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C/EBPα Induces PU.1 and Interacts with AP-1 and NF-κB to Regulate Myeloid Development

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Abstract

C/EBPα and PU.1 are key regulators of early myeloid development. Mice lacking C/EBPα or PU.1 have reduced granulocytes and monocytes. Consistent with a model in which induction of PU.1 by C/EBPα contributes to monocyte lineage specification, mice with reduced PU.1 have diminished monocytes but retain granulocytes, C/EBPα directly activates PU.1 gene transcription, and exogenous C/EBPα increases monocytic lineage commitment from bipotential myeloid progenitors. In addition to C/EBPα, AP-1 proteins also have the capacity to induce monocytic maturation. C/EBPα:c-Jun or C/EBPα:c-Fos leucine zipper heterodimers induce monopoiesis more potently than C/EBPα or c-Jun homodimers or c-Fos:c-Jun heterodimers. C/EBPs and NF-κB cooperatively regulate numerous genes during the inflammatory response. The C/EBPα basic region interacts with NF-κB p50, but not p65, to induce bcl-2, and this interaction may be relevant to myeloid cell survival and development.

Keywords

C/EBPα; PU.1; c-Jun; NF-κB; myeloid

The focus of this report is our laboratory's work on the role of C/EBPα as a regulator of myeloid (granulocyte versus monocyte) lineage commitment. A comprehensive overview of the regulation of myeloid development may be found in two published reviews [1,2].

Myeloid defects in mice with reduced or absent C/EBPα or PU.1

C/EBPα(−/−) newborn mice lack neutrophils and have reduced hepatic monocytes, and their marrow CFU-M numbers are decreased 2-fold [3]. Deletion of C/EBPα in the marrow of adult mice inhibits the common myeloid progenitor (CMP) to granulocyte-monocyte progenitor (GMP) transition [4]. Consistent with these findings, analysis of an independently derived C/EBPα(−/−) line demonstrates reduced CFU-GM, neutrophils, and monocyte/macrophages [5]. A dominant-inhibitory C/EBP, KRAB-C/EBPα-ER, inhibits murine CFU-G, CFU-M, and CFU-GM formation in IL-3 or GM-CSF [