

Upregulation of Interleukin 6 and Granulocyte Colony-Stimulating Factor Receptors by Transcription Factor CCAAT Enhancer Binding Protein α (C/EBP α) Is Critical for Granulopoiesis

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Summary

Cytokines stimulate granulopoiesis through signaling via receptors whose expression is controlled by lineage-specific transcription factors. Previously, we demonstrated that granulocyte colony-stimulating factor (G-CSF) receptor mRNA was undetectable and granulocyte maturation blocked in CCAAT enhancer binding protein α (C/EBP α)-deficient mice. This phenotype is distinct from that of G-CSF receptor^{-/-} mice, suggesting that other genes are likely to be adversely affected by loss of C/EBP α . Here we demonstrate loss of interleukin 6 (IL-6) receptor and IL-6-responsive colony-forming units (CFU-IL6) in C/EBP α ^{-/-} mice. The observed failure of granulopoiesis could be rescued by the addition of soluble IL-6 receptor and IL-6 or by retroviral transduction of G-CSF receptors, demonstrating that loss of both of these receptors contributes to the absolute block in granulocyte maturation observed in C/EBP α -deficient hematopoietic cells. The results of these and other studies suggest that additional C/EBP α target genes, possibly other cytokine receptors, are also important for the block in granulocyte differentiation observed in vivo in C/EBP α -deficient mice.

Key words: CCAAT enhancer binding protein • knockout mice • colony-forming unit • hematopoiesis • myelopoiesis

Multipotential hematopoietic stem cells differentiate into all of the different lineages of blood cells. The processes of distinct lineage differentiation is regulated by distinct transcription factors such as GATA-1 for erythroid cells, PU.1 for myeloid and B cells, and CCAAT enhancer binding protein α (C/EBP α)¹ for granulocytes (1, 2). The molecular basis for how these factors regulate hematopoietic stem cell differentiation is still not clear. Interestingly, all of these factors can positively regulate the expression of lineage-specific growth factor receptors. For example, GATA-1 activates the erythropoietin receptor promoter (3), PU.1 is critical for M-CSF receptor promoter activity

(4), and C/EBP α is an important regulator of G-CSF receptor promoter function (5). Therefore, one possible mechanism of transcription factor regulation of lineage differentiation is through activation of cell type-specific cytokine receptors.

C/EBP α is a member of the C/EBP transcription factor family (6). Other members of this family include C/EBP β , or NF-IL6, which is critical for macrophage activation (7, 8), and C/EBP ϵ , which regulates granulocyte maturation at a very terminal stage (9). C/EBP α has been shown to regulate a number of hepatocyte and adipocyte genes (10, 11). Targeted deletion of the C/EBP α gene in mice disrupts normal glucose metabolism, and homozygous newborn animals die from hypoglycemia within 8 h of birth (12, 13). In the hematopoietic system, C/EBP α is expressed specifically in both human and murine myeloid cells (14). It upregulates several myeloid gene promoters, such as G-CSF receptor (5), myeloperoxidase (15, 15a), and neutrophil

¹Abbreviations used in this paper: C/EBP, CCAAT enhancer binding protein; CFU-GM, GM-CSF-responsive CFU; CFU-IL3 and -IL6, IL-3 and IL-6-responsive CFU, respectively; RT, reverse transcription; SCF, stem cell factor.

elastase (15a, 16). Recent studies have demonstrated that C/EBP α expression is selectively maintained during granulocytic differentiation, but is markedly downregulated with monocytic differentiation (17).

Previously, we reported that granulocyte maturation was completely blocked in C/EBP α -deficient mice, but T cell, B cell, and macrophage subsets were normal. Expression of G-CSF receptor mRNA was profoundly and selectively reduced, whereas levels of M-CSF receptor, GM-CSF receptor α , and IL-3 receptor α mRNA were all comparable to wild-type (18). No defects were observed in C/EBP α ^{+/-} mice. However, mice with a targeted disruption of the G-CSF receptor only have a quantitative defect in granulopoiesis; mature granulocyte are still detected at a level 12% of wild-type (19). These results suggest that in addition to G-CSF receptor, other genes were likely to be affected by loss of the C/EBP α gene.

Multiple cytokines are involved in the regulation of granulopoiesis, including G-CSF, GM-CSF, IL-3, and IL-6 (20, 21). Mice that have been treated with IL-6 have an increased number of peripheral neutrophils (22). Double transgenic mice which coexpress human IL-6 and soluble IL-6 receptor demonstrate dramatic increases of white blood cells consisting mainly of neutrophilic granulocytes compared with wild-type, single transgenic mice (23). Coadministration of recombinant human IL-6 and G-CSF to irradiation-induced myelosuppressed mice caused a synergistic increase in the GM-CSF-responsive CFU (CFU-GM) in peripheral blood and bone marrow (24). Loss of IL-6 in G-CSF receptor-deficient mice leads to a significant further reduction in the number of mature neutrophils in peripheral blood and bone marrow (25). All of these data indicate that IL-6 also regulates granulopoiesis, and that IL-6 and G-CSF may have a synergistic effect on myelopoiesis.

The biological effects of IL-6 are known to be mediated through their unique cell surface receptors (21). IL-6 receptors are expressed in many cell types, including plasmacytoma cells, macrophages, T cells, B cells, and pre-B cells (26). During embryogenesis, it is expressed constitutively at readily detectable levels that do not change significantly during in vitro embryoid body development and blastocyst development (27). In the hematopoietic system, the expression of IL-6 receptor rapidly decreases to undetectable levels during erythroid differentiation, and slowly decreases during granulocyte differentiation (28). IL-6 has been shown to bind to the IL-6 receptor with low affinity (29). However, the presence of gp130, a signal transducer not only for IL-6 but also for leukocyte inhibition factor (LIF), Oncostatin M, IL-11, and ciliary neurotrophic factor, together with IL-6 will result in high-affinity IL-6 binding and subsequent signal transduction (30). The cytoplasmic domain of the IL-6 receptor is apparently not required for high-affinity IL-6 binding and signal transduction, since the soluble form of the IL-6 receptor, when complexed to IL-6, can also trigger high-affinity IL-6 binding and signaling on target cells lacking IL-6 receptor but expressing gp130 (31). Interestingly, G-CSF receptor and gp130 share significant similarity within their cytoplasmic domains (21, 30).

Here, we demonstrate that the IL-6 receptor is markedly downregulated in C/EBP α ^{-/-} mice, whereas the expression level of gp130 in C/EBP α ^{-/-} mice is comparable with the level in wild-type mice. C/EBP α ^{-/-} progenitors do not respond to IL-6 alone in vitro, but a small number of precursors can differentiate to metamyelocytic granulocytes by the addition of soluble IL-6 receptor to the culture. Addition of G-CSF together with soluble IL-6 receptor and IL-6 induces formation of mature segmented granulocytes. C/EBP α ^{-/-} fetal liver hematopoietic cells also can be rescued in vitro by transduction of G-CSF receptors into the cells by using retroviral infection. These studies demonstrate that the IL-6 receptor is a second major target gene for C/EBP α . Restoration of expression of either receptor can restore granulopoiesis in vitro, demonstrating that they are important functional targets for C/EBP α .

