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C/EBP epsilon directs granulocytic versus monocytic lineage determination and confers chemotactic function via Hlx

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Abstract

Objective—Mutations in the C/EBPε gene have been identified in the cells of patients with neutrophil specific granule deficiency (SGD), a rare congenital disorder marked by recurrent bacterial infections. Their neutrophils, in addition to lacking specific granules required for normal respiratory burst activity, also lack normal phagocytosis and chemotaxis. Although the SGD phenotype has been replicated in C/EBPε$^{-/-}$ (KO) mice, the mechanisms by which C/EBPε mutations act to decrease neutrophil function are not entirely clear.

Methods—In order to determine the role of C/EBPε in neutrophil differentiation and migration, we generated immortalized progenitor cell lines from C/EBPε KO and wild type (WT) mice and performed expression and flow cytometric analysis and functional studies.

Results—Expression of lineage specific cell surface antigens on our in vitro differentiated cell lines revealed persistent expression of monocytic markers on KO granulocytes. We verified this in primary murine peripheral blood and bone marrow cells. In addition, KO BM had an increase in immature myeloid precursors at the common myeloid progenitor (CMP) and granulocyte monocyte progenitor (GMP) level suggesting a critical role for C/EBPε not only in granulocyte maturation beyond the promyelocyte stage, but also in the monocyte/granulocyte lineage decision. We found that restoration

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No financial interest/relationships with financial interest relating to the topic of this article have been declared.

of Hlx (H2.0-like homeo box 1) expression, which was decreased in C/EBPε KO cells, rescued chemotaxis, but not the other defects of C/EBPε KO neutrophils.

Summary—We show two new regulatory functions of C/EBPε in myelopoiesis: in the absence of C/EBPε, there is not only incomplete differentiation of granulocytes, but myelopoiesis is disrupted with the appearance of an intermediate cell type with monocyte and granulocyte features, and the neutrophils have abnormal chemotaxis. Restoration of expression of Hlx provides partial recovery of function; it has no effect on neutrophil maturation, but can completely ameliorate the chemotaxis defect in C/EBPe KO cells.

Keywords

granulopoiesis; transcription

Introduction

The pluripotent hematopoietic stem cell gives rise to mature neutrophils via a series of commitment steps that produce the myeloblast, promyelocyte, myelocyte, and band stages of development. The secondary granule protein genes lactoferrin, transcobalamin I, neutrophil collagenase, and neutrophil gelatinase are expressed in a stage-specific manner during late neutrophil differentiation. The appearance of secondary granules at the transition from the promyelocyte to myelocyte stage marks the commitment to terminal neutrophil maturation. The secondary granule proteins (SGPs) contained in secondary granules are absent SGD[1, 2], and in other disorders of myelopoiesis, such as myelodysplastic syndromes [3,4] and acute myelogenous leukemia[5,6].

The CCAAT enhancer binding protein (C/EBP) family has 6 members, including C/EBPabgde and z, each characterized by highly conserved basic DNA binding and leucine zipper dimerization domains.[7–9] The specificity of C/EBP protein activity is determined by the cell type- and stage-specific expression of different gene family members. C/EBPε expression is restricted to hematopoietic cells, and its upregulation correlates with granulocytic differentiation, at which stage it is a key regulator of secondary granule protein gene expression that occurs during neutrophil development.[10] In addition to stage-specific gene expression, the capacity of C/EBPε and other C/EBPs to differentially regulate stage-specific gene expression is controlled by complex modulations of homo- and heterodimerization at the binding sites of target gene promoters.[11–15] There are at least 2 murine isoforms of C/EBPε, a 36 kDa and 34 kDa isoform, [16] while in humans 4 isoforms have been identified that are expressed as activation domain containing 32kDa, 30kDa, and 27kDa proteins, plus a shorter 14 kDa isoform that lacks transcriptional activity.[8,10,17,18]

Patients with SGD attributable to CEBPE mutations that abrogate either all or specifically the p32 and p30 isoforms have been previously described.[19–21] The phenotypic and functional defects of neutrophils in C/EBPε KO mice closely parallel those in patients with SGD[22]. C/EBPε KO neutrophils have bilobed nuclei, lack specific granules as well as the mRNA for the secondary and tertiary granule proteins, have abnormal respiratory burst activity, and have impaired chemotaxis and bactericidal activity. The mice die prematurely of infection or from complications of myeloproliferation.[22], [23]While it is clear that SGP genes are direct targets of C/EBPε, the mechanisms underlying the loss of chemotactic function in C/EBPε KO cells is not yet known. Also, although C/EBPε is upregulated during myeloid differentiation towards granulocytes but not monocytes[24], the monocytes/macrophages from patients with SGD and from the C/EBPε KO mice have phenotypic and functional defects[23], [25], suggesting a role for C/EBPε in both the granulocyte and monocyte lineages. C/EBPε KO mice also have

defective eosinophil differentiation and as shown in human blood-derived CD34+ cells, the four C/EBPε isoforms differentially regulate eosinophil differentiation. [26]

A convenient model for assessing regulators of neutrophil development and function is the EML cell line, which is immortalized by transduction of bone marrow with the dominant negative retinoic acid receptor alpha, RARa403, and that shows differentiation potential towards erythroid, myeloid and lymphoid lineages (hence the name EML).[27] To elucidate the mechanisms underlying the functional defects of C/EBPε KO neutrophils, we generated EML-like cell lines from the bone marrow of C/EBPε KO mice and show that their differentiation *in vitro* mimics the *in vivo* findings of abnormal neutrophil maturation and function including abnormal chemotaxis. Using these new cell lines as well as primary cells from C/EBPε KO mice, we show that loss of C/EBPε leads to expansion of an immature population of cells with shared expression of monocyte and neutrophil markers and a skewing towards monocytic differenitation. We also demonstrate that C/EBPε KO cells have reduced expression of the Hlx transcription factor, which has been implicated in myeloid maturation and migration[28]. Restoration of Hlx expression rescued the chemotactic defect of KO cells, but not the the other defects of granulocytic differentiation.

Matenrials and Methods

Generation of EML like cell line

C/EBPε$^{-/-}$ mice[22] (a gift of Julie Lekstrom-Himes and Helene Rosenberg, NIH) were backcrossed to the C57Bl/6 strain. C/EBPε KO and WT littermates were treated with 150 mg/kg 5-fluorouracil (SoloPak Laboratories, Franklin Park, IL). EML like cell lines were generated as described in [27]. In brief, whole bone marrow was harvested and cultured for 48 hours in IMDM/20% horse serum, muGM-CSF (2.5ng/ml; Peprotech), huIL-6 (20ng/ml; Peprotech), and huIL-1b (10ng/ml; Peprotech). Spinfection with ecotropic L-RARalpha403-SN retrovirus [27] was performed twice 12 hours apart over 2 hours at 1000g at 30°C in presence of polybrene (8μg/ml; Sigma, St. Louis, MO). Cells were cultured in IMDM/20% horse serum, muSCF (100ng/ml; Peprotech), Wehi-3B conditioned medium (0.25%) as a source of IL-3 (2.5–5ng/ml) and human erythropoietin (8U/ml; Ortho-Biotech, Bridgewater, NJ). LRARalphaSN [29] supernatants were derived from a stable GP+E86 producer line. Within 1–2 months, rapidly proliferating cell lines were generated and cultured in medium containing only SCF.

Induction of Differentiation

EPRO cells were derived from EML cells by induction with all-*trans*-retinoic acid (ATRA, 10μM, Sigma) and IL-3 (15ng/ml) for 3 days, washed and maintained in IMDM/20% Horse Serum and 10% BHK/HM5-conditioned medium as a source of GM-CSF[27]. EPRO cells were differentiated by addition of ATRA (10μM) for 2–4 days.

