into all myeloerythroid cells. CMPs can generate granulocyte/monocyte progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs) (Akashi et al., 2000). In these purified stem and progenitor subsets, C/EBPα is expressed at low levels in HSCs, and upregulated with development of CMPs and GMPs, but is shut down in MEPs or lymphoid cells (Akashi et al., 2000; Traver et al., 2001; Miyamoto et al., 2002).

Previous studies of C/EBPα in myelopoiesis have been done in vivo solely during fetal liver hematopoiesis due to the perinatal mortality of nonconditional knockouts from hypoglycemia (Wang et al., 1995). The importance of studying the role of transcription factors in adults has been emphasized by recent studies utilizing conditional targeting. At least two factors thought to regulate stem cells based on nonconditional knockout models, AML1 and SCL, have been shown to have a much more limited role in adult hematopoiesis (Ichikawa et al., 2002; Mikkola et al., 2003; Hall et al., 2003). Therefore, several questions regarding the role of C/EBPα in hematopoiesis remain unclear. First, will disruption of C/EBPα in adult mice result in accumulation of blasts blocked in differentiation? Second, at what stage is granulopoiesis blocked in C/EBPα$^{-/-}$ mice? Third, because C/EBPα is expressed in HSCs, is C/EBPα involved in self-renewal or lineage commitment of stem cells? Fourth, will accumulation of such blasts result in development of AML?

We analyzed hematopoietic development in both C/EBPα$^{-/-}$ fetal liver and bone marrow from conditional C/EBPα$^{-/-}$ mice. Here, we demonstrate that disruption of the C/EBPα gene blocks the transition from CMP to GMP and results in accumulation of >30% blasts in the adult bone marrow, resembling human AML. Furthermore, C/EBPα$^{-/-}$ HSCs express increased levels of the polycomb gene Bmi-1, which can enhance HSC self-renewal (Iwama et al., 2004 [this issue of *Immunity*]) and demonstrate a competitive advantage over wild-type (wt) HSC. These results indicate that C/EBPα normally serves to limit HSC self-renewal.

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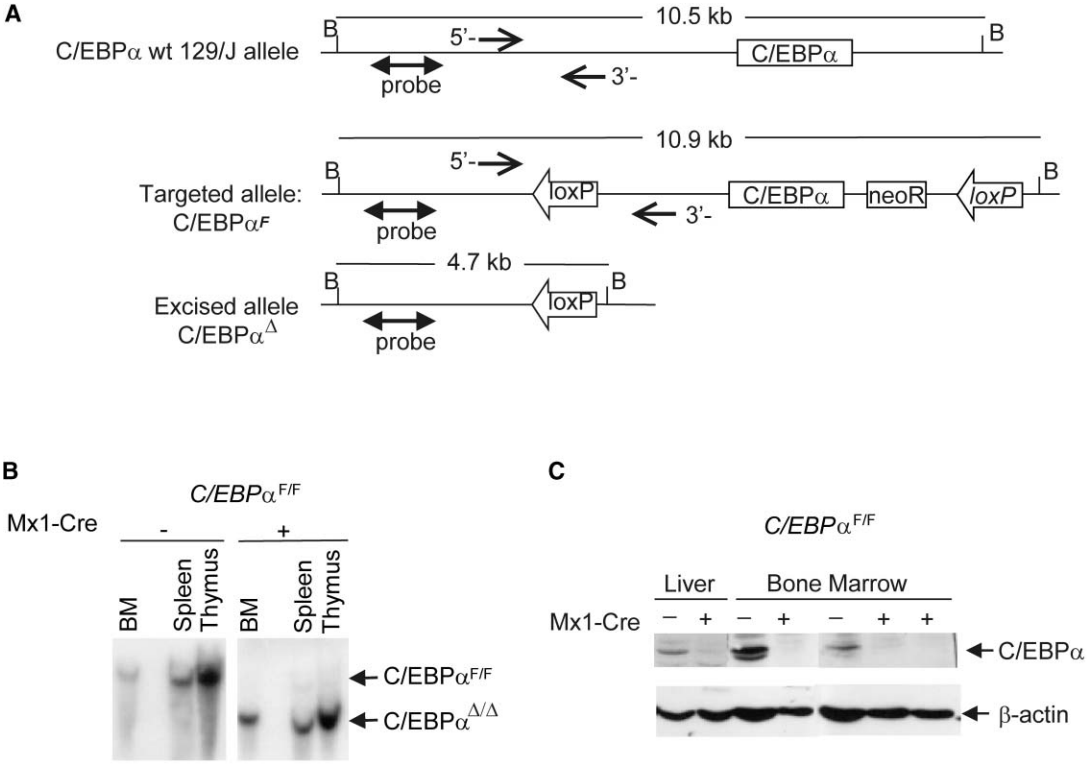

Figure 1. Generation of a Conditional C/EBPα Knockout Mouse Lines

(A) Top, the genomic C/EBPα locus; middle, after targeted insertion of loxP sequences flanking the single C/EBPα exon and neomycin resistance gene (neoR); bottom, genomic structure after C/EBPα and neoR are deleted by Cre-mediated gene excision. Double-headed arrows, probe used in assessment of excision efficiency by Southern blot analysis. B, BamHI restriction enzyme site. Expected sizes of bands detected on Southern blots are indicated for wt, floxed, and targeted alleles. Single-headed arrows, primers used in PCR reactions for genotyping the C/EBP$\alpha^{F/F}$ mice.

(B) The C/EBPα gene was efficiently excised from bone marrow, spleen, and thymus in C/EBP$\alpha^{\Delta/\Delta}$ mice. Southern blot analysis of genomic DNA from poly I:C treated C/EBP$\alpha^{F/F}$ and Mx1-Cre x C/EBP$\alpha^{F/F}$ (C/EBP$\alpha^{\Delta/\Delta}$) mouse bone marrow, spleen, and thymus were digested with BamHI and hybridized with the probe shown in Figure 1A. Arrows on the right indicate the targeted allele (10.9 kb) and excised allele (4.7 kb).

(C) C/EBPα protein expression is not detectable in the bone marrow of C/EBP$\alpha^{\Delta/\Delta}$ mice. Western blot analysis of whole-cell lysates of adult liver and total bone marrow cells from two C/EBP$\alpha^{F/F}$ and C/EBP$\alpha^{\Delta/\Delta}$ mice.

Results

Generation of Mice with Conditional Disruption of the C/EBPα Gene

Nonconditional C/EBP$\alpha^{-/-}$ mice die perinatally of hypoglycemia (Wang et al., 1995). In order to analyze the role of C/EBPα in the adult, we generated conditional C/EBPα knockouts (Figure 1A). There were no notable abnormalities in the hematopoietic system among C/EBP$\alpha^{+/+}$, C/EBP$\alpha^{F/+}$, and C/EBP$\alpha^{F/F}$ mice (Table 1, Supplemental Figure S1 available online at http://www.immunity.com/cgi/content/full/21/6/853/DC1/, and data not shown), indicating that the floxed C/EBPα allele functions like the wt gene. We bred C/EBP$\alpha^{F/F}$ mice with Mx1-Cre transgenic mice, in which Cre can be induced with interferons or synthetic double-stranded RNA polyinosinic-polycytidylic acid (poly I:C) (Kuhn et al., 1995). Studies have demonstrated near complete recombination of floxed alleles in bone marrow by using Mx1-Cre strain, including early progenitors (Radtke et al., 1999; Higuchi et al., 2002).