Materials and Methods

Hematological Analysis. Human recombinant IL-6 (500 ng/ml), soluble IL-6 receptor (100 ng/ml), and G-CSF (1,000 U/ml), which are all active on murine cells, and murine recombinant IL-3 (300 U/ml) and GM-CSF (10 ng/ml) were used in the CFU assays. Methylcellulose culture of single cell suspensions of fetal liver cells was performed in IMDM medium containing 0.8% methylcellulose and either 30% heat-inactivated fetal bovine serum for IL-6 and G-CSF CFU assays, or 20% heat-inactivated serum for the GM-CSF and IL-3 CFU assays. 10% Chinese hamster ovary cell-conditioned medium from cells stably transfected with a stem cell factor (SCF) expression vector was used as a source of SCF, and 10% WEHI-3-conditioned medium was used as a source of IL-3 for liquid cultures. Colonies were counted at days 7–9. Two to four colonies were pooled and cytocentrifuged onto glass slides daily after day 7. The slides were stained with Wright-Giemsa (Diff-Quik; Baxter Healthcare Corp., Deerfield, IL) according to the manufacturer's protocol.

Northern Blot Analysis. Total RNA was isolated from mouse fetal liver, adult bone marrow, spleen, thymus, and peritoneal cells 48 h after thioglycollate stimulation, at which time 90% of the cells are macrophages. Total RNA was purified by guanidine isothiocyanate extraction and cesium chloride gradient. Poly A⁺ RNA was purified by oligo-(dT) chromatography. Northern blot analysis was performed as described previously (32). The probe used was a 1.6-kb SacI fragment of murine IL-6 receptor cDNA (26).

Quantitative PCR. Quantitative PCR analysis to determine receptor mRNA levels was performed as described previously (33). In brief, total RNA was purified from day 19 C/EBP α wild-type or ^{-/-} fetal liver. In vitro transcription of plasmid pSPCR1 mRNA was used as a standard. The standard mRNA concentration was measured by UV absorption after purification by oligo-(dT) chromatography. The length of the IL-6 receptor RNA molecules obtained from transcription of pSPCR1 as the template was 520 bp, and 1 μ g of RNA equals 1.76 \times 10¹² molecules. First-strand cDNA from fetal liver RNA was synthesized by reverse transcription (RT) according to the manufacturer's protocol (Boehringer Mannheim Corp., Indianapolis, IN). The reaction contained 2 μ g of total cellular RNA and decreasing amounts of standard RNA starting at 10⁸ molecules. 1 μ l of RT product was used for PCR. The annealing temperature for G-CSF receptor was 52°C; for the IL-6 receptor, 52°C; and for

gp130, 54°C. The oligonucleotides used were as follows: for the IL-6 receptor, 5'-primer, ACCACGAGGATCAGTACGAA, and 3'-primer, TGTTGTCATAAGGGCTCTGT; for gp130, 5'-primer, TTGAAGTCTGGGTGGAAGCA, and 3'-primer, GGTGGTCTGGATGGTCTGTC; for the G-CSF receptor, 5'-primer, TCCGTCACCCTAAACATCTC, and 3'-primer, TGGAAGGTTTCCTCTGTCAT. PCR amplification was continued for 30 cycles. 5 µl of each PCR product was electrophoresed in 5% polyacrylamide gels in 1× TBE buffer. Quantitation of autoradiography of dried gels was performed using a PhosphorImager and ImageQuant densitometry software (Molecular Dynamics, Sunnyvale, CA).

Flow Cytometry (FACS®). Human recombinant G-CSF and IL-6 were biotinylated by using NHS-LC-biotin (Pierce Chemical Co., Rockford, IL) following the manufacturer's procedure. Single cell suspensions of fetal liver and spleen cells were washed twice with PBS and then incubated at 4°C for 1 h with biotinylated G-CSF (25 ng/10⁶ cells) or IL-6 (25 ng/10⁶ cells). After washing twice with PBS, the cells were incubated with PE-conjugated streptavidin. Cells were analyzed on a FACScan® flow cytometer (Becton Dickinson, San Jose, CA). G-CSF and IL-6 (without biotinylation) were used as controls.

Retrovirus Infection of G-CSF Receptor-expressing Retroviruses. For retroviral transduction experiments, the murine G-CSF receptor cDNA (the EcoRI/XhoI fragment) was inserted into the MND-X-SN retroviral plasmid (34). This construct was stably transfected into the GP+E86 packaging cell line (35). Cells were maintained in HXM (10% fetal bovine serum, 15 µg/ml of hypoxanthine, 250 µg/ml xanthine, 25 µg/ml mycophenolic acid) and 0.8 mg/ml G418. The titer of the retrovirus from this pro-

ducer line was 5 × 10⁵ PFU/ml. For infection of fetal liver, producer cells were plated the day before cocultivation. The following day, when the cells were 80–90% confluent, they were irradiated with a single dose of 3,000 rad from a ¹³⁷Cs source. Single cell suspensions from C/EBPα wild-type, +/-, or -/- fetal livers were washed with PBS. After lysis of red blood cells with ACK (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.3), the cells were resuspended in IMDM medium containing 10% SCF (Chinese hamster ovary cell conditioned-medium) and 8 µg/ml polybrene. The cells were divided into two cultures, one with retrovirus producer cells, one without. 20 h later, cells were harvested and plated in methylcellulose.