Expression Analysis by Northern Blotting and QRT-PCR

Northern blot analysis was performed as described previously[30]. Northern blots were probed with ^{32}P-labeled cDNA for previously described mouse LF, mouse neutrophil collagenase, mouse neutrophil gelatinase and β-actin [32]. The mouse C/EBPε probe was generated with primer pair A (5′-GACCTACTATGAGTGCGAGCCT-3′ and 5′-TGGTACTGCAGGGGATTGTAG-3″). For semiquantitative RT-PCR, cDNA was synthesized from total RNA by random priming using SuperScript II (Invitrogen). Primer pair B (5′-GACCTACTATGAGTGCGAGCCT and 5′-ACACCCTTGATGAGGGTAGCAG) was used for PCR amplification of C/EBPε and primer pair C (5′-CGGACTCACTACTATGC-3′ and 5′-GTCCTTCTGTCCCGAGG-3′) for lactoferrin and primer pair D (5′-GTGGGCCGCTCTAGGCACCA-3′ and 5′-CGGTTGGCCTTAGGGTTCAGGGGGG-3′) for b-actin. PCR reactions were carried out in

a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA) for the indicated number of cycles using the following conditions: 94°C × 30 seconds, 60°C × 30 seconds, and 72°C × 1 minute for 28–32 cycles. Reaction products were analyzed on ethidium bromide stained 1% agarose gels. For QRT-PCR analysis of Hlx1 Taqman primers and probes (Applied Biosystems) were used and analyzed according to manufacturer's protocol on a CFX96 C1000 thermal cycler (Biorad).

Respiratory Burst

For respiratory burst assays using luminol enhanced chemiluminescence, cells (1×10^6) were resuspended in 400 μL of Hank's Balanced Salt Solution containing Mg^{2+} and Ca^{2+} supplemented with 0.1% D-glucose, and added to 100 μl of Diogenes reagent (National Diagnostics, Atlanta, GA). Following incubation at 37°C for 15 minutes, cells were stimulated with 3.2 μM PMA and chemiluminescence was read for 10 seconds in a luminometer (Berthold, Perkin-Elmer, Gaithersburg, MD) immediately after PMA addition and at 1 minute intervals.

Analysis of cell surface marker expression

Cell surface 7/4 (Cedarlane, Burlington, NC), Gr-1, Mac-1 (BD Biosciences, San Jose, CA), and F4/80 (Ebiosciences, San Diego, CA) expression of EML derived cells was performed according to standard protocol with respective isotype controls. Cells were analyzed on a FACS Calibur or LSRII (BD Biosciences). Analysis of bone marrow progenitor cells was performed as described by Akashi et al.[33]. Briefly bone marrow was harvested as described above and stained with lineage Cell Detection Cocktail-Biotin and PE-streptavidin (Miltenyi biotech, Auburn, CA), APC-Cy7 conjugated anti-CD117, Alexa 647-conjugated anti-sca1 (BD), PeCy7-conugated anti FcgRII/III, and FITC-conjugated anti-CD34 (Ebiosciences).

Retroviral transduction

EPRO cells were transduced with retroviral vectors in the presence of polybrene (8μg/ml) by spinfection at 1000×g at room temperature for 60 minutes. C/EBPε containing retroviral vectors/producer lines were L-C/EBPε-eGFP-SN, L-C/EBPε-SH in PA317. MIGR-1 and L-X-SH were used as control vectors. L-C/EBPε-eGFP-SN and MIGR-1 were packaged with ecotropic envelope by transient transfection of Phoenix cells with Fugene (Roche, Indianapolis, IN) using standard protocol as described by the manufacturer. RNA was isolated from the transduced cells as described above and subjected to RT-PCR analysis using primers specific for C/EBPε and lactoferrin (see above). The human HLX1 gene was cloned from a human MGC verified FL cDNA library (NIH_MGC_116) clone IRAT 5184776 in pCMV-SPORT6 (Open Biosystems, Huntsville, AL) into MSCV as an EcoR1-Xho1 fragment. Expression was verified by RT-PCR and Western Blot.

Chemotaxis Assays

Chemotaxis assays were performed in a transwell assay using 96 well plates with 3.2mm diameter wells, accepting a volume of 30μL/well, and 3μm pore size polycarbonate membranes (ChemoTx 101-3, Neuroprobe, Gaithersburg, MD). Chemoattractant-containing medium (rhu-IL-8 at 0, 1, 10, or 50nM, or muKC, at 0, 10, 50, or 250 ng/ml R&D Systems, Minneapolis, MN) was pipetted in the bottom well. The framed filter was placed on top of the plate according to manufacturer's instructions. Subsequently 25μl of the respective cell suspensions at 2×10^6/ml were pipetted on top of each membrane. The plate was incubated at 37°C in humidified air with 5% CO_2 for 2 hours. The cell number in each bottom well was counted and normalized.

Results

KO EML cells lack C/EBPε and fail to upregulate lactoferrin expression when induced to differentiate

EML cells differentiate down the granulocytic lineage in two stages; first, in response to ATRA at supraphysiological concentrations in the presence of SCF and IL-3, they become GM-CSF dependent promyelocytes (EML-derived promyelocytes, or EPRO), and second, in response to ATRA in the presence of GM-CSF, they differentiate predominantly into mature neutrophils with a minor admixture of monocytes/macrophages. [27,34] As shown in Figure 1, our newly generated wild type EML cells gave rise to mature granulocytes as described for the original EML cell line.[27] In contrast, the C/EBPe knockout EML cells differentiated into EPROs, but failed to differentiate into normal, mature neutrophils. Instead, the cells became dysmorphic, immature appearing neutrophils, which closely resemble the abnormal neutrophils in the KO mice[22] (Figure 1).

By Northern blot analysis (Figure 2), induction of WT EML cells resulted in upregulation of C/EBPε and the secondary granule protein genes lactoferrin (LF), murine neutrophil collagenase (MNC) and murine neutrophil gelatinase (MNG). C/EBPε KO cells lacked C/EBPε expression, as expected, and also failed to express LF. Expression of MNC and MNG was detected in the KO, suggesting that their expression can be induced independently of C/EBPε, possibly due to induction with ATRA [35]. We validated these findings by producing an alternative cell line that is not dependent on ATRA for differentiation. These cell lines (pBIM (*pB*abe-puro *I*mmortalized *M*yeloid cells)) were produced by immortalization via retroviral insertional mutagenesis, leading to development of a SCF/IL-3-dependent myeloid progenitor cell line that is immortalized but not transformed or leukemogenic, and that can be differentiated with G-CSF (supplementary data Figure 1)[36]. Northern blot analysis of these cell lines after induction with G-CSF also showed absent C/EBPe and LF expression, as well as reduced levels of gelatinase and collagenase expression in C/EBPe KO neutrophils compared to WT cells (supplementary data Figure 2). Therefore, while C/EBPe is required for LF expression, alternative C/EBPe-independent mechanisms allow expression of gelatinase and collagenase. Because the mechanism of differentiation block in EML cell lines is known and well studied, we chose to continue to use the EML cell lines for further studies of C/EBPε-dependent mechanisms of neutrophil function.

Functional properties of the KO and WT cell lines mimic the in vivo phenotype

We next sought to determine whether the functional defects in the C/EBPε$^{-/-}$ mouse neutrophils could be reproduced in our cell line model. We assessed respiratory burst activity of WT and KO EPRO cells (EML derived promyelocytes) and of ATRA-induced WT and KO EPRO cells (neutrophils) prior to ("0 min") and after ("3min" stimulation with PMA, by measurement of luminol-enhanced chemiluminescence. As shown in Figure 3, undifferentiated EPROs produced a small amount of superoxide after stimulation with PMA, which was greatly enhanced in WT EPRO cells induced to differentiate with ATRA. In contrast, ATRA-induced KO EPRO cells produced significantly lower levels of superoxide in the presence or absence of ATRA.

"Rescue" of C/EBPε KO cells by transduction with C/EBPε

To confirm that the abnormalities described in the KO cell lines are indeed due to lack of C/EBPε, rather than the presence of the dnRAR403a or retroviral insertional mutagenesis, we tested whether the phenotype could be rescued by transduction with a functional C/EBPε gene. EPRO cells were transduced with an empty (CTRL) vector or a C/EBPε cDNA containing retroviral vector, transduced cells were sorted for eGFP, and RT-PCR for C/EBPε and lactoferrin was performed. Restoration of C/EBPε expression in the KO EPRO cells led to up-

regulation of lactoferrin expression (Figure 4A). Re-expression of C/EBPε in KO EPRO cells and subsequent differentiation with ATRA also led to rescue of the oxidative burst in response to PMA (4min) as shown in Figure 4B.