Mx1-Cre^{+} × C/EBP$\alpha^{F/F}$ mice were injected with poly I:C intraperitoneally once 2 days after birth as described (Kuhn et al., 1995). Excision of the floxed gene is reportedly 35% of bone marrow cells 1 week after poly I:C

Table 1. Peripheral Blood Differential Counts of C/EBP$\alpha^{F/F}$ and C/EBP$\alpha^{\Delta/\Delta}$ Mice

	C/EBP$\alpha^{F/F}$	C/EBP$\alpha^{\Delta/\Delta}$
RBC (M/μl)	8.4 ± 1.5	9.2 ± 0.5
Hemoglobin (g/dl)	14.0 ± 1.3	14.6 ± 0.6
HCT (%)	42.3 ± 2.9	44.3 ± 2.0
Platelet x (K/μl)	509.57 ± 295.2	980.4 ± 544.8
Total WBC (K/μl)	3.9 ± 0.6	4.4 ± 1.3
Lymphocyte (%)	78.6 ± 1.4	99.7 ± 0.5
Neutrophils (%)	16.8 ± 4.2	0
Monocyte (%)	2.2 ± 1.4	0.3 ± 0.5
Eosinophils (%)	2.4 ± 1.4	0

RBC, Hgb, HCT, platelet counts, and total WBC were determined with an automated cell counter (Hemovet) at 3 weeks of age after treatment with poly I:C 2 days after birth. WBC differentials were determined by morphology of 200–300 cells stained with Wright-Giemsa. Shown are the numbers from ten mice with each genotype ± standard deviation.

Table 2. Analysis of Differential Counts, Hematopoietic Stem Cell and Progenitor Numbers in Nonconditional Fetal Liver, and Conditional Bone Marrow Derived from Mice with Disruption of C/EBPα

Genotype	Blast	Promyelocyte	Myelocyte	Neutro	Mono	Eosinophil	Erythroblast	Lymphoid
[a]C/EBPα$^{F/F}$	1.0 ± 0.3	1.6 ± 0.6	9.8 ± 5.4	37.0 ± 2.8	1.7 ± 1.9	2.6 ± 2.0	36.4 ± 5.5	12.6 ± 7.1
C/EBPα$^{\Delta/\Delta}$	32.7 ± 4.8	1.0 ± 0.6	1.1 ± 0.3	1.4 ± 3.4	0.2 ± 0.1	0	41.4 ± 6.0	21.2 ± 4.0

	HSC	CMP	GMP	MEP
[b]FL C/EBPα$^{+/+}$	0.25% ± 0.043%	0.62% ± 0.015%	0.49% ± 0.11%	1.80% ± 0.11%
FL C/EBPα$^{-/-}$	0.30% ± 0.068%	0.32% ± 0.017%*	0.0% ± 0.03%*	3.80% ± 0.69%*
rBM C/EBPα$^{+/+}$	0.09%	1.04%	0.72%	1.18%
rBM C/EBPα$^{-/-}$	2.89%*	5.5%*	0.04%*	4.78%*

[a] Six mice were evaluated for each genotype 3–4 weeks after induction of deletion with poly I:C. Shown are the percentages of total bone marrow cells as assessed by Wright-Giemsa staining ± SD.
[b] FL refers to embryonic day 14.5 fetal liver from C/EBPα$^{+/+}$ and nonconditional C/EBPα$^{-/-}$ knockout mice. rBM refers to recipient bone marrow from C/EBPα$^{+/+}$ and C/EBPα$^{-/-}$ fetal liver transplanted mice. Shown are the percentages ± SD of total fetal liver hematopoietic cells (FL) or total bone marrow cells (rBM) derived from 6 (FL) and 3 (rBM) mice in each category, respectively. An asterisk (*) indicates $p < 0.05$ compared to C/EBPα wt.

and 85% at 2 weeks (Higuchi et al., 2002). Therefore, we analyzed mice 3 to 4.5 weeks after injection. As shown in Figure 1B, C/EBPα gene deletion efficiency is higher than 95% in all hematopoietic tissues tested. C/EBPα protein in Mx1-Cre^{+} × C/EBPα$^{F/F}$ mice was decreased to undetectable levels in bone marrow after excision (Figure 1C). Therefore, Mx1-Cre^{+} × C/EBPα$^{F/F}$ mice represent an excellent model to study the role of C/EBPα during adult hematopoietic development. We shall refer to poly I:C treated Mx1-Cre^{+} × C/EBPα$^{F/F}$ mice as C/EBPα$^{\Delta/\Delta}$ for the sake of brevity, with the realization that a small fraction (<5%) of C/EBPα$^{\Delta/\Delta}$ hematopoietic cells have likely not deleted the C/EBPα gene.

Disruption of the C/EBPα Gene in Adult Mice Selectively Blocks Granulocytic Development

As granulopoiesis is selectively blocked in the fetal and newborn liver in C/EBPα nonconditional knockout mice (Zhang et al., 1997), we tested whether we would see the same phenotype in C/EBPα$^{\Delta/\Delta}$ adult bone marrow cells. As shown in Table 1, the number of erythroid cells in peripheral blood was unaffected in C/EBPα$^{\Delta/\Delta}$ mice as compared to poly I:C treated C/EBPα$^{F/F}$ controls. Peripheral blood platelets were elevated, as was the case in C/EBPα$^{-/-}$ fetal livers (Zhang et al., 1997); this difference was not statistically significant. Although the total white blood cell count was similar, no mature granulocytes were observed in C/EBPα$^{\Delta/\Delta}$ mice, and the percentage of lymphocytes was slightly increased. We observed no accumulation of immature myeloid cells in the peripheral blood. In the bone marrow, C/EBPα$^{\Delta/\Delta}$ mice displayed a significant differentiation block in myeloid maturation. Myelocytes, neutrophils, monocytes, and eosinophils were rarely seen in the C/EBPα$^{\Delta/\Delta}$ bone marrow, whereas immature myeloblasts were increased up to 32% (Table 2), which is one of the diagnostic criteria for human AML. However, in C/EBPα$^{\Delta/\Delta}$ bone marrow, the percentages of erythroblasts were normal (Table 2).

Consistent with these morphological observations, C/EBPα$^{\Delta/\Delta}$ mice displayed a dramatic loss of Gr-1^{+} Mac-1^{+} neutrophils/monocytes on FACS analyses in bone marrow (Supplemental Figure S1), spleen, and peripheral blood (Supplemental Table S1). T and B cells remained normal (Supplemental Table S1). These data in adult C/EBPα$^{\Delta/\Delta}$ mice are compatible with that seen in C/EBPα$^{-/-}$ fetal liver cells. The lack of granulopoiesis in all hematopoietic organs demonstrates that loss of granulocytes in the peripheral blood of C/EBPα$^{\Delta/\Delta}$ mice is not due to lack of migration from the bone marrow to spleen or peripheral blood.

The Block in Differentiation Resulting from Disruption of the C/EBPα Gene in Mice Occurs at the Transition from CMP to GMP in Fetal Liver and Adult Hematopoiesis

In order to locate the stage of differentiation block, we characterized the change in the distribution of myeloid progenitors by using multiple cell surface markers (Akashi et al., 2000). As shown in Figure 2, GMPs were almost completely absent in C/EBPα$^{-/-}$ fetal liver hematopoiesis, and relative increases in MEPs were observed (Figure 2A and Table 2). This phenotype was identical in adult bone marrow lacking C/EBPα (Supplemental Figure S1).