Results

C/EBPα^{-/-} Murine Myeloid Progenitors Do Not Respond to IL-6. Because of the differences in phenotype between C/EBPα^{-/-} and G-CSF receptor^{-/-} mice, we suspected that other cytokine signaling pathways in addition to G-CSF may be adversely affected in C/EBPα^{-/-} mice. Therefore, to investigate the response of C/EBPα-deficient cells to various cytokines, methylcellulose CFU assays were performed using liver hematopoietic cells. As shown in Table 1, the total colony numbers from -/- mice are quantitatively equal to those from wild-type mice in the presence of GM-CSF and IL-3. We also observed mature granulocytes in colonies derived from C/EBPα^{-/-} mice in the presence of IL-3 or GM-CSF, as shown in Fig. 1. The

Table 1. *In Vitro Hematopoiesis and Differentiation of Wild-type and C/EBPα^{-/-} Cells*

CSF	Colonies		Segmented neutrophils % wild-type
	Wild-type	-/-	
IL-6	134 ± 25	2.0 ± 1.4	<1
IL-6 + soluble IL-6 receptor	200 ± 49	26 ± 11	3.4 ± 1.8
IL-6 + soluble IL-6 receptor + G-CSF	266 ± 50	82 ± 40	12 ± 4.8
G-CSF	150 ± 70	0.5 ± 1	<1
G-CSF + G-CSF receptor	148 ± 66	63 ± 28	66 ± 26
IL-6 + soluble IL-6 receptor + G-CSF	227 ± 58	37 ± 11	39 ± 23
IL-6 + soluble IL-6 receptor + G-CSF + G-CSF receptor retrovirus	192 ± 23	46 ± 11	84 ± 12
IL-3	249 ± 25	217 ± 86	35 ± 18
GM-CSF	181 ± 81	163 ± 57	40 ± 5.7

Fetal liver cells (10⁵ cells for CFU-G and CFU-IL6, and 2 × 10⁴ cells for CFU-IL3 and CFU-GM) were plated in methylcellulose medium containing the indicated cytokines. The concentration of cytokines is as described in Materials and Methods. Colonies containing >50 cells were counted after 6–7 d. Three to five animals of each genotype were analyzed. Wild-type animals (+/+) gave a result similar to that obtained from heterozygous animals (+/-). The number of CFU-IL6 from C/EBPα^{-/-} livers was derived from only three colonies in one plate, which morphologically showed all macrophages after cytocentrifugation of the colonies onto slides and staining with Wright-Giemsa. Differential counts were performed as follows: two to four colonies were pooled from each plate on two successive days and cytocentrifuged onto glass slides. The slides were stained with Diff-Quik solution. The percentages of segmented granulocytes were determined by counting 200–300 cells from each slide, and calculating the percentage of granulocytes in knockout colonies compared with that in wild-type. Each number was derived from 3 to 4 individual animals and a total of at least 16 colonies for CFU-G and CFU-IL6.

Table 2. *Differential Counts of CFU Assays*

Growth factor	Genotype	Segmented neutrophils	Macrophage	Immature
		%	%	%
GM-CSF	+/-	53.8 ± 13.8	6.5 ± 3.5	39.8 ± 10.2
	-/-	20.7 ± 2.3	7.2 ± 1.9	72.2 ± 0.8
IL-3	+/-	51.0 ± 6.2	11.7 ± 1.5	40.7 ± 4.9
	-/-	17.3 ± 7.0	7.2 ± 3.3	75.5 ± 10.3
IL-6 + soluble IL-6 receptor	+/-	38.7 ± 8.4	33.0 ± 6.3	28.3 ± 2.3
	-/-	1.2 ± 0.3	48.3 ± 8.7	50.5 ± 9.0
G-CSF + G-CSF receptor	+/-	43.0 ± 14.7	23.1 ± 11.7	34.3 ± 3.3
	-/-	26.0 ± 5.4	22.3 ± 7.6	51.7 ± 3.3
G-CSF + IL-6 + soluble IL-6 receptor	+/-	46.1 ± 9.4	31.6 ± 11.4	22.3 ± 4.6
	-/-	16.5 ± 6.4	37.2 ± 6.8	46.3 ± 8.1
G-CSF + IL-6 + G-CSF receptor + soluble IL-6 receptor	+/-	48.7 ± 8.7	33.1 ± 2.5	18.1 ± 11.4
	-/-	40.6 ± 5.5	33.3 ± 7.0	22.5 ± 8.3

Using the colonies generated as described in the legend to Table 1, percentages of different types of cells were determined by counting 200–300 cells from each slide. Each number was derived from a total of 3 to 4 individual animals, and at least 16 colonies for CFU-G and CFU-IL6. Immature cells include myeloblasts and metamyelocytes; segmented neutrophils include band forms.

percentage of segmented neutrophils in IL-3-responsive CFU (CFU-IL3) and CFU-GM-CSF derived from C/EBP α ^{-/-} fetal liver is much lower than those from wild-type mice. In contrast, the percentage of myeloblasts in C/EBP α ^{-/-} mice is much higher than in wild-type mice in CFU assays performed in the presence of IL-3 and GM-CSF, as shown in Table 2. In cultures of C/EBP α wild-type hematopoietic progenitors, IL-6-stimulated colonies contained immature and mature granulocytic cells and mature macrophages. However, C/EBP α -deficient cells did not respond to IL-6 (Table 1). These results indicate that C/EBP α ^{-/-} mice have myeloid precursors that responded to IL-3 and GM-CSF in vitro, but that there is no such population that responded to IL-6.

Mutation of C/EBP α Results in Drastically Decreased Expression of IL-6 Receptor and G-CSF Receptor mRNA. To examine whether IL-6 receptor expression is defective in C/EBP α ^{-/-} mice, we first performed Northern blot analysis. As shown in Fig. 2 A, IL-6 receptor mRNA was detected from the liver of day 19 C/EBP α ^{+/-} embryos. The signal is weaker than that detected in RNA from adult bone marrow, peritoneal cells after thioglycollate stimulation, spleen, and liver (data not shown). We did not detect any hybridization from total RNA from the liver of day 19 C/EBP α ^{-/-} embryos (Fig. 2 A, lane 2). However, we could detect IL-6 receptor using poly A⁺ mRNA from the liver of -/- day 19 embryos (Fig. 2 A, lane 4). To quantitate the expression level of IL-6 receptor mRNA, we performed quantitative PCR using RNA from livers of C/EBP α ^{-/-} and wild-type mice. The results of the quantitative PCR are shown in Fig. 2 B. The number of IL-6 receptor molecules per microgram of total liver RNA from wild-type mice was very similar to that published previ-

ously (33). The number of IL-6 receptor mRNA molecules was dramatically reduced in -/- mice, 170-fold lower than in wild-type animals. The number of gp130 molecules from -/- mice was comparable to the number found in wild-type mice, and the number of G-CSF receptor molecules from -/- livers was 17-fold lower than in wild-type livers (data not shown). These results further support the notion that C/EBP α regulates G-CSF receptor not only in vitro (5), but also in vivo. Our results also suggest that C/EBP α directly or indirectly regulates IL-6 receptor but not gp130 mRNA expression in vivo.