Expression of cell surface markers reveals predominance of granulocyte/macrophage mixed population in C/EBPε KO mice

Bone marrow (BM, Figure 5A, left) and peripheral blood cells (PB, Figure 5A, right) from WT littermates showed a much higher percentage of Gr-1+ cells than C/EBPε$^{-/-}$ mice (92% and 62% versus 89% and 41%, respectively). In addition, the Gr-1 intensity on C/EBPε$^{-/-}$ cells was significantly lower than that in WT cells (Figures 5A). Similarly, in the cell lines, the increase in Gr-1 expression upon differentiation was significantly decreased in the C/EBPε$^{-/-}$ cells (Figure 5B, black line, EML: 0.3%; EML D3 IL-3+ATRA: 1%; EPRO: 49%; and EPRO+ATRA D3: 55%) compared to WT EML cells (Figure 5B, green line, EML: 2%; EML D3 IL-3+ATRA: 20%; EPRO: 90%; and EPRO+ATRA D3: 97%), and Gr-1 fluorescence intensity was reduced by more than a log.

Interestingly, Gr-1 positive differentiated EPRO C/EBPε$^{-/-}$ cells showed persistent expression of F4/80, a cell surface marker of monocytes and macrophages[37] (Figure 6A, thick black line), whereas this was not observed on WT cells (Figure 6A, red). To ensure that this was not unique to the cell lines and therefore an artifact, we further analyzed BM and peripheral blood (PB) of WT and KO mice. Combined staining for 7/4 antigen, a specific marker for neutrophils [38], and Gr-1 captures virtually all myeloid cells in BM and PB. In WT BM (Figure 6B), most of the myeloid cells were 7/4 positive and F4/80 negative, indicating a high percentage of granulocytes, with only one third representing a 7/4^{+}F4/80^{+} granulocyte/macrophage mixed population. In C/EBPe$^{-/-}$ bone marrow, more than 80% of the 7/4^{+} cells were also F4/80 positive, with a small increase in the 7/4^{dim} F4/80^{+} monocyte population. A similar pattern was seen in the PB; in WT mice there are distinct granulocyte and monocyte populations, while in KO mice the blood retains a large percentage of 7/4^{+}F4/80^{+} double positive cells and a small increase in monocytes (Figure 6C). When analyzing murine BM for myeloid subsets according to Gr-1 and 7/4 expression (Figure 6D and E), it was evident that, as with the 7/4^{+} population, the Gr-1^{+} cells coexpressed F4/80 in the KO mice (Figure 6E). It is of interest that the Gr-1^{dim} 7/4^{dim} population in the WT mice consisted of a F4/80 negative as well as a F4/80 bright populations (Figure 6E rightmost panel). This suggests that in C/EBPε$^{-/-}$ mice myeloid progenitors remain arrested in a mono-vs-granulocyte indecisive state. This is corroborated by morphological appearance (Figure 8B) and the higher percentage of PB monocytes in C/EBPε$^{-/-}$ (11% ± 0.22) versus $^{+/+}$ (0.32% ± 0.11) mice ($p < 0.01$)(data not shown). Analysis of the myeloid progenitor pool in the BM revealed expansion of the GMP compartment (Supplementary Figure 3) and colony formation assays revealed an increase in CFU-M at the expense of CFU-G (Supplementary Figure 4). This suggests that C/EBPε is not only involved in terminal differentiation of neutrophils, but that it may also play a role in the complex process of monocyte versus granulocyte lineage resolution.

Chemotaxis is defective in C/EBPe$^{-/-}$ neutrophils

Neutrophils from C/EBPε$^{-/-}$ mice are known to have defects in their ability to migrate in response to chemotactic stimuli; early (4.5 hrs) after thioglycollate is injected i.p., WT mice have approximately 5-fold higher levels of neutrophils in their peritoneal fluid than KO mice. [39] Also, in WT mice assessed at 4.5 and 24 hours after injection, the peritoneal exudate contains mostly mature neutrophils. In contrast, in C/EBPε KO mice, myelocytes and metamyelocytes represent the majority of cells with only few band neutrophils.[22] We tested whether loss of C/EBPε expression also disrupts chemotaxis in our cell lines. As shown in Figure 7, ATRA-induced EPRO KO cells showed little transmigration even to high IL-8 concentrations (white bar), while WT ATRA-induced EPRO cells have a higher baseline level

of migration (black bar, 0nM) with a further increase in response to elevated IL-8 concentrations. While the lack of secondary granules and their content proteins, which are known direct targets of C/EBPε, clearly can be the cause of the decreased oxidative burst in C/EBPε$^{-/-}$ granulocytes, the cause of the underlying defect in chemotaxis is not yet known.

HLX1 is differentially expressed in C/EBPε WT versus KO neutrophils and restores chemotaxis

To identify genes, which may function downstream of C/EBPε in regulating neutrophil migration, we performed microarray analysis of KO and WT ATRA-induced EPRO cells and screened the data set for genes, and in particular transcription factors, potentially implicated in neutrophil migration (complete data available upon request). Analysis of the data identified many known targets of C/EBPε including the secondary granule genes, (Supplementary data table 1 and reviewed in[40] and refs therein), further validating the cell line model and expression analysis. We found that the H2.0-like homeobox gene (Hlx1, also called HB24) was downregulated 4–5 fold in KO versus WT ATRA induced EPRO cells. Hlx1 is a highly conserved homeobox transcription factor that is expressed in cells of the myeloid lineage and at early stages of B- lymphocyte development.[41, 42] Its homeobox sequence is divergent from those of the known vertebrate homeotic genes, but is closely related to that of the *Drosophila H2.0* gene. [43] To verify that differences in Hlx1 expression in our cell lines also occur in vivo, we assessed Hlx1 mRNA levels in primary BM cells as well as immature and mature neutrophils, which were isolated from WT and C/EBPε −/− mice based on side scatter, and expression of Gr-1 and 7/4 antigens as shown in Figure 8A. The purity of the sorted populations was confirmed by Wright Giemsa staining (Figure 8B). Using these primary BM cells and primary granulocytes isolated from PB of WT and KO mice, we confirmed that the KO mice have dramatically decreased levels of Hlx1 mRNA in their “mature” granulocytes and that Hlx1 is upregulated with granulocyte maturation in C/EBPε WT cells (Figure 8C).

Because published data suggest that overexpression of Hlx1 in myeloid progenitor cell lines promotes cell migration, [28] we tested whether overexpression of Hlx1 could rescue the defective chemotaxis in our C/EBPε$^{-/-}$ cell line. We transduced KO EPRO cells with MSCV-HLX1-ires-eGFP or control retroviral vector, sorted cells for GFP expression, and assayed chemotaxis to human IL-8 or its murine homologue KC in the transwell assay after induction of differentiation with ATRA. As shown in figure 9, transduction with Hlx1, but not with control vector, rescued chemotaxis compared to control transduced, ATRA-induced WT cells. Hlx1 over-expression was verified by QRT-PCR on GFP sorted cell populations (data not shown).