The lack of GMPs could either represent a true absence of this population in the absence of C/EBPα or else an artifact as a result of dysregulation of one of the markers used to define this population. In order to verify that the phenotypic CMP isolated from C/EBPα$^{-/-}$ mice function as CMP, we performed colony-forming unit (CFU) assays. CMPs were isolated by FACS and single-cell CFU assays performed in methycellulose. CMPs purified from C/EBPα$^{-/-}$ fetal liver cells and C/EBPα$^{\Delta/\Delta}$ bone marrow exhibited clonogenicity similar to that of wt and had no significant change in the number of mixed, erythroid, megakaryocytic and mixed erythroid/megakaryocytic colonies (Figure 2B and Supplemental Figure S1), demonstrating their normal erythroid and megakaryocytic potentials. However, no mature granulocyte-macrophage (GM), macrophage (M), or granulocytic (G) colonies could be generated from C/EBPα$^{\Delta/\Delta}$ or C/EBPα$^{-/-}$ CMPs. Instead, C/EBPα$^{\Delta/\Delta}$ and C/EBPα$^{-/-}$ CMPs gave rise to an equivalent number of blastic colonies, which did not display myeloid maturation (Figure 2B and Supplemental Figure S1). The myeloid component in C/EBPα$^{\Delta/\Delta}$ or C/EBPα$^{-/-}$ CMP-derived mix colonies also displayed blastic immature morphology (Figure 2B and data not shown). Therefore, CMP derived from

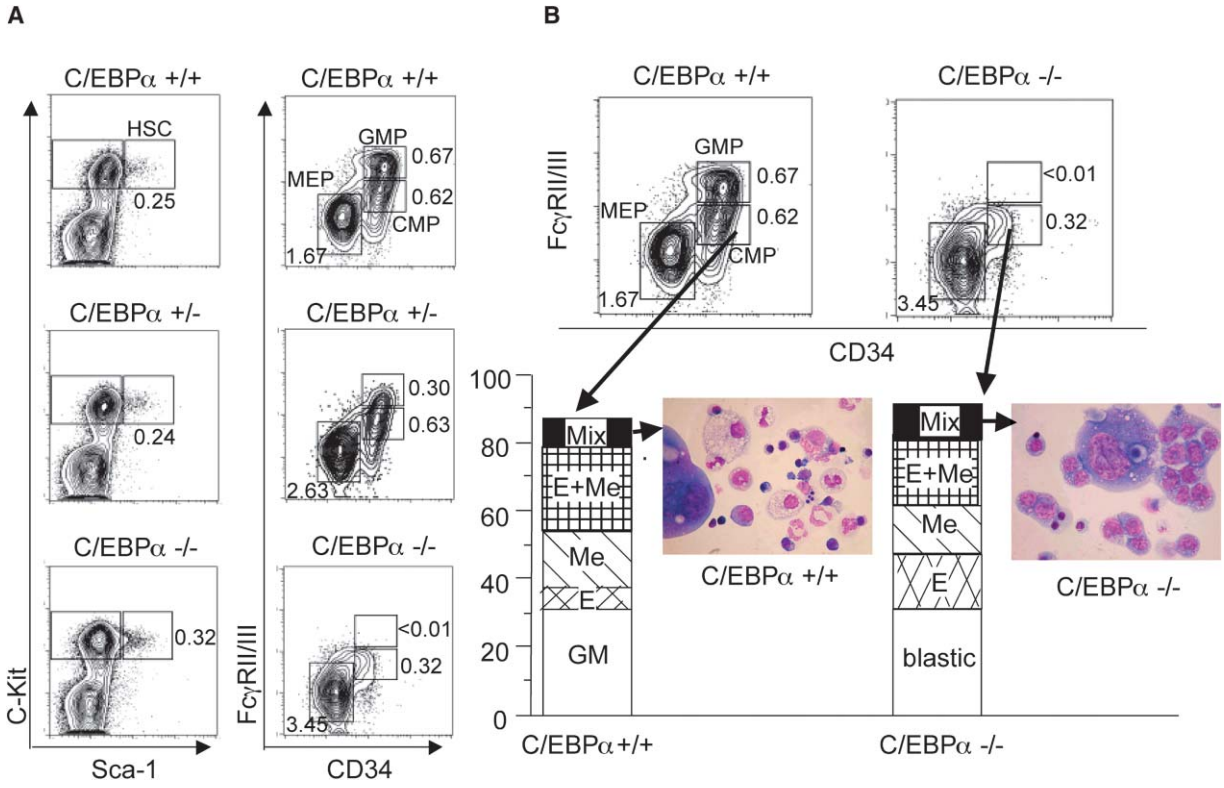

Figure 2. Disruption of the C/EBPα Gene in Fetal Liver Blocks the Transition from CMP to GMP

$Lin^-IL\text{-}7R\alpha^-Sca\text{-}1^-c\text{-}Kit^+$ cells isolated by multicolor flow cytometry on the left (A) were further analyzed with CD34 and FcγRII/III on the right (B). A similar pattern was observed in 10 different C/EBP$\alpha^{-/-}$ mice. Numbers in the figures indicate the percentage of total fetal liver hematopoietic cells.

(B) CMPs derived from C/EBP$\alpha^{-/-}$ fetal livers differentiated into multiple lineages in vitro in CFU assays. 96 individual CMPs from C/EBP$\alpha^{+/+}$ wt and C/EBP$\alpha^{-/-}$ mice were sorted and distributed one cell per well into 96-well plates containing methylcellulose. The bar graph demonstrates the numbers of each type of colony observed in the plate. Shown to the right of each bar graph is a Wright-Giemsa stained CFU-mix colony, showing mature granulocytic and monocytic cells derived from C/EBP$\alpha^{+/+}$ fetal livers not observed in those derived from C/EBP$\alpha^{-/-}$ embryos. Mix colonies from C/EBP$\alpha^{-/-}$ CMP are defined as those including normal appearing megakaryocytes and erythroid cells and immature myeloid blasts.

C/EBPα deficient fetal liver and adult bone marrow do possess multilineage differentiation potential but are incapable of differentiating to form myeloid CFU or granulocytic cells. In conclusion, disruption of the C/EBPα gene blocks the transition from CMP to GMP in vivo and the granulocytic maturation of CMPs in vitro.

C/EBPα Is Not Required for Terminal Granulocytic Maturation beyond the GMP Stage

The experiments described above demonstrate that C/EBPα is required for the transition from CMP to GMP but did not address whether continued expression of C/EBPα beyond the GMP stage is required for granulocytic maturation. We isolated GMP from C/EBP$\alpha^{F/F}$ mice and transduced them with a retrovirus expressing Cre recombinase and EGFP. Polymerase chain reaction (PCR) analysis of genomic DNA revealed excision only in the $EGFP^+$ (Cre+) population (Figure 3A), demonstrating that the absence or presence EGFP was an accurate marker of floxed or excised alleles, respectively. We then asked whether deletion of C/EBPα in the GMP population would affect myeloid colony formation in vitro. The distribution of GM, G, and M colonies derived from Cre^+ (C/EBP$\alpha^{\Delta/\Delta}$) GMP was similar to that of Cre^- (C/EBP$\alpha^{F/F}$) GMP (Figure 3B), and the cells in these colonies were just as differentiated (Figures 3C and 3D). Therefore, C/EBPα is required for differentiation of CMP to GMP. However, once GMP have been produced, C/EBPα is no longer required for maturation to more differentiated myeloid cells (granulocytes and monocytes), indicating a role early, but not late, in granulocytic differentiation.