Hematopoietic Cells in C/EBP α Mutant Mice Have Undetectable IL-6 Receptor and G-CSF Receptor Protein Levels. Our quantitative PCR results demonstrated very low levels of G-CSF receptor and IL-6 receptor mRNA, and showed that progenitor cells from C/EBP α ^{-/-} mice did not respond to G-CSF and IL-6 in vitro or in vivo (18). To determine the protein levels of IL-6 receptor and G-CSF receptor expression, hematopoietic cells from the liver of C/EBP α wild-type and -/- mice were labeled separately with biotinylated G-CSF and IL-6 followed by PE-conjugated streptavidin. After staining, the cells were analyzed by FACS[®]. As shown in Fig. 3, A, C, and E, G-CSF receptor-positive and IL-6 receptor-positive cell populations are easily detected in C/EBP α ^{+/-} fetal liver and spleen, but there are no such populations detected in -/- mice (Fig. 3, B, D, and F). Therefore, the expression of G-CSF receptor and IL-6 receptor on the surface of C/EBP α ^{-/-} cells is undetectable. The number of G-CSF receptors on mature granulocytes is much higher than on immature myeloid cells (36), and C/EBP α ^{-/-} animals do not make mature granulocytes. One possible explanation for the low level of G-CSF receptors detected in C/EBP α ^{-/-} mice is

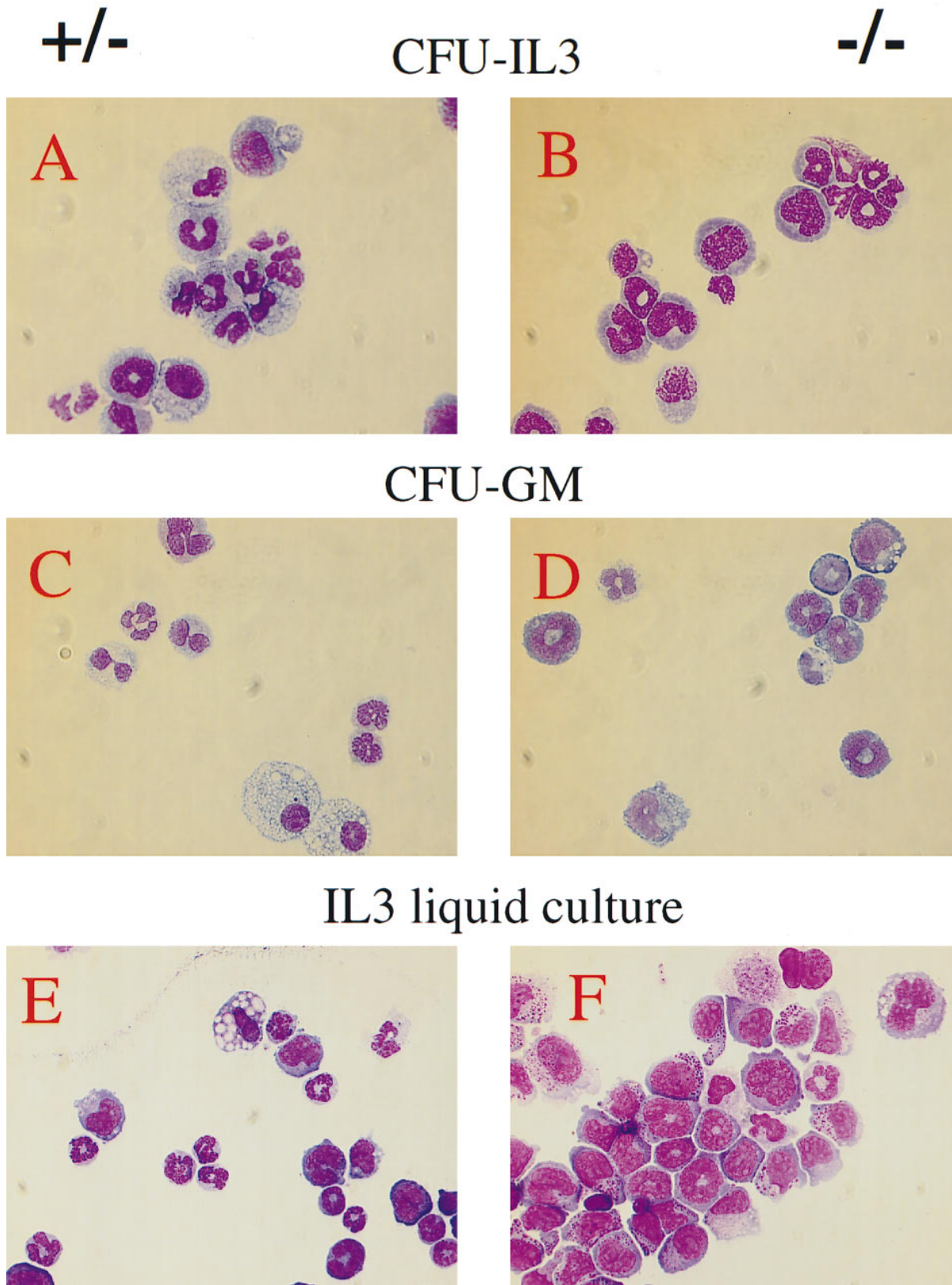


Figure 1. GM-CSF and IL-3 can stimulate $C/EBP\alpha^{-/-}$ fetal liver cells to differentiate to mature granulocytes. (A–D) Representative views of cells from colonies obtained in CFU assays (Table 1) from $C/EBP\alpha^{+/-}$ (A and C) and $-/-$ (B and D) fetal liver in the presence of GM-CSF or IL-3 after 7 d in methylcellulose culture. Two to three colonies from each plate were pooled, cytocentrifuged onto slides, and stained with Wright-Giemsa. (E and F) The same cells were grown in liquid culture in the presence of IL-3 and SCF for 5 d. E shows cells derived from $+/-$ mice, and F from $-/-$ mice. Identical results were obtained after culture with GM-CSF. We were also not able to distinguish differences between cells obtained from wild-type and heterozygous animals in any of the experiments in this study.

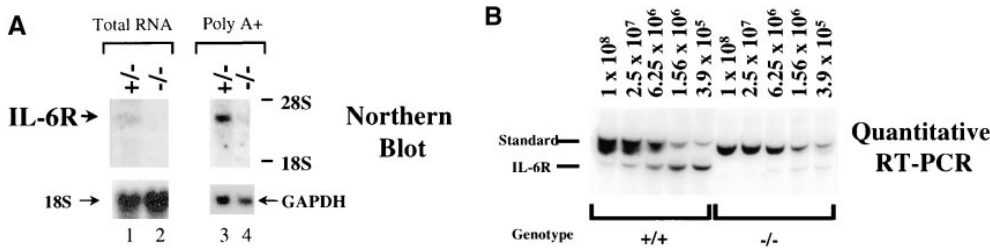


Figure 2. IL-6 receptor mRNA is markedly decreased in *C/EBPα^{-/-}* mice. (A) Northern blot analysis of IL-6 receptor mRNA. 15 μg of total RNA from *C/EBPα^{+/-}* day 19 fetal liver (lane 1) and *C/EBPα^{-/-}* day 19 fetal liver (lane 2), and 2 μg of poly A⁺ RNA from *C/EBPα^{+/-}* day 19 fetal liver (lane 3) and *-/-* day 19 fetal liver (lane 4) were electrophoresed in 1% agarose/formaldehyde gels, transferred to nylon membranes, and hybridized to a murine IL-6 receptor cDNA (reference 26). The position of the IL-6 receptor mRNAs (left) and the 28S and 18S RNAs (right) are shown. Below the total RNA blot (left) are shown the results of hybridization of an 18S ribosomal RNA oligonucleotide to the blot (5'-TCGGGCCTGCTTTGAACA-3' [reference 51]), and below the poly A⁺ blot (right) are shown the results of hybridization of a GAPDH probe (reference 52). (B) Quantitative RT-PCR analysis of IL-6 receptor mRNA. Above the autoradiograph are indicated the numbers of molecules of competitor RNA. The first five lanes are sample RNAs (2 μg/lane) from *C/EBPα* wild-type fetal liver, and the last five lanes are from *C/EBPα^{-/-}* fetal liver RNA. The locations of the standard and IL-6 receptor-amplified PCR products are indicated (left).