Discussion

Neutrophil specific granule deficiency is a rare congenital disorder marked by recurrent bacterial infections of the skin and respiratory system. Neutrophils from SGD patients lack secondary and tertiary granules and their content proteins and exhibit defects in chemotaxis and bactericidal activity. A mouse model deficient for the transcription factor C/EBPε manifests a similar phenotype to SGD patients[22], and functional mutations in the C/EBPε gene have been identified in two patients with SGD. The mutations described either abrogate all functional C/EBPε isoforms or the activating p32/p30 isoforms only.[19,20]

We have generated cell lines from C/EBPε$^{-/-}$ and C/EBPε$^{+/+}$ mice in order to further study the functional defects in SGD. The EML cell lines were immortalized by the dominant negative retinoic acid receptor RARa403 and can be differentiated towards neutrophils via the intermediary EPRO stage[27]. WT EML/EPRO cells have well described functional properties [44], which are replicated in our cell lines, making this approach applicable to other transgenic mouse models as well.[45] Upon induction of myeloid maturation, WT EML cells undergo

normal differentiation and the C/EBPε$^{-/-}$ cells show abnormal differentiation consistent with that observed in KO mice, as verified by morphology, RNA expression analysis, cell surface marker expression, and assays of respiratory burst activity and chemotaxis. Furthermore, we demonstrate restoration of lactoferrin expression and respiratory burst activity following expression of C/EBPε in the KO background.

During the initial generation of the C/EBPε$^{+/+}$ and $^{-/-}$ EML cell lines, prior to establishment of dnRARa403 immortalized clones, we noted an increase in generation of monocytes/macrophages in the C/EBPε$^{-/-}$ cell cultures suggesting an increased monocyte/macrophage potential of C/EBPε$^{-/-}$ progenitors. The role of C/EBPε in monocyte/macrophage development appears to be ambiguous. Previous studies have shown that C/EBPε is expressed in WT monocytes and macrophages and that monocytes/macrophages from C/EBPε$^{-/-}$ mice were dysfunctional.[46] Overexpression of C/EBPε in vitro caused upregulation of genes related to macrophage maturation, [16] and macrophage genes were differentially expressed in C/EBPε$^{+/+}$ versus C/EBP$^{-/-}$ macrophages. [47] Shiohara et al. also demonstrated maturation defects in monocytes/macrophages derived from SGD patients, indicating that loss of C/EBPε impacts monocytic cell development in humans. [25] However, during monocytic differentiation of human bipotential cell lines such as NB4 and HL60, C/EBPε mRNA levels decrease, suggesting that monocytic differentiation may require downregulation of C/EBPε or that it is independent of C/EBPε.[8,10] It is possible that different concentrations of C/EBPε are required for monocytic versus granulocytic differentiation.

Flow cytometric analysis of ATRA induced C/EBPε$^{-/-}$ EPRO cells revealed persistent expression of F4/80. This was not only an in vitro phenomenon of our cell lines, but also found through our analysis of mouse BM and PB that revealed a predominance of a 7/4^{+}F4/80^{+} mixed population at the expense of mature 7/4^{+}F4/80^{-} (and Gr-1+) neutrophils. The BM of KO mice has an expanded GMP compartment and colony formation assays revealed an increase in CFU-M at the expense of CFU-G, suggesting that C/EBPε is not only involved in terminal differentiation of neutrophils beyond the promyelocyte stage[22], but that it may also play a role in the complex process of monocyte versus granulocyte lineage resolution. This is reminiscent of the phenotype seen in mice lacking the Growth factor independent 1 (GFI1) gene, a model for severe congenital neutropenia.[48] Interestingly, in myeloid cells from GFI1$^{-/-}$ mice, C/EBPε expression is not affected, the defect in granulocyte maturation is mediated by lack of repression of colony stimulating factor 1 (CSF1) and its receptor CSF1R.[49] We have previously described a patient with SGD whose myeloid cells show decreased expression of GFI1 while carrying a hemizygous C/EBPε mutation.[45] Functional studies revealed critical interactions between GFI1 and C/EBPε in granulocytic differentiation, suggesting that GFI1 and C/EBPε activate parallel pathways in myeloid maturation.[45] PU.1 is an ets-domain transcription factor, with key functions in lineage decision between the myeloid and lymphoid lineage as well as induction of the monocytic fate. In eosinophil differentiation, C/EBPε directly inhibits PU.1 function via protein-protein interactions.[50] Similarly, C/EBPε mediated repression of PU.1 may be required in granulocytic versus monocytic differentiation. Additionally, the different C/EBPε isoforms may exert differential effects, as recently demonstrated in eosinophil lineage fate decisions by Bedi et al.[26]

In order to further understand the functional defects of C/EBPε$^{-/-}$ neutrophils, we identified transcription factors that were potential downstream targets of C/EBPε. The H2.0-like homeobox 1 *(HLX1, HB24, Hlx)* gene encodes a highly conserved putative homeobox transcription factor[41,51,52]. It is expressed during embryonic development, is essential for fetal liver development, and is expressed in bone marrow where it is upregulated during myeloid differentiation with high expression levels in granulocytes and monocytes, suggesting a role in myeloid differentiation. [42] When overexpressed in immature myeloid cell lines, immature cell surface markers are lost and an altered colony appearance suggests an increased

migratory potential.[28] Divergent functions have been described for Hlx1 in other hematopoietic cells, such as T- and NK cells, and HLX1 polymorphisms have been implicated in Asthma disease severity as well as risk of treatment-related myeloid leukemia.[53–60] Our studies show that Hlx1 expression is decreased in C/EBPε KO versus WT neutrophils derived from the EML cell line, as well as in primary neutrophils from KO and WT mice. Expression of Hlx1 in WT mature neutrophils is higher than in immature neutrophils and in whole bone marrow, confirming its upregulation with granulocytic differentiation. When we overexpress Hlx1 in C/EBPε KO neutrophils, chemotaxis is rescued to near WT levels. However, no change in neutrophil nuclear morphology, lactoferrin expression, or respiratory burst was evident (Supplementary Figure 5).

In summary, we have generated an in vitro cell line model for C/EBPε mediated specific granule deficiency that has allowed us to further understand the role of C/EBPε in myeloid differentiation and to identify potential downstream targets of C/EBPε, including HLX1, that play a role in neutrophil function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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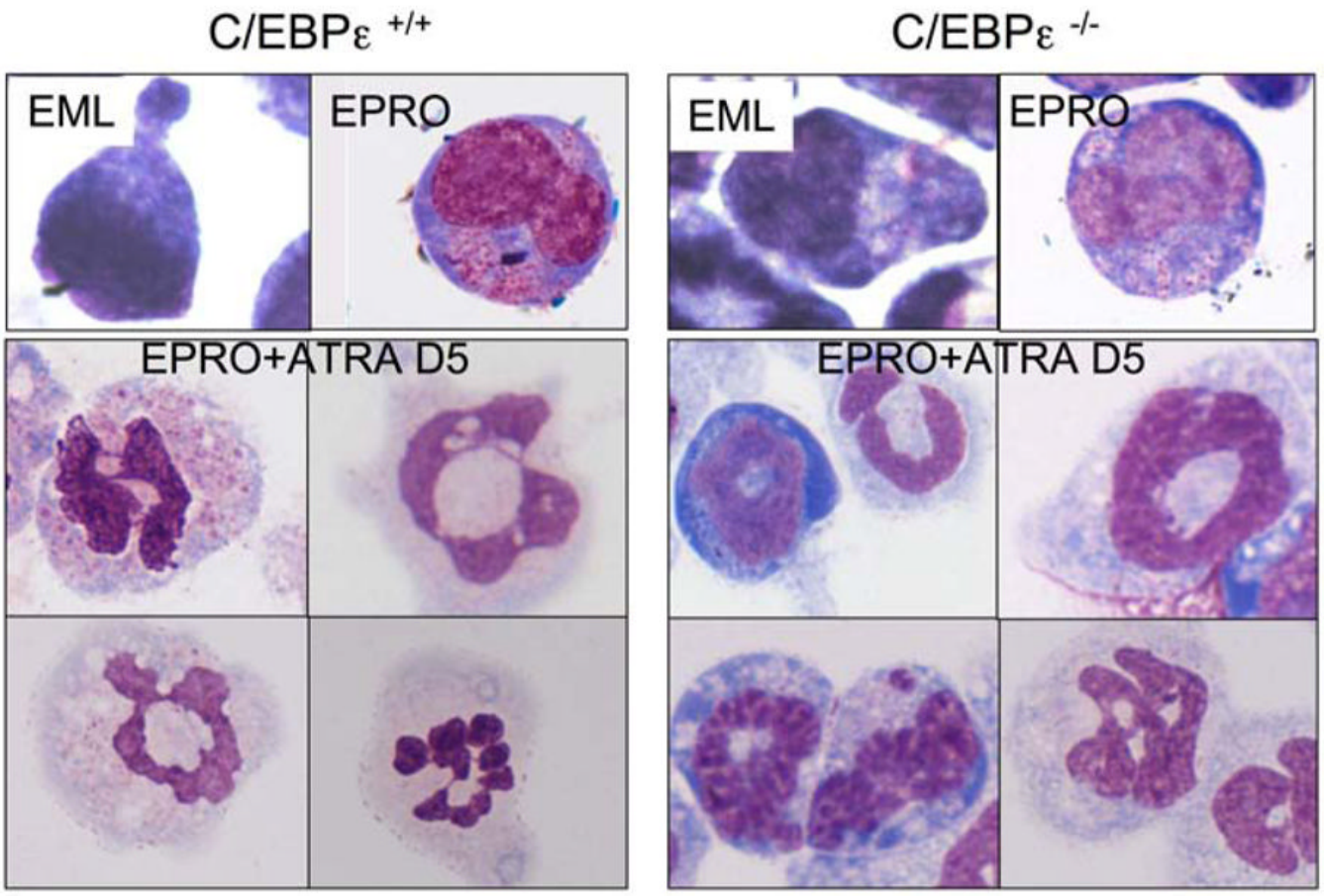