C/EBPα Deficiency Leads to Augmentation of Hematopoietic Stem Cell Function

We have shown that the C/EBP$\alpha^{-/-}$ fetal liver hematopoietic cells that reconstituted lethally irradiated recipients could differentiate into mature T and B cells, but not mature granulocytes (Zhang et al., 1997). However, these experiments were not definitive because they utilized total fetal liver cells and not purified progenitor populations as donors, and the recipient mice did not survive beyond 8 weeks. Therefore, in order to determine whether the specific differentiation block from

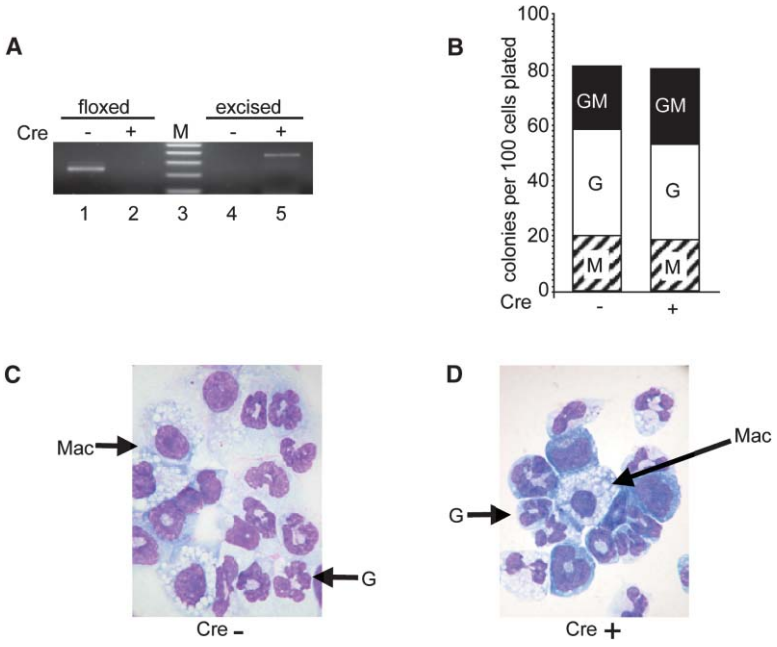

Figure 3. C/EBPα Is Not Required after the GMP Stage of Myeloid Differentiation

(A) Complete excision of both C/EBPα$^{F/F}$ alleles after retroviral transduction of Cre recombinase. GMP isolated from C/EBPα$^{F/F}$ mice were transduced with Cre-IRES-EGFP retrovirus, sorted as EGFP^{-} (Cre^{-}, lanes 1 and 4) and EGFP^{+} (Cre^{+}, lanes 2 and 5) and then assessed for presence of the C/EBPα^{F} (floxed, lanes 1 and 2) or C/EBPα$^{\Delta}$ (excised, lanes 4 and 5) allele. Lane 3, DNA size markers (M).

(B) Myeloid colony formation in unexcised (Cre^{-}) and excised (Cre^{+}) C/EBPα$^{F/F}$ GMP. The chart indicates the number of CFU-GM, CFU-G, and CFU-M per 100 GMP plated.

(C and D) Morphology of cells derived from Cre^{-} (C) and Cre^{+} (D) CFU-GM; note the presence of both macrophages (Mac) and granulocytes (G).

CMP to GMP stages in C/EBPα-deficient mice is cell autonomous, we transplanted 200 total fetal liver HSCs consisting of various ratios of Ly5.2^{+} C/EBPα$^{-/-}$ or C/EBPα$^{+/+}$ and Ly5.1^{+} C/EBPα$^{+/+}$ HSCs into lethally irradiated Ly5.1^{+} C57BL/6 congenic mice. Surprisingly, C/EBPα$^{-/-}$ HSCs were able to out compete C/EBPα$^{+/+}$ HSCs. As shown in Figure 4A, left, the number of Ly5.2 (donor) cells increased steadily over time, even when four times as many wt HSCs were utilized. All of the Ly5.2^{+} cells in the recipients receiving C/EBPα$^{-/-}$ HSCs were CD3^{+} or B220^{+} lymphoid cells, and no Ly5.2^{+} granulocytes (Gr-1^{+}/Mac-1^{+}) could be detected (data not shown). Most of the HSCs in those recipients with a high degree of peripheral blood chimerism were derived from C/EBPα$^{-/-}$ HSC, as assessed by both phenotypic analysis (Figure 4B) or the ability to repopulate the hematopoietic system in secondary (Figure 4A, right) and tertiary transplantation experiments (data not shown). Analysis of the bone marrow in the primary recipients also confirmed that C/EBPα$^{-/-}$ fetal liver HSC conferred the same distinct block in differentiation at the CMP to GMP stage and that a proportional number of HSCs, CMPs, and MEPs, but not GMPs, were derived from the Ly5.2^{+} C/EBPα$^{-/-}$ fetal liver HSC (Figure 4B). In addition, B220^{+} B cells, but not Mac-1^{+} myeloid cells, were derived from C/EBPα$^{-/-}$ fetal liver HSCs. Therefore, loss of C/EBPα appeared to augment competitive repopulation activity of HSCs.

We sought to determine the mechanism through which loss of C/EBPα led to this effect. C/EBPα has been demonstrated to inhibit the cell cycle through multiple mechanisms (McKnight, 2001). However, BrdU labeling experiments failed to demonstrate any differences in cell cycle status between HSC derived from C/EBPα$^{+/+}$, C/EBPα$^{+/-}$, and C/EBPα$^{-/-}$ mice (data not shown), making effects on cell cycle an unlikely reason for the enhanced HSC self-renewal. Because increased expression of the homeobox gene HoxB4 has been demonstrated to lead to an expansion in HSC (Sauvageau et al., 1995), we measured HoxB4 RNA levels through quantitative real-time PCR. Again, no differences could be detected among HSC derived from C/EBPα$^{+/+}$, C/EBPα$^{+/-}$, and C/EBPα$^{-/-}$ mice (Figure 4C, bottom). A final candidate gene that we investigated was the polycomb gene Bmi-1. Loss of Bmi-1 leads to a profound defect in HSC self-renewal (Park et al., 2003; Lessard and Sauvageau, 2003). Furthermore, increased expression of Bmi-1 leads to increased self-renewal and marked enhancement of HSC repopulating activity in vivo (Iwama et al, 2004 [this issue of *Immunity*]). In contrast to what we observed with HoxB4, Bmi-1 RNA was increased an average of 3.5-fold in HSC derived from C/EBPα$^{-/-}$ mice compared to those purified from C/EBPα$^{+/-}$ (Figure 4C, top). Therefore, the enhancement of HSC repopulating activity in C/EBPα$^{-/-}$ HSC was accompanied by a significant increase in expression of Bmi-1.

We also asked whether HSC derived from adult bone marrow deficient in C/EBPα could confer the same phenotype after transplantation into congenic recipient mice by transplanting mixtures of Ly5.2^{+} Mx1-Cre × C/EBPα$^{F/F}$ and Ly5.1^{+} C/EBPα$^{+/+}$ bone marrow cells, establishing chimerism after transplantation, and then inducing deletion of the C/EBPα gene by administration of poly I:C 2.5 months after transplantation. Southern blot analysis demonstrated that we could achieve >90% deletion of C/EBPα after multiple injections of poly I:C in 3-month-old Mx1-Cre^{+} × C/EBPα$^{F/F}$ mice (data not shown), similar to that after newborn injection (Figure 1). As shown in Figure 5A, analysis of the bone marrow of these mice revealed that a block at the CMP to GMP transition could be detected in bone marrow precursors arising from Ly5.2^{+} donor C/EBPα$^{\Delta/\Delta}$ bone marrow cells after long term transplantation. Furthermore, analysis of