(lane 4) were electrophoresed in 1% agarose/formaldehyde gels, transferred to nylon membranes, and hybridized to a murine IL-6 receptor cDNA (reference 26). The position of the IL-6 receptor mRNAs (left) and the 28S and 18S RNAs (right) are shown. Below the total RNA blot (left) are shown the results of hybridization of an 18S ribosomal RNA oligonucleotide to the blot (5'-TCGGGCCTGCTTTGAACA-3' [reference 51]), and below the poly A⁺ blot (right) are shown the results of hybridization of a GAPDH probe (reference 52). (B) Quantitative RT-PCR analysis of IL-6 receptor mRNA. Above the autoradiograph are indicated the numbers of molecules of competitor RNA. The first five lanes are sample RNAs (2 μg/lane) from *C/EBPα* wild-type fetal liver, and the last five lanes are from *C/EBPα^{-/-}* fetal liver RNA. The locations of the standard and IL-6 receptor-amplified PCR products are indicated (left).

the absence of mature granulocytes in the liver and spleen. Therefore, hematopoietic cells from *C/EBPα* heterozygous and *-/-* fetal liver were cultured in suspension in the presence of SCF and IL-3 to stimulate both heterozygous and *-/-* cells towards mature granulocytic differentiation. As shown below, SCF alone does not promote granulocyte differentiation or development, but was added to the cultures to promote viability in the absence of other growth factors. After 5 d in culture with IL-3, the cells were cytocentrifuged onto slides and subsequently stained with Wright-Giemsa. Mature granulocytes appeared in both heterozygous and *-/-* cultures, but the percentage of mature granulocytes in heterozygous cultures was higher than that observed in the *-/-* cultures, similar to what was observed in the CFU assays (Fig. 1, E and F). The cells were harvested and then stained with biotinylated G-CSF and IL-6. As shown in Fig. 3, G-J, we still failed to observe G-CSF receptor and IL-6 receptor-positive cells from *-/-* compared with wild-type mice, even after culture in IL-3 induced morphologic maturation in which ~30% of the

cells were metamyelocytes or segmented granulocytes. These results indicated that although mature segmented granulocytes are not present in vivo in *C/EBPα* mice, stimulation of *C/EBPα^{-/-}* hematopoietic cells with cytokines like GM-CSF and IL-3 can induce the formation of cells which have the morphologic appearance of mature cells yet still do not express G-CSF receptor and IL-6 receptor. Therefore, the absence of G-CSF receptor and IL-6 receptor is not simply a result of absence of mature cells, and the G-CSF receptor and IL-6 receptor are truly regulated, either directly or indirectly, by *C/EBPα* in vivo.

IL-6 and Soluble IL-6 Receptor Together Can Rescue C/EBPα Granulopoiesis In Vitro. These results suggest that lack of IL-6 and G-CSF signaling due to loss of both IL-6 and G-CSF receptors contributes to the block of granulocyte maturation in *C/EBPα^{-/-}* mice. This phenotype could potentially be rescued by restoration of the receptors on the surface of the cells. The cytoplasmic domain of the IL-6 receptor is not required for IL-6 binding and signal transduction, as IL-6 can bind to soluble IL-6 receptor and

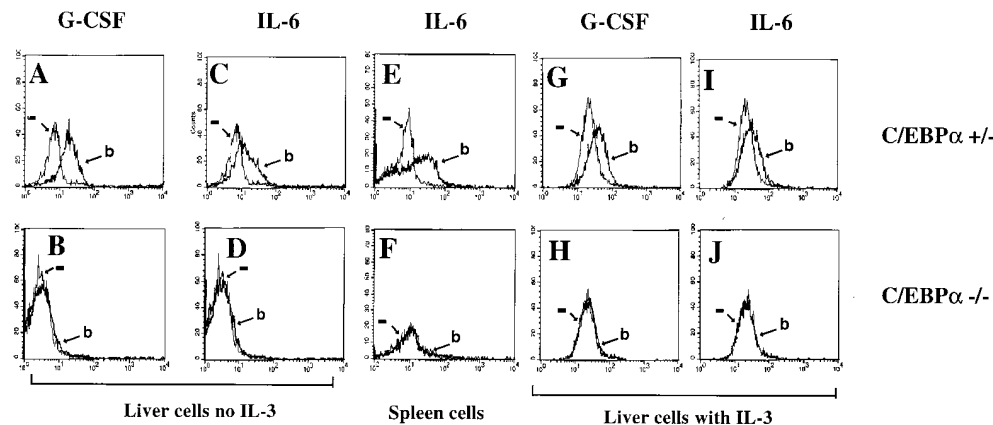
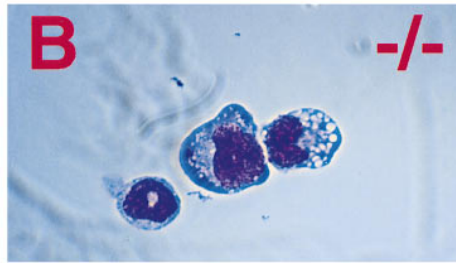
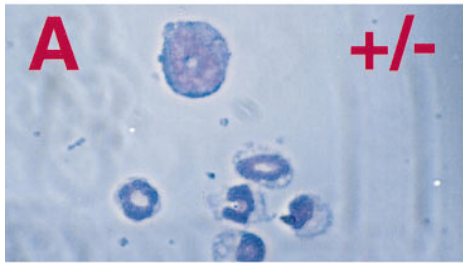
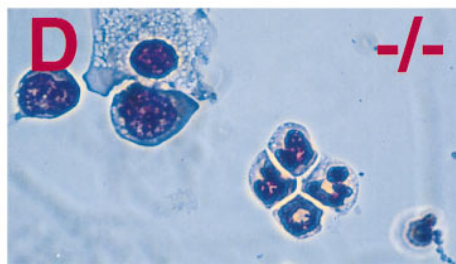
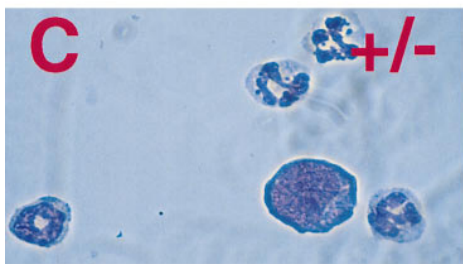