FIGURE 1. C/EBPε$^{-/-}$ cells fail to become fully mature neutrophils

EML cells derived from C/EBPε WT (left panels) and KO (right panels) bone marrow are sequentially induced to the promyelocyte (EPRO) and neutrophil stage (EPRO + ATRA) and stained with Wright-Giemsa stain. C/EBPε cells lack secondary granules and nuclei do not fully mature.

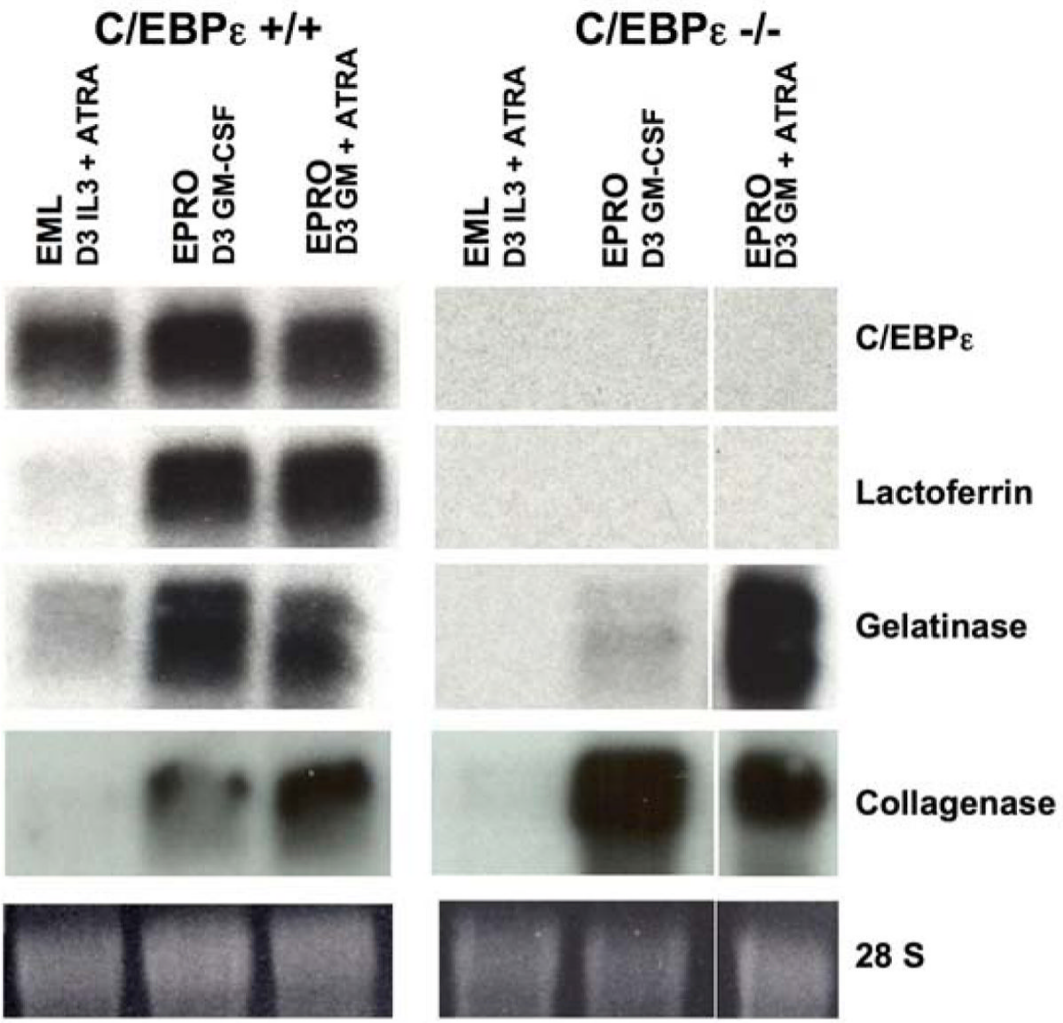

FIGURE 2. C/EBPε$^{-/-}$ cells fail to express lactoferrin

RNA was harvested from C/EBPε WT and KO EML clones after differentiation into neutrophils. The Northern blots were probed with cDNA probes as labeled. Equal RNA loading was verified by 28S band on the ethidium bromide-stained gel (bottom).

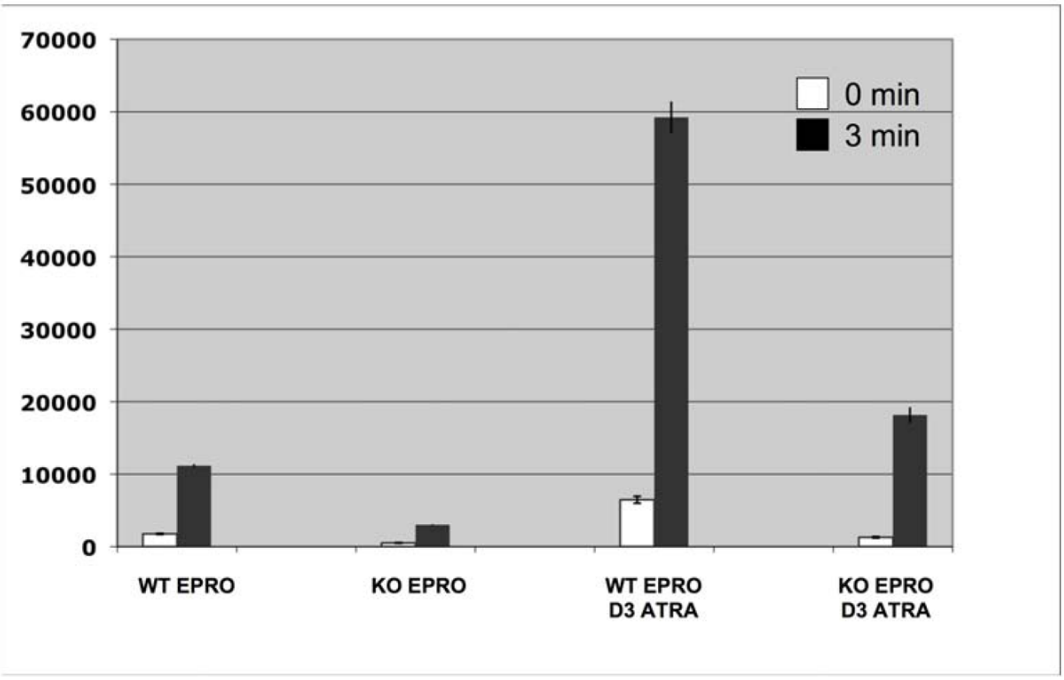

FIGURE 3. Respiratory burst is abnormal in C/EBPε $^{-/-}$ cells

Respiratory burst was assayed by the luminol enhanced chemiluminescence method on uninduced EPRO and on EPRO cells before ("0 min") and after ("3min") stimulation with PMA. A representative experiment of at least 3 is shown.