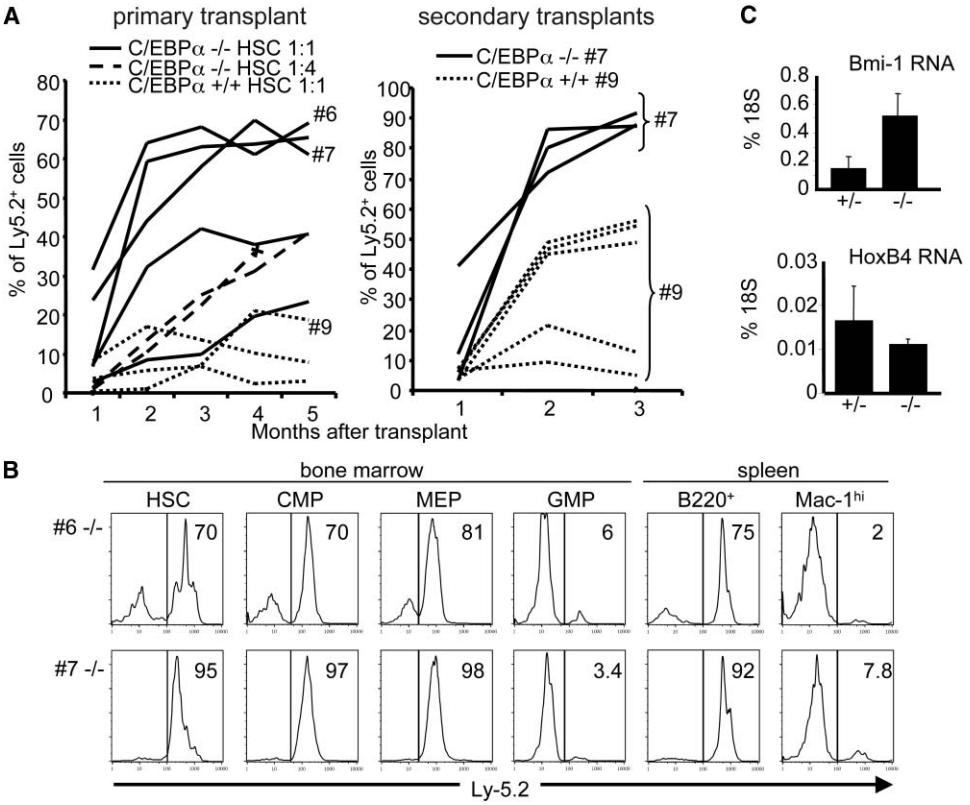

Figure 4. C/EBPα$^{-/-}$ Fetal Liver Hematopoietic Stem Cells Have a Competitive Advantage over Wt

(A) Left, fetal liver HSCs consisting of Ly5.2$^+$ C/EBPα$^{-/-}$ and Ly5.1$^+$ C/EBPα$^{+/+}$ at ratios of 1:1 and 1:4, or Ly5.2$^+$ C/EBPα$^{+/+}$ and Ly5.1$^+$ C/EBPα$^{+/+}$ at a ratio of 1:1 were transplanted into lethally irradiated Ly5.1$^+$ congenic recipients, and the percentage of donor Ly5.2$^+$ cells in peripheral blood is plotted as a function of time after transplantation. A total of 200 fetal liver HSCs were transplanted into each recipient. Solid lines denote Ly5.2$^+$ C/EBPα$^{-/-}$: Ly5.1$^+$ C/EBPα$^{+/+}$ HSCs at a ratio of 1:1 (100 Ly5.2$^+$ C/EBPα$^{-/-}$ plus 100 Ly5.1$^+$ C/EBPα$^{+/+}$ HSCs as donors) and the dashed lines 1:4. Dotted lines denote Ly5.2$^+$ C/EBPα$^{+/+}$: Ly5.1$^+$ C/EBPα$^{+/+}$ HSC at a ratio of 1:1 (100 Ly5.2$^+$ C/EBPα$^{+/+}$ plus 100 Ly5.1$^+$ C/EBPα$^{+/+}$ HSCs as donors). The cross indicates unexpected death of one of the recipients. #6, #7, and #9 refer to recipient mice shown in Figure 4B. Right, secondary transplantation. 2 × 10^6 total bone marrow cells from mouse #7 (Ly5.2$^+$ C/EBPα$^{-/-}$ and Ly5.1$^+$ C/EBPα$^{+/+}$ cells) and #9 (Ly5.2$^+$ C/EBPα$^{+/+}$ and Ly5.1$^+$ C/EBPα$^{+/+}$ cells) were transplanted into Ly5.1$^+$ C/EBPα$^{+/+}$ recipients. The graph demonstrates the percentage of Ly5.2$^+$ peripheral blood cells in three different recipient mice for donor #7 and five for donor #9.

(B) Relative numbers of HSC, progenitors, and B220$^+$ B cells and Mac-1^{hi} myeloid cells in bone marrow and spleen of recipient mice #6 and #7 from primary transplantation experiments (Figure 4A, left). All populations are mainly derived from C/EBPα$^{-/-}$ Ly5.2$^+$ donor cells with the exception of GMP and Mac-1^{hi} cells representing granulocytes and their precursors.

(C) Bmi-1 RNA is increased in C/EBPα$^{-/-}$ HSC. Bmi-1 (top) and HoxB4 (bottom) RNA was determined by quantitative real-time PCR and shown as percentage of 18S RNA. Data for this figure are derived from two embryonic day 15.5 C/EBPα$^{-/-}$ and C/EBPα$^{+/-}$ fetal livers, respectively. Similar data was obtained by using pooled RNA derived from four knockout and heterozygote mice. Bmi-1 RNA was increased 3.5-fold in C/EBPα$^{-/-}$ versus C/EBPα$^{+/-}$ HSCs.

bone marrow and peripheral blood of long-term recipient mice indicated that C/EBPα$^{\Delta/\Delta}$ bone marrow could contribute to production of HSC, CMP, MEP, and peripheral blood B220$^+$ B cells, but not GMP or peripheral blood Mac-1$^+$ granulocytic cells (data not shown). These results demonstrate that persistent block in granulocytic differentiation at the CMP to GMP transition is indeed a cell autologous effect.

C/EBPα Deficiency in Adult Mice Leads to Persistence of Immature Myeloid Blasts without the Development of Malignant AML

Because disruption of C/EBPα leads to a block in myeloid maturation, increased stem cell repopulating function, and accumulation of immature myeloid blasts in the bone marrow, we asked whether loss of C/EBPα would lead to development of AML. Three weeks after injection of poly I:C at birth, C/EBPα$^{\Delta/\Delta}$ animals demonstrated a phenotype with some characteristics resembling AML, including no mature peripheral blood granulocytes (Table 1 and Supplemental Table S1) and the presence of >30% immature myeloid cells in the bone marrow (Figure 5B and Table 2), but without anemia or thrombocytopenia (Table 1). Most of these blasts have phenotypic markers of CMP (Supplemental Figure S1). We therefore asked the question of whether this hematopoietic state would be transient, whether it would persist, or alternatively whether it would progress to include other characteristics of leukemia. We observed that the animals rarely survived beyond 4 to 5 weeks of age due to sepsis as a result of granulocytopenia (Table 1). To date, 8 C/EBPα$^{\Delta/\Delta}$ mice have been followed for 2 to 5 months. One animal appeared to succumb from disseminated intravascular coagulation and likely had sep-