Figure 3. Flow cytometry analysis with biotinylated IL-6 and G-CSF. Cells from *C/EBPα^{+/-}* (A and C) or *C/EBPα^{-/-}* (B and D) day 17 fetal liver or from *+/-* (E) or *-/-* (F) day 17 fetal spleen were stained with biotinylated G-CSF (A and B) or biotinylated IL-6 (C-F). Cells from *C/EBPα^{+/-}* (G and I) or *C/EBPα^{-/-}* (H and J) were cultured in IL-3 and SCF for 5 d and then stained with biotinylated G-CSF (G and H) or biotinylated IL-6 (I and J). b, The FACS[®] profiles using biotinylated factor (G-CSF or IL-6); bar, the profiles using unbiotinylated factor, which serves as a control. In the bottom row, the profiles using biotinylated and unbiotinylated factor are superimposable. y-axis, Number of events (10,000 events counted in each panel); x-axis, log₁₀ fluorescence.

sIL6 receptor + IL6



sIL6 receptor + IL6 + G-CSF



G-CSF receptor + G-CSF

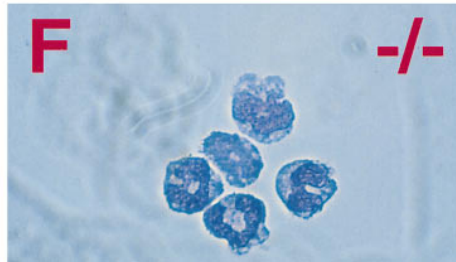
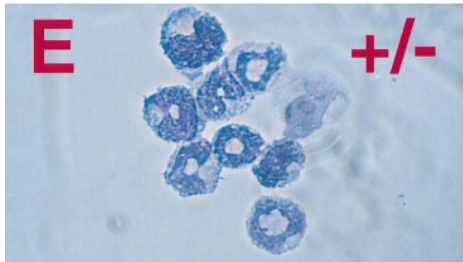


Figure 4. Mature granulocytes are observed after rescue by soluble IL-6 receptor and G-CSF receptor retrovirus. (A, C, and E) Cells from $C/EBP\alpha^{+/-}$ fetal liver. (B, D, and F) Cells from $C/EBP\alpha^{-/-}$ fetal liver. A and B show cells from CFU assays with IL-6 and soluble IL-6 receptor in the medium; C and D, CFU with IL-6, soluble IL-6 receptor, and G-CSF in the medium. E and F show CFU after infection of fetal liver cells with the G-CSF receptor retrovirus described in the legend to Fig. 5 followed by culture in methylcellulose in the presence of G-CSF only.

this complex can trigger gp130 to transduce a signal on cells that lack the IL-6 receptor (31). Therefore, fetal liver suspension cells from both wild-type and $-/-$ day 14 to day 16 embryos were used in CFU assays in the presence of IL-6 plus soluble IL-6 receptor, and IL-6 plus soluble IL-6 receptor plus G-CSF, respectively (Table 1). There were significant numbers of colonies generated from wild-type mice in the presence of IL-6 plus soluble IL-6 receptors which were morphologically CFU-GM. When G-CSF was also added, both the colony number and the percentage of mature granulocytes increased as well (Tables 1 and 2; Fig. 4, A and C). Compared with those from wild-type animals, the colony numbers from $C/EBP\alpha^{-/-}$ cells in the presence of IL-6 and soluble IL-6 receptor were lower, but a small number of metamyelocytes and segmented neutrophils appeared from the colonies after day 6 as shown in Fig. 4 B. Most of cells in the $-/-$ colony

were immature cells. Interestingly, increases in colony numbers and percentage of more mature segmented granulocytes were observed in $C/EBP\alpha^{-/-}$ mice after the addition of G-CSF to the IL-6 plus soluble IL-6 receptor culture as shown in Fig. 4 D. These results indicated that IL-6 plus soluble IL-6 receptor can partially rescue the $C/EBP\alpha$ phenotype in vitro but does not efficiently induce terminal maturation of granulocytes.

Restoration of G-CSF Receptor Expression Can Restore the Production of Mature Granulocytes In Vitro. Granulocyte maturation was completely blocked in $C/EBP\alpha^{-/-}$ mice in vivo. This phenotype can only be partially rescued by IL-6 signaling in vitro. Since G-CSF receptors were undetectable in $C/EBP\alpha^{-/-}$ mice, rescue experiments were performed by transduction of G-CSF receptors into the cells using retrovirus infection. Day 15 fetal liver hematopoietic cells from $C/EBP\alpha^{-/-}$ and heterozygous mice were cul-

tured with or without murine G-CSF receptor retrovirus producer cells in the presence of SCF (added to promote cell viability) and polybrene. After 20–22 h, cells were harvested and were plated in methylcellulose in the presence of G-CSF (Table 1). In addition, cells were maintained in the same culture conditions without polybrene for an additional 4 d and stained with biotinylated G-CSF to demonstrate induction of high levels of G-CSF receptor by the retrovirus (Fig. 5). Colony numbers from C/EBP α heterozygous mice were not significantly different with or without G-CSF receptor retrovirus infection. No colonies were observed in cultures from C/EBP $\alpha^{-/-}$ mice without retrovirus infection, or with retrovirus infection but cultured in the absence of added G-CSF. Infected cells from C/EBP α heterozygous mice also yielded no colonies when cultured without G-CSF. Colonies were observed from G-CSF receptor retrovirus-infected C/EBP $\alpha^{-/-}$ cells in the presence of G-CSF, but less than the number from C/EBP α heterozygous hematopoietic cells after retrovirus infection (Table 1). Mature granulocytes could be detected in the $-/-$ colonies (Fig. 4 F). Interestingly, when infected cells were plated in medium in the presence of IL-6 and soluble IL-6 receptor in addition to G-CSF, the colony numbers were only slightly increased, but the percentage of mature granulocytes from C/EBP $\alpha^{-/-}$ cells was increased to almost the same level as that from C/EBP α heterozygous mice (Tables 1 and 2). Therefore, restoration of both IL-6 receptor and G-CSF receptor levels restored granulocyte production in vitro.