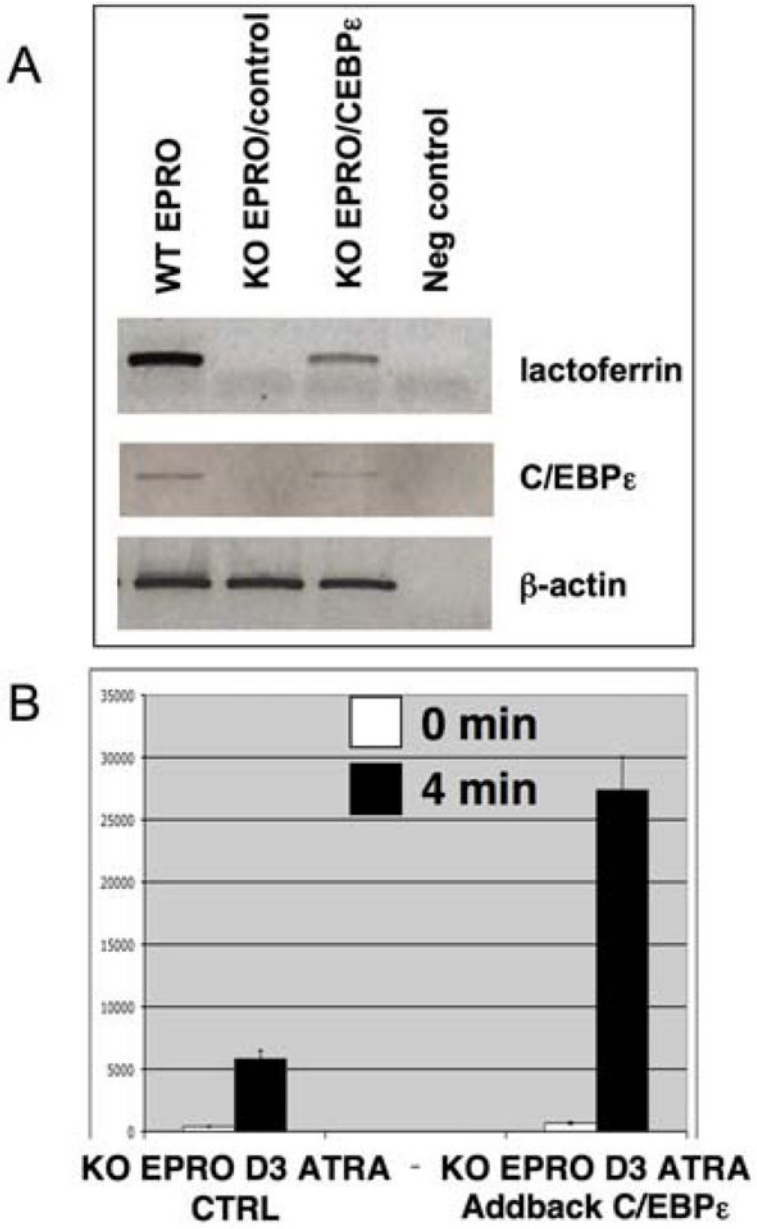

FIGURE 4. Functional defects of C/EBPε $^{-/-}$ cells can be rescued by restoration of C/EBPε expression

KO EPRO cells were transduced with C/EBPε containing and control vectors and assayed for C/EBPε and lactoferrin expression by RT-PCR (A). Respiratory burst was assayed by the luminol enhanced chemiluminescence method before ("0 min") and after ("4 min") stimulation with PMA (B).

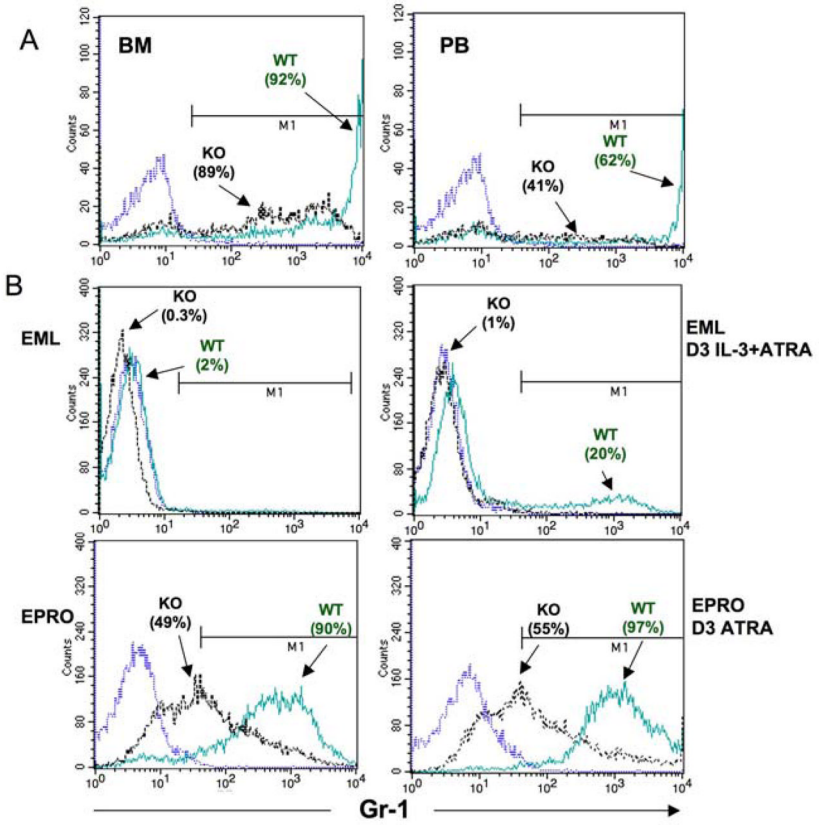

FIGURE 5. Cell Surface marker expression is abnormal in C/EBPε KO neutrophils

(A) Gr1 expression on BM (top left) and PB (top right) from C/EBP**ε** WT (green) and KO (black) mice, and (B) on WT (green) and KO (black) uninduced EML cells (top left), ATRA/IL-3 induced EML cells (top right), EPRO cells (bottom left), and ATRA induced EPRO cells (bottom right). Percentages of Gr1+ cells are shown. Gating was based on the appropriate isotype (blue). A representative experiment of at least 3 independent experiments is shown.