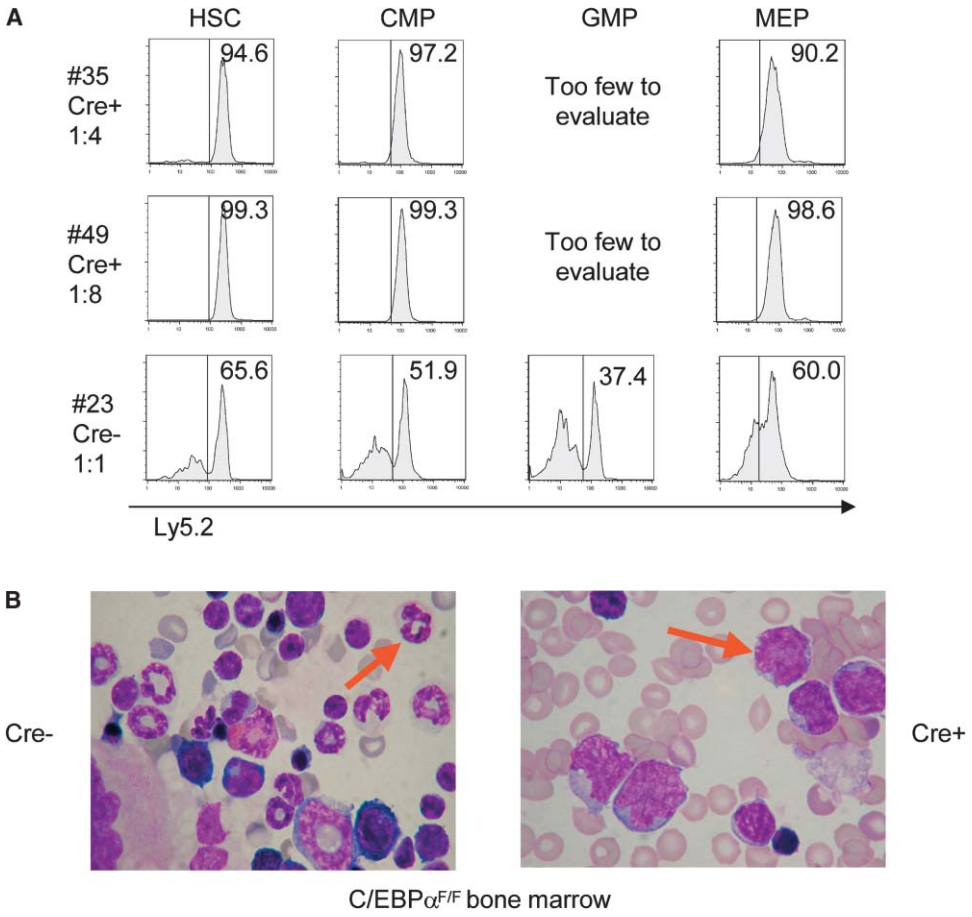

Figure 5. Excess Blasts Persist in C/EBPα$^{\Delta/\Delta}$ Bone Marrow without Transition to Myeloid Leukemia

(A) The differentiation block in C/EBPα deficient adult bone marrow is cell autonomous. Different mixtures derived from Ly5.2^{+} C/EBPα$^{F/F}$ (#23 Cre^{-}) or Ly5.2^{+} Mx1-Cre × C/EBPα $^{F/F}$ (#35 and #49) and Ly5.1^{+} C/EBPα$^{+/+}$ bone marrow cells (total 4 × 10^{5} cells) were used as donor cells and transplanted into lethally irradiated Ly5.1^{+} congenic recipients. 1:4, 1:8, and 1:1 indicate the ratios of Ly5.2^{+} to Ly5.1^{+} donor cells. 2.5 months after transplantation, poly I:C was administered and the mice were analyzed 6 months later, a total of 8.5 months after transplantation. The figure demonstrates donor Ly5.2^{+} HSCs, CMPs, GMPs, or MEPs as a percentage of the total of each population. There were ≤1% Ly5.2+ GMPs in Cre+ mice.

(B) Wright-Giemsa staining of bone marrow from 6-week-old Mx1-Cre^{-} × C/EBPα$^{F/F}$ (left) and C/EBPα$^{\Delta/\Delta}$ mice (right) after poly I:C induction 2 days after birth. Note mature neutrophils in the left (arrow) and their absence in the right and the presence of myeloid blasts (right, arrow). Similar results were observed in over 20 C/EBPα$^{\Delta/\Delta}$ mice. Poly I:C-treated wt and Mx1-Cre^{-} mouse bone marrow demonstrated morphology similar to that shown on the left.

sis. Another animal was necropsied shortly after death and had an elevated peripheral blast count (>60%), but based on peripheral blood smear did not appear anemic or thrombocytopenic, and a third animal had 8% immature blasts in the peripheral blood. The remaining five mice demonstrated a persistence of the same hematologic picture seen at 3 weeks without development of other characteristics of leukemia. This state was characterized by excess blasts in the bone marrow and absence of peripheral blood granulocytes, but normal hematocrit, hemoglobin, and platelet count, and lack of malignant infiltration of other tissues. Furthermore, we have not detected progression to leukemia after long-term (>9 months) monitoring of chimeric mice generated by transplantation of adult bone marrow cells lacking C/EBPα (Figure 5A). In summary, at least over this time period, this AML-like state consisting of an excess number of myeloid blasts persists but does not commonly progress to a true myeloid leukemia. The persistence of excess C/EBPα$^{-/-}$ blasts in the bone marrow of mice lacking C/EBPα supports the notion that loss of C/EBPα leads to an increase in HSC self-renewal compared to that of wt HSC.

Discussion

We previously described that granulocytic differentiation is blocked along with an accumulation of immature myeloid blasts in nonconditional C/EBPα$^{-/-}$ fetal liver hematopoiesis (Zhang et al., 1997). To study the role of C/EBPα during adult hematopoiesis, we utilized the Mx1-Cre strain of mice. We, and others, (Higuchi et al., 2002) have not observed abnormalities in myelopoiesis in Mx1-Cre mice with the dosage of poly I:C utilized in these studies. In addition, the effects observed in double Mx1-Cre^{+} × C/EBPα$^{F/F}$ mice induced with poly I:C are

similar in many respects to those observed in the fetal liver of nonconditional C/EBP$\alpha^{-/-}$ mice and are transplantable (Figure 5). Therefore, we believe that hematopoietic effects are specifically due to loss of the C/EBPα gene.

C/EBPα Function in Myeloid Progenitors

In the present study, by using loss of function studies, we have demonstrated that C/EBPα is not required for commitment of HSCs to CMPs but has a very specific role in the transition of the CMP to the GMP stage. In addition, the enforced expression of C/EBPα in CLPs induces granulocyte/monocyte development, indicating that C/EBPα can activate silenced myeloid programs to convert CLPs into GMPs (H.I. and K.A., unpublished data). These studies establish that C/EBPα is an instructive and indispensable regulator of GMP commitment. However, once myeloid maturation has reached the GMP stage, C/EBPα is no longer required (Figure 3). This observation is consistent with the findings that upon induction of myeloid differentiation, C/EBPα DNA binding activity is rapidly replaced by that of other C/EBP family members, such as C/EBPβ and/or C/EBPϵ (H.S. Radomska and D.G.T., unpublished data); that C/EBPβ can rescue the granulocytic defect in C/EBP$\alpha^{-/-}$ mice (Jones et al., 2002); and that C/EBPβ and C/EBPϵ can induce myeloid maturation in cell line models (Park et al., 1999; Nakajima and Ihle, 2001; Duprez et al., 2003) and in vivo (Truong et al., 2003). An alternative is that once GMP develop, further myeloid maturation can proceed in the absence of C/EBPα due to the actions of cytokines such as IL-3 and GM-CSF (Zhang et al., 1998, 2002). Further experiments utilizing compound C/EBP family member knockouts will help distinguish these possibilities. Interestingly, GMP lacking PU.1 are unable to differentiate under the identical conditions (H.I., C. Somoza, H. Shigematsu, E.A. Duprez, J.I.-A., S.-i. Mizuno, K. Geary, P.Z., T. Dayaram, M. Fenyus, S. Chan, C.S.H., R. Murray, D.G.T., and K.A., unpublished data), suggesting that whatever synergy (Petrovick et al., 1998) or antagonism (Reddy et al., 2002; Dahl et al., 2003) these two transcription factors demonstrate in myeloid differentiation PU.1, and not C/EBPα, is required after the GMP stage.