Discussion

Previously, we had reported that granulocyte differentiation and maturation were selectively blocked, and the expression of G-CSF receptor mRNA could not be detected by Northern blot analysis in cells from mice with a targeted disruption of the C/EBP α gene. However, mice with a targeted disruption of either G-CSF (37) or G-CSF receptor (19) show a decrease only in the number of peripheral

blood neutrophils but are not blocked in maturation. Therefore, other mechanisms in addition to G-CSF are involved for induction of granulopoiesis. In this paper, we show that hematopoietic cells from C/EBP $\alpha^{-/-}$ fetal liver can form CFU-GM in the presence of IL-3 or GM-CSF in vitro. However, the percentage of mature granulocytes is lower in $-/-$ than in wild-type cells. These results indicate that immature myeloid progenitors are present in C/EBP $\alpha^{-/-}$ mice, and those progenitors respond to GM-CSF and IL-3. Interestingly, these cells do not mature in response to retinoic acid, a potent inducer of granulocytic differentiation (38), given both in vivo and in vitro (data not shown). These immature cells also do not respond to IL-6 and G-CSF in vitro. There are several possible explanations for why GM-CSF and IL-3 could stimulate myeloid precursors to differentiate into mature granulocytes in vitro, but granulocytes are not observed in vivo. First, GM-CSF and IL-3 are not normally produced in the bone marrow, but by activated T cells and mast cells (39). Fetal T cells and mast cells are not normally activated by pathogens because of the protection of the placental barrier. Consistent with this hypothesis, neither GM-CSF nor IL-3 could be detected in adult bone marrow, during embryonic stem cell differentiation by RT-PCR or during in vitro culture of embryonic blastocysts (40, 41). These studies suggest that the fetus does not produce its own IL-3 and GM-CSF. To investigate whether IL-3 could rescue granulopoiesis in vivo, we administered IL-3 into a C/EBP $\alpha^{+/-}$ pregnant female that had mated with a C/EBP $\alpha^{+/-}$ male. Day 19 embryos were analyzed, but no mature granulocytes could be detected in C/EBP $\alpha^{-/-}$ peripheral blood or fetal liver. However, since no changes were detected in C/EBP α wild-type fetuses as well, it is possible that IL-3 and GM-CSF may, like erythropoietin (42) but not G-CSF (43), be unable to cross the placental barrier. Mice deficient in the entire IL-3/GM-CSF/IL-5 signaling pathway revealed normal numbers of total peripheral leukocyte counts and differential counts except for eosinophils (44). These results suggest that GM-CSF and IL-3 are not necessary for

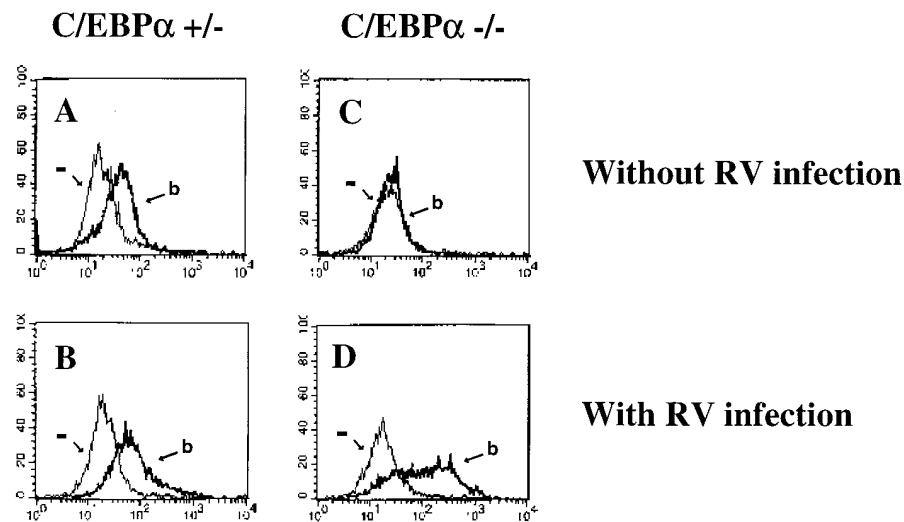


Figure 5. Transduction of G-CSF receptor using retroviral infection of C/EBP $\alpha^{-/-}$ fetal liver cells. The histograms represent analysis of surface expression of G-CSF receptors on cells from C/EBP α heterozygous (A and B) and C/EBP $\alpha^{-/-}$ (C and D) without (A and C) or after (B and D) infection with a murine G-CSF receptor expressing retrovirus, followed by culture in the presence of SCF only for 5 d, then FACS[®] analysis after staining with biotinylated G-CSF. *b* and *bar*, Profiles obtained with biotinylated and unbiotinylated G-CSF, respectively. *y*-axis, Number of events (10,000 events counted in each panel); *x*-axis, log₁₀ fluorescence. *RV*, Retrovirus.

routine production of mature neutrophils. These two factors may play a major role in expansion of hematopoietic cells in emergency situations (39). IL-3 and GM-CSF share a common β chain receptor which signals by inducing protein phosphorylation of similar proteins (45); the two cytokines can also compete with each other in binding to their high-affinity receptors (46). All of these studies suggest that there may be a cell population in mice that expresses both GM-CSF and IL-3 receptors on the cell surface. This population apparently exists in the $C/EBP\alpha^{-/-}$ mice, but because there is no GM-CSF and IL-3 in the fetus, this population might not differentiate in vivo but could nevertheless differentiate in vitro after stimulation with IL-3 and GM-CSF.

The absence of G-CSF signaling alone could not explain the phenotype of $C/EBP\alpha$ -deficient mice, and IL-6 is another important growth factor for myeloid cell proliferation and differentiation (20–22). Our previous studies suggested that G-CSF receptor and IL-6 receptor stimulated the same myeloid progenitors (19). Therefore, in addition to the G-CSF receptor, the IL-6 receptor could be a candidate target for the $C/EBP\alpha$ transcription factor, and indeed, the number of CFU-IL6 is drastically reduced in $C/EBP\alpha^{-/-}$ mice. The phenotype of mice deficient in the IL-6 signal transducer gp130 is distinct from that of $C/EBP\alpha^{-/-}$ mice (47), so we focused our studies on the expression of the IL-6 receptor. Like G-CSF receptor, IL-6 receptor could not be detected by Northern blot analysis of total fetal liver RNA from $C/EBP\alpha^{-/-}$ mice. By quantitative RT-PCR, IL-6 receptor could be detected, but IL-6 receptor mRNA in $C/EBP\alpha^{-/-}$ mice is 170-fold lower than in wild-type mice. As expected, the expression of gp130 is normal in $C/EBP\alpha^{-/-}$ compared with wild-type mice. Staining of hematopoietic cells from $C/EBP\alpha$ wild-type and $-/-$ fetal liver with biotinylated G-CSF and IL-6 demonstrated no surface G-CSF receptor and IL-6 receptor-positive cell populations in $C/EBP\alpha^{-/-}$ mice. When $C/EBP\alpha^{-/-}$ cells were induced to mature granulocytes with IL-3 and SCF or GM-CSF and SCF, the morphologically mature cells are still IL-6 and G-CSF receptor-negative. These findings demonstrate that $C/EBP\alpha$ is a major regulator for both IL-6 receptor and G-CSF receptor in vivo, and mature cells from IL-3 and GM-CSF stimulation in vitro do not express IL-6 and G-CSF receptors. The proliferation and survival of these cells depend on IL-3 and GM-CSF but not IL-6 and G-CSF. Mice with a disruption of the IL-6 gene fail to efficiently control infection with vaccinia virus and *Listeria monocytogenes*, a facultative intracellular bacterium, and in these animals the inflammatory acute-phase response after tissue damage or infection is severely compromised (48). Consistent with this report and our current findings is the additional observation that the acute-phase response is completely lacking in these same $C/EBP\alpha^{-/-}$ mice (Burgess-Beusse, B.L., and G.J. Darlington, manuscript in preparation), perhaps due in part to the loss of IL-6 receptor expression.