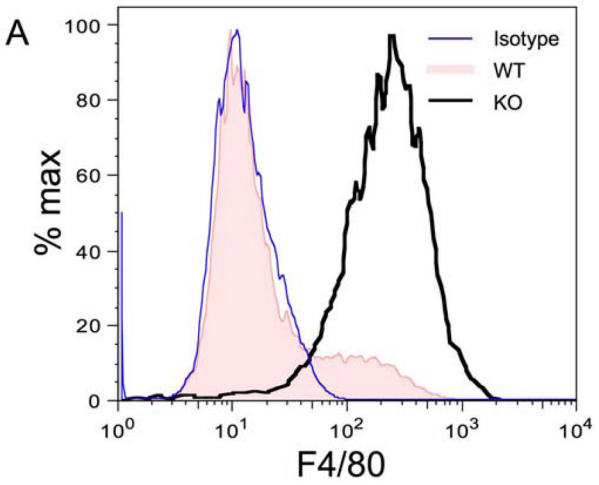

Figure 6A

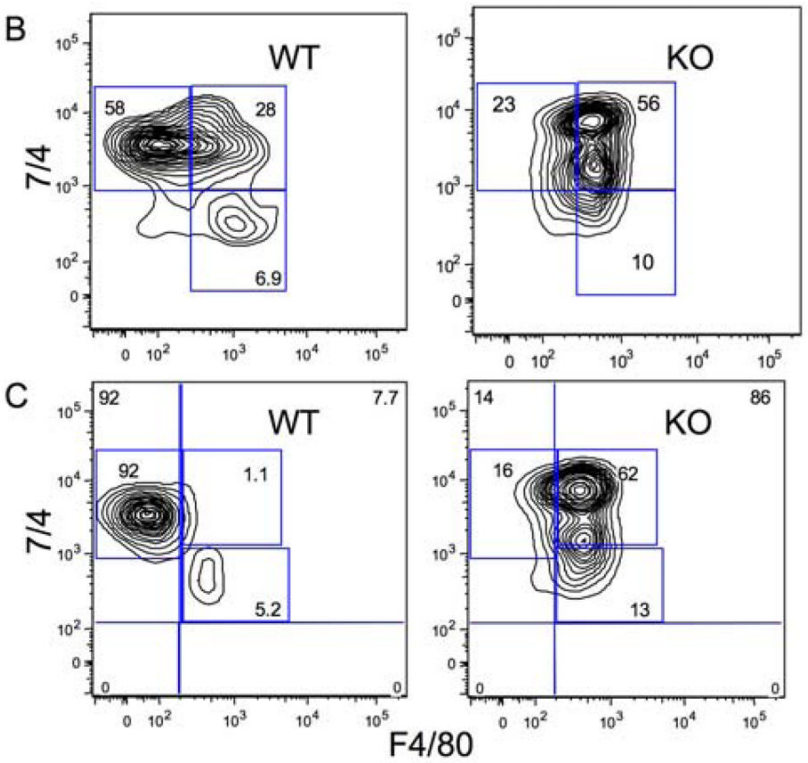

Figure 6B, C

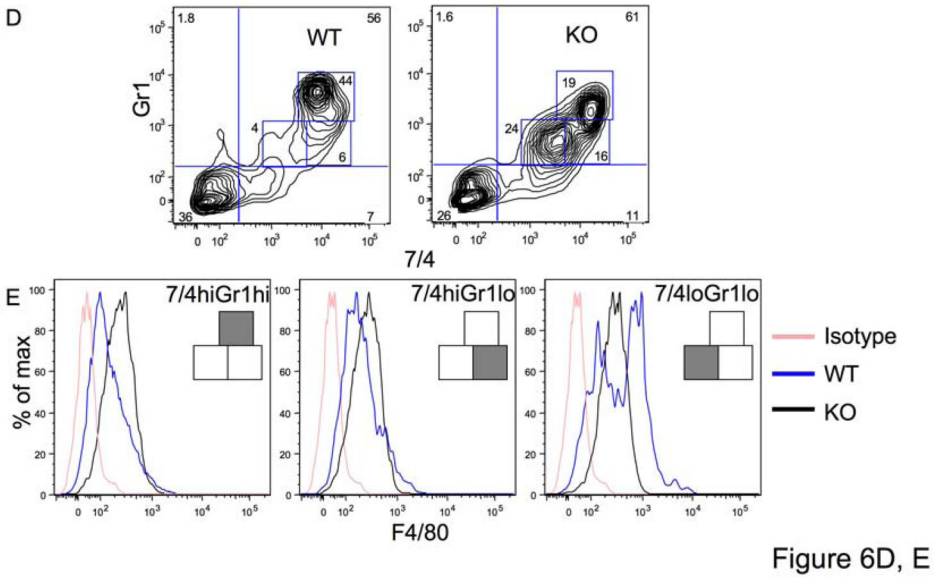

FIGURE 6. ATRA induced C/EBPε KO EPRO cells abnormally retain monocytic cell surface marker expression

(A) C/EBPε WT and KO EPRO cells were differentiated with ATRA and stained for Gr-1 and F4/80. Analysis of F4/80 expression on Gr-1 positive cells is shown for C/EBPε WT (filled histogram) and C/EBPε KO (thick black line) compared to isotype (thin blue line). Analysis of F4/80 and 7/4 co-expression in BM (B) and PB (C) from WT (left) and KO (right) mice. Analysis of 7/4 and Gr1 expression in BM from WT (left) and KO (right) mice (D). F4/80 expression in Gr1/7/4 subsets as shown in D are analyzed for F4/80 expression (E): $7/4^{hi}Gr1^{hi}$ (left), $7/4^{hi}Gr1^{lo}$ (center), and $7/4^{lo}Gr1^{lo}$ (right) myeloid cells in BM from WT (blue) and KO (black) mice. Gating is based on the appropriate isotype controls (pink).

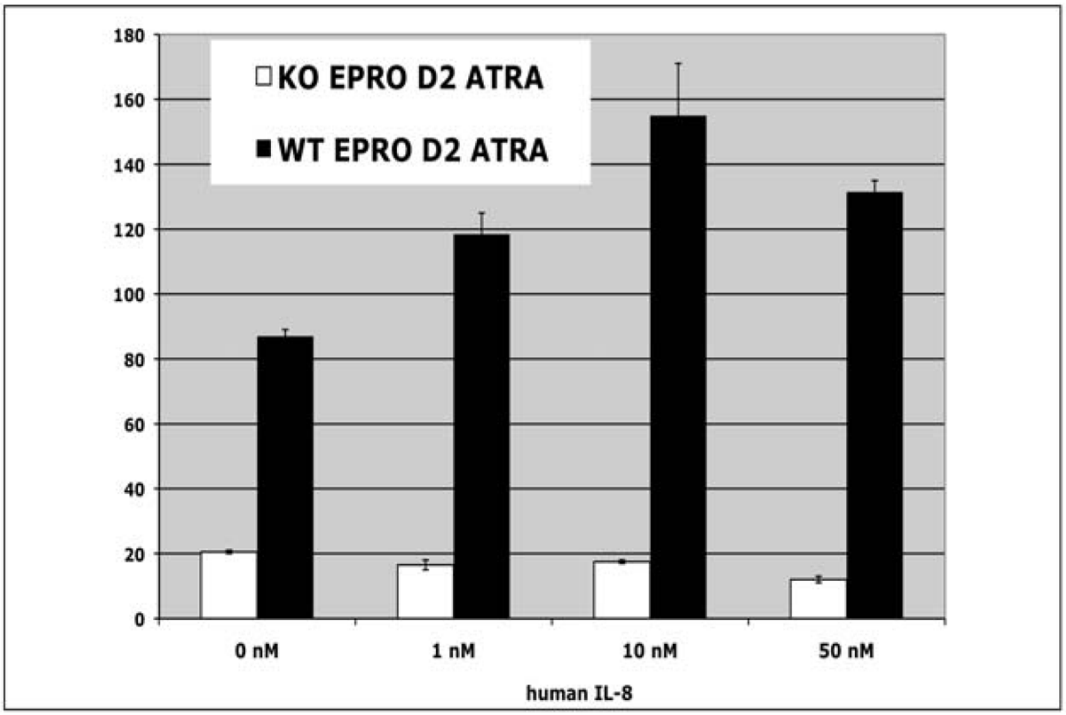

FIGURE 7. Chemotaxis C/EBPε WT and KO neutrophils

ATRA induced KO and WT EPRO cells were assayed for chemotaxis in response to human IL-8 using the transwell assay. The cell number migrated to the bottom well was counted and normalized as described.