An Additional Function of C/EBPα in Hematopoietic Stem Cells

Surprisingly, loss of C/EBPα led to an effect on stem cell function. CEBPα is expressed at low levels in HSCs and is upregulated in CMPs and GMPs (Akashi et al., 2000; Traver et al., 2001). By using more precise real-time RT-PCR methods, we have found that C/EBPα is expressed at about 0.1% of 18S ribosomal RNA levels in HSC, increases 2-fold during the transition from HSC to CMP, subsequently increases 10-fold as CMPs differentiate to GMP, and decreases 10-fold as CMPs differentiate into MEPs (P.Z. and E.L., unpublished data). Our results demonstrate the importance of the transcription factor C/EBPα in HSCs; relatively very low levels of expression are important in HSC functions such as competitive reconstitution activity. Furthermore, we suggest a possible mechanism for the enhanced HSC self-renewal observed in C/EBP$\alpha^{-/-}$ mice: increased expression of Bmi-1. In the accompanying manuscript, Iwama et al. (2004) demonstrate that increased expression of Bmi-1 in HSC leads to marked enhancement of HSC repopulating activity in vivo, a phenotype remarkably similar to what we observe in C/EBP$\alpha^{-/-}$ HSC. A somewhat surprising finding was that we could not detect differences in cell cycle status of HSC derived from C/EBP$\alpha^{-/-}$ mice, but Iwama et al. (2004) also reported that disruption of Bmi-1 did not affect HSC cell cycle, supporting the idea that upregulation of Bmi-1 could be the basis of augmented repopulating ability of C/EBP$\alpha^{-/-}$ HSC. Further experiments testing HSC derived from mice with targeted disruption of both C/EBPα and Bmi-1 will be required to confirm this hypothesis. Strikingly, Bmi-1 expression in HSC and progenitors (Iwama et al., 2004) is reciprocal to that of C/EBPα. Bmi-1 is expressed in HSC and multipotential progenitors, maintained in lymphocytes, and markedly downregulated in Gr-1$^+$ myeloid cells, whereas the expression of C/EBPα is upregulated as HSCs mature to GMPs, expressed at highest levels in Gr-1$^+$ granulocytes (Pabst et al., 2001a), and not expressed in either early progenitors or mature cells from the lymphoid lineage (Akashi et al., 2000; Miyamoto et al., 2002). Therefore, future experiments will be directed at determining whether Bmi-1 is a direct target of C/EBPα and that one of the normal functions of C/EBPα is to inhibit HSC self-renewal in myeloid progenitors by downregulating Bmi-1.

Furthermore, the role of C/EBPα in HSCs appears to be quite distinct from its function to induce CMPs to differentiate into GMPs. In HSC, C/EBPα plays a role in self-renewal and repopulating activity, whereas, in contrast, in the transition from CMPs to GMPs, the function of C/EBPα appears to be to promote myeloid differentiation. As a transcription factor, C/EBPα has a crucial role in regulating the balance between cell proliferation and differentiation (McKnight, 2001; Johansen et al., 2001; Porse et al., 2001). Taken together, these results suggest that loss of C/EBPα leads to not only a block in myeloid cell differentiation but may selectively promote proliferation. Whereas our data suggests that loss of C/EBPα does not measurably affect the cell cycle of HSC, a recent report demonstrated increased proliferation of C/EBP$\alpha^{-/-}$ progenitors in vitro (Heath et al., 2004). Therefore, it is possible that these discrete functions of C/EBPα in HSCs compared to progenitors may be due to differences in effects on cell cycle.

Loss in peripheral blood granulocytes and increase in blasts in the bone marrow persisted for at least 5 months in C/EBP$\alpha^{\Delta/\Delta}$ mice that were able to avoid infections and survive for this period of time. This is quite distinct from our results in Mx1-Cre$^+$ $\times$ PU.1$^{F/F}$ animals, in which PU.1-deficient hematopoiesis could be maintained for only a few weeks (H.I., C. Somoza, H. Shigematsu, E.A. Duprez, J.I.-A., S.-i. Mizuno, K. Geary, P.Z., T. Dayaram, M. Fenyus, S. Chan, C.S.H., R. Murray, D.G.T., and K.A., unpublished data). Assuming deletion of C/EBPα and PU.1 are equally efficient in HSC in the two strains, these results suggest that loss of C/EBPα in the stem cell provides a selective advantage over wt HSC, whereas loss of PU.1 results in defective HSC function, compatible with previous reports utilizing progenitor populations (Scott et al., 1997). PU.1-deficient fetal liver also exhibited a differentiation block from

HSCs to CMPs as well as CLPs (H.I., C. Somoza, H. Shigematsu, E.A. Duprez, J.I.-A., S.-i. Mizuno, K. Geary, P.Z., T. Dayaram, M. Fenyus, S. Chan, C.S.H., R. Murray, D.G.T., and K.A., unpublished data). Thus, although both C/EBPα and PU.1 are critical for differentiation of myeloid cells at their progenitor stages, the loss of C/EBPα and PU.1 in HSCs resulted in opposite effects on their repopulating activity and showed that they both play distinct roles in HSCs and CMPs. One possible mechanism leading to the marked enhancement in C/EBPα$^{-/-}$ and not PU.1$^{-/-}$ HSC is due to selective upregulation of Bmi-1. This hypothesis is strengthened by the finding that whereas Bmi-1 is upregulated in C/EBPα$^{-/-}$ HSC (Figure 4C), we cannot detect any differences in Bmi-1 RNA comparing PU.1$^{-/-}$ and PU.1$^{+/+}$ HSC by real-time quantitative PCR assays (H.I., B.M.O., and D.G.T., unpublished data).

Relevance of These Findings to AML

Given the importance of loss of C/EBPα function in human myeloid leukemias (Tenen, 2003), we asked whether loss of C/EBPα function in the adult would lead to the development of leukemia. Whereas C/EBPα induced differentiation block, and enhanced HSC repopulating activity may cooperate to induce a persistent accumulation of myeloblasts in the bone marrow (Figure 5), these effects are not sufficient for development of AML. Whereas it is possible that the anemia and thromobocytopenia characteristic of human AML is not frequently observed in murine models due to utilization of the spleen as a hematopoietic organ, we did not observe malignant infiltration of other tissues. Furthermore, we did not observe the development of frank leukemia in mice that had been transplanted with either C/EBPα$^{-/-}$ fetal liver HSC (Figure 4) or adult C/EBPα$^{\Delta/\Delta}$ bone marrow (Figure 5), in which the animals have survived for over 1 year in the presence of neutrophils contributed by cotransplanted Ly5.1^{+} C/EBPα$^{+/+}$ donor HSC. Thus, although we cannot exclude the possibility of development of leukemia beyond this observation period (up to 1 year), our results suggest that additional genetic events will be required for final leukemic transformation. Accordingly, by crossing conditionally targeted C/EBPα mice to other strains with specific gene disruptions, as well as insertional mutagenesis, we are attempting to identify the specific genetic pathways resulting in leukemic transformation.

Experimental Procedures

C/EBPα Nonconditional Knockout Mice

C/EBPα nonconditional +/− mice were bred and genotyped by Southern blot analysis as previously described (Zhang et al., 1997). Mice utilized in these studies were backcrossed at least six generations in a purebred C57BL/6 background. No difference in C/EBPα$^{-/-}$ fetal liver hematopoiesis was noted compared to the 129 × C57BL/6 background used in our previous studies (Zhang et al., 1997).