Because hematopoietic cells from $C/EBP\alpha^{-/-}$ mice lack normal numbers of IL-6 receptors, no colonies were ob-

served in CFU assays from $C/EBP\alpha^{-/-}$ cells when stimulated with IL-6 only. IL-6 can bind to the soluble form of the IL-6 receptor, and can trigger gp130 signaling on target cells lacking the IL-6 receptor but expressing gp130. When soluble IL-6 receptor and IL-6 were added to $C/EBP\alpha^{-/-}$ cells, CFU-IL6 were observed at a level 30% that of wild-type cells. Interestingly, the addition of G-CSF to the cultures with IL-6 and soluble IL-6 receptor resulted in increased numbers of colonies and percentage of mature granulocytes in the colonies. Previously, it has been reported that IL-6 stimulates stem cells in their ability to respond to IL-3, and when IL-6 is combined with other recombinant hematopoietic factors, including G-CSF, it can augment responses to these factors or even modify them (49). The results presented here suggest that G-CSF signaling may be one of the growth factor pathways whose function can be augmented by IL-6.

The block of granulocytic differentiation in $C/EBP\alpha^{-/-}$ mice can only be partially rescued in vitro by restoration of IL-6 receptor. Therefore, we infected fetal liver cells from $C/EBP\alpha$ wild-type and $-/-$ mice with a retrovirus expressing the wild-type G-CSF receptor. When the cells were placed in culture without added growth factors, no colonies were detected after retrovirus infection from both wild-type and $-/-$ mice. When infected cells from the $C/EBP\alpha^{-/-}$ mice were cultured in the presence of G-CSF, cells from $-/-$ liver generated half the number of colonies compared with wild-type liver, and these colonies contained mature granulocytes in addition to macrophages and immature myeloid cells. With the addition of IL-6 and soluble IL-6 receptor to the same cultures, the colony number was still lower than in wild-type. Furthermore, our recent

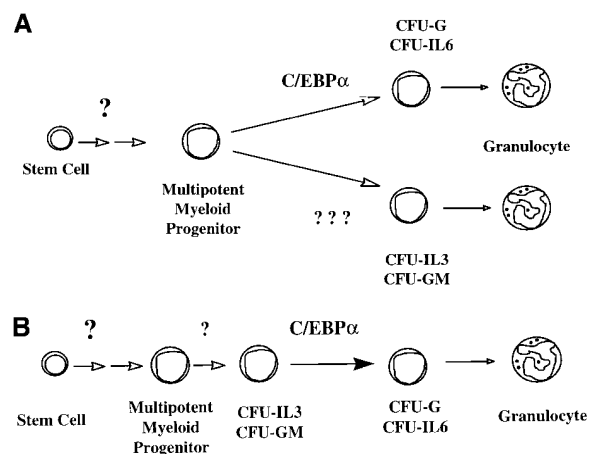


Figure 6. Two models of granulocytic development. (A) A pluripotential stem cell differentiates into an immature multipotent myeloid progenitor, perhaps under the influence of transcription factors such as PU.1 (reference 2). This cell can then further differentiate to a mature granulocyte, either via a CFU-G/CFU-IL6 precursor which is dependent upon $C/EBP\alpha$ function, or via a CFU-IL3/CFU-GM precursor which is $C/EBP\alpha$ independent. (B) The multipotent progenitor differentiates into an early granulocytic precursor that can respond to IL-3 and/or GM-CSF, which itself is induced to differentiate further under the influence of $C/EBP\alpha$ to a precursor that responds to G-CSF and/or IL-6.

studies have shown that the IL-6 and G-CSF receptor double knockout phenotype is similar to that of the G-CSF receptor alone and, unlike the *C/EBP α ^{-/-}* mouse, still makes mature granulocytes. These results suggest that in addition to the IL-6 and G-CSF receptor, some other genes are affected in the *C/EBP α ^{-/-}* mice (15a). Since the G-CSF receptor and gp130 have a similar intracellular domain and activate similar signaling pathways, our combined data suggest that there is a G-CSF and IL-6 receptor-positive cell population in mice. The generation of mature granulocytes from this population of cells is blocked due to the lack of IL-6 and G-CSF responsiveness, but another population can respond, at least in vitro, to IL-3 and GM-CSF. Whether such a population exists in vivo can potentially be addressed once mice with selective restoration of *C/EBP α* expression in liver and not blood, and with longer viability, are produced.

In summary, we demonstrate that *C/EBP α* may directly or indirectly regulate the IL-6 receptor. In the absence of *C/EBP α* , we observed a marked decrease in IL-6 and

G-CSF receptor expression. Neither IL-6 nor G-CSF signaling is required for myeloid cell lineage commitment, but they are likely to play an important role in proliferation and/or viability of myeloid precursors. Our results raise the very interesting possibility (Fig. 6 A) that two distinct populations of granulocyte-macrophage progenitors are present in the murine hematopoietic system, one G-CSF and IL-6 dependent and the other IL-3 and GM-CSF dependent. Alternatively, granulocyte development occurs in several stages, beginning with an IL-3 and GM-CSF-responsive precursor, which itself can further differentiate to become responsive to G-CSF and IL-6 (Fig. 6 B). This latter model is more consistent with the hierarchical expression of CSF receptors (50). Our data tend to support the first model (Fig. 6 A). At least in vitro, loss of *C/EBP α* mainly affects the G-CSF/IL-6-dependent precursor, and stimulation of either IL-3 or GM-CSF can induce granulocyte formation. If such a model is correct, it remains to be determined why this latter pathway is incapable of inducing formation of mature granulocytes in *C/EBP α* knockout animals.

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