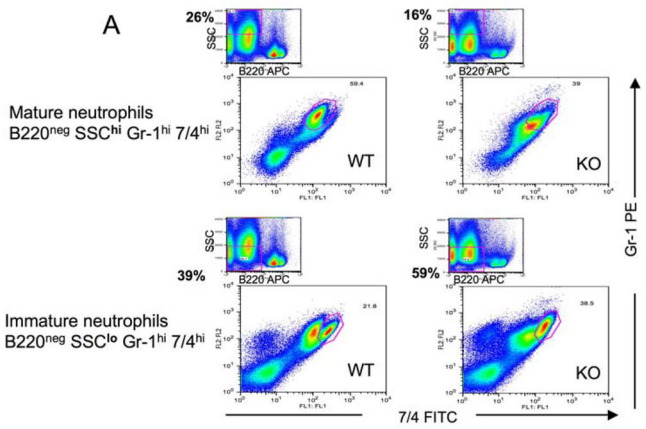

Figure 8A

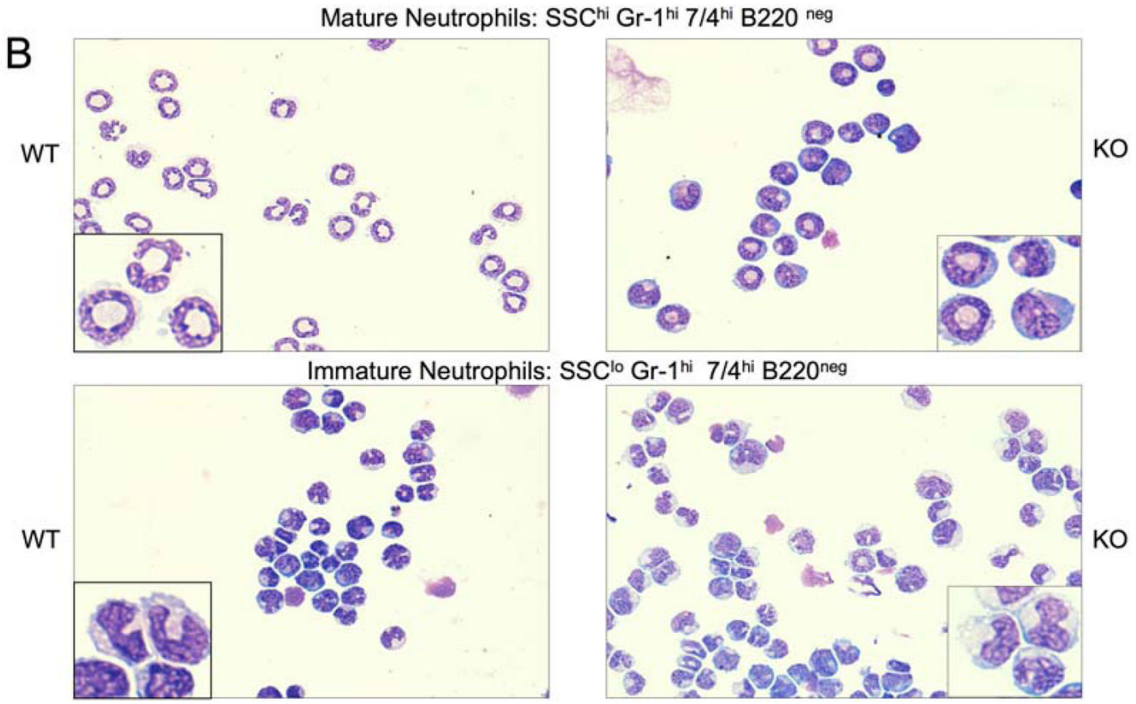

Figure 8B

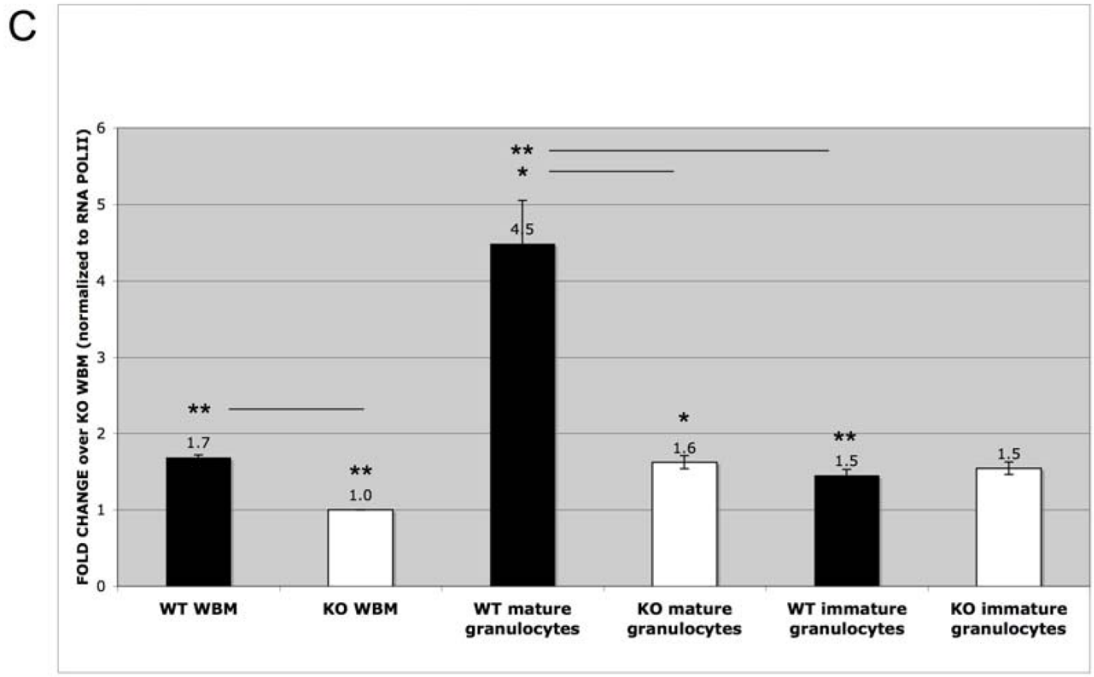

Figure 8C

FIGURE 8. Granulocytes from C/EBPε KO mice fail to fully differentiate and do not upregulate expression of *Hlx1*

Mature ($B220^{-}SSC^{hi}7/4^{hi}Gr1^{hi}$) and immature ($B220^{-}SSC^{lo}7/4^{hi}Gr1^{hi}$) cells from C/EBPε WT and KO BM was sorted (A) and cell purity and cellular morphology verified by Wright Giemsa staining (B). QRT-PCR of *Hlx1* in whole bone marrow (WBM) and sorted mature ($B220^{-}SSC^{hi}7/4^{hi}Gr1^{hi}$) and immature ($B220^{-}SSC^{lo}7/4^{hi}Gr1^{hi}$) granulocytes from C/EBPε WT and KO bone marrow.

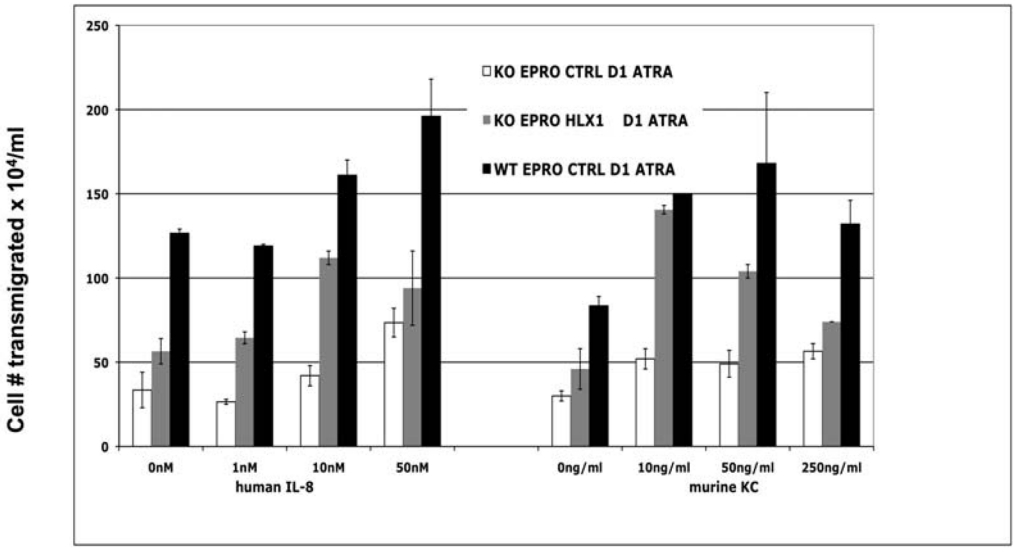

FIGURE 9. HLX1 over-expression rescues chemotaxis in C/EBPε KO ATRA induced EPRO cells

KO and WT EPRO cells were transduced with Hlx1 expressing or control retroviral vectors and assayed for chemotactic response to human IL-8 (left) and murine KC (right) in the transwell assay.