Generation of Loxp-C/EBPα Mice

The 5′ homologous genomic region in the targeting vector was a 7 kb EcoRI genomic fragment encompassing the single C/EBPα exon and extending 3.3 kb upstream. A NotI-loxP-NotI linker (38 bp) was inserted into a NotI site located 600 bp upstream of the C/EBPα exon. The 3′ homologous region was isolated from a 1.6 kb 3′ C/EBPα genomic fragment and subsequently ligated into the pPNTloxP targeting vector backbone (Kimura et al., 2002) directly after the loxP site. The targeting plasmid was linearized with NotI and introduced into TC1 ES. Genomic DNA was digested with BamHI and NotI and hybridized to an 875 bp Pst I-Xba I fragment derived from the 7 kb EcoRI 5′ C/EBPα genomic fragment described above. The targeted allele generated a 10.9 kb band, whereas the wt allele generated a 5 kb band. Selected ES cell clones were injected into C57BL/6 blastocysts, implanted into (CBA × C57BL/6)F1 foster mothers, and propagated as described (Yamanaka et al., 1997).

Genotyping Mx1-Cre and C/EBPα$^{F/+}$ Mice

Mx1-Cre mice (kindly provided by Klaus Rajewsky) (Kuhn et al., 1995) were genotyped by PCR yielding a 300 bp product with primers 5′-ATGTTCAATTTACTGACCG-3′ (sense primer) and 5′-CGC CGCATAACCAGTGAAAC-3′ (antisense primer). C/EBPα$^{F/+}$ mice were genotyped using 5′-TGGCCTGGAGACGCAATGA-3′ (sense primer) and 5′-CGCAGAGATTGTGCGTCTTT-3′ (antisense primer), which gave rise to a 269 bp band for the loxP targeted allele and a 235 bp band for the wt allele, respectively (Figure 1A). To analyze excision efficiency, genomic DNAs were digested with BamHI and probed with the 875 bp PstI-XbaI fragment described above (Figure 1A). The excised allele generated a 4.7 kb band whereas the nonexcised but targeted allele generated a 10.9 kb band. To induce excision of C/EBPα$^{F/F}$, day 2 newborn mice were given a single intraperitoneal injection of 500 μg poly I:C in a volume of 50 μl of PBS, or alternatively adult mice received 450 μg per injection every other day for a total of seven injections (Mikkola et al., 2003).

Western Blot Analysis

Single cell suspensions were harvested from liver and bone marrow 1 month after poly I:C induction, lysed with modified RIPA buffer, and 50 μg whole-cell lysate separated on 10% SDS-PAGE gels. Immunoblots were stained with rabbit polyclonal anti-C/EBPα antibody (Santa Cruz), stripped, and then stained with monoclonal anti-murine β-actin antibody (Sigma).

Flow Cytometry and Cell Sorting

HSC and progenitors were isolated from fetal liver cells and mouse bone marrow cells as described previously (Akashi et al., 2000; Kondo et al., 1997). For analysis of peripheral blood, white blood cells were separated from red blood cells through dextran sedimentation followed by lysis of residual red blood cells and stained with anti-CD45 (Ly5.2) and lineage specific markers. For single-cell clonogenic assays, cells were deposited into 96-well plates after a second round of sorting as described (Akashi et al., 2000).

Clonogenic Assay to Determine Differentiation Potential of Fetal Liver Progenitors

Myeloid progenitors were cultured in Methocult H4100 methylcellulose media (Stemcell Technologies) with cytokines including murine SCF, IL-3, IL-11, TPO, and eythropoietin. Single cells were deposited into 96-well plates as described above. Colonies were enumerated under an inverted microscope consecutively from days 4–7.

Induction of Disruption of C/EBPα$^{F/F}$ GMP by Transduction with a Retrovirus Expressing Cre Recombinase

To induce a disruption of C/EBPα at the GMP stage, we used an MSCV based retrovirus encoding the Cre recombinase followed by an IRES and EGFP. GMPs were isolated from C/EBPα$^{F/F}$ bone marrow and cultured with virus supernatant for 48 hr in the presence of SCF and IL-11. Average transduction efficiency of GMP was 30%. After transduction, cells were isolated into EGFP^{+} and EGFP^{-} populations and assessed for recombination by PCR using the set described for genotyping C/EBPα$^{F/+}$ mice (see sequences above) for the C/EBPα^{F} allele and 5′-GCCTGGTAAGCCTAGCAATCCT-3′ and 5′-TGGAAACTTGGGTTGGGTGT-3′ for the C/EBPα$^{\Delta}$ allele.

Transplantation Experiments

For transplantation of total fetal liver hematopoietic cells (Table 2), 6- to 8-week-old recipients (Ly5.1 C57BL/6) were irradiated with 550 rads followed by a 4 hr interval and then a second dose of 400 rads of γ irradiation from a ^{137}Cs source. 2 × 10^{6} total fetal liver

hematopoietic cells from day 14.5 gestation Ly5.2$^+$ C57BL/6 C/EBP$\alpha^{+/+}$ or C/EBP$\alpha^{-/-}$ mice were used as donor cells. Reconstitution by donor cells was detected by staining peripheral white blood cells with anti-CD45.2 (Ly5.2) antibody. Progenitors populations were analyzed 6 to 9 months after transplantation.

For transplantation of purified fetal liver HSC, KSL fetal liver cells from day 14.5 gestation Ly5.2$^+$ C/EBP$\alpha^{-/-}$ or Ly5.2$^+$ C/EBP$\alpha^{+/+}$ embryos and from Ly5.1$^+$ C/EBP$\alpha^{+/+}$ mice were isolated and a total of 200 HSCs were injected by tail vein injection into each irradiated Ly5.1 recipient. Donor origin cells were detected by flow cytometry analyses performed every 2 weeks between 2 and 6 months after transplantation. For secondary transplantation, 2×10^6 total bone marrow cells were harvested from mice 7.5 months after primary transplantation and injected into Ly5.1+ C57BL/6 mice irradiated with a total dose of 950 rads as described above. The percentage of donor-derived cells was measured in peripheral blood as described.

For transplantation of adult total bone marrow cells (Figure 5), Ly5.1$^+$ recipients were irradiated with a total of 950 rads. 2×10^5 total bone marrow cells were harvested from Ly5.2$^+$ Mx1-Cre $\times$ C/EBP$\alpha^{F/F}$ or C/EBP$\alpha^{F/F}$ mice and mixed with Ly5.1$^+$ C57BL/6 bone marrow cells in the ratios indicated in Figure 5A. Reconstitution of Ly5.2$^+$ cells was monitored at 1 and 2.5 months after transplantation, followed by administration of poly I:C (450 μg/mouse every other day for a total of seven injections) 2.5 months after transplant. Recipient mice were sacrificed and donor derived bone marrow progenitor populations analyzed 6 months after poly I:C administration.

Quantitative Real-Time PCR Assay of HoxB4 and Bmi-1 RNA in C/EBP$\alpha^{-/-}$ HSC

2000 to 5000 KSL HSCs were isolated by FACS and then resorted directly into 500 μl trireagent. RNA was reverse transcribed and subsequently amplified with an AbiPrism 7700 Sequence Detector by the following parameters: 48°C (30 min), 95°C (10 min), followed by 40 cycles of 95°C (15 s), and 60°C (60 s). For Bmi-1, the forward primer was 5′-CCAGCAAGTATTGTCCTATTTGTGA-3′, the reverse primer was 5′-ATATCTTGAAGAGTTTTATCTGACCTTATGTT-3′, and the probe was 5′-FAM-TCCAGGTTCACAAAACCAGACCACTCCT-TAMRA-3′. For measurement of HoxB4 RNA, the forward primer was 5′-CCTGGATGCGCAAAGTTCA-3′, the reverse primer was 5′-CGTCAGGTAGCGGTTGTAGTGA-3′, and the probe was 5′-FAM-TGAGCACGGTAAACCCCAATTACGCC-TAMRA-3′.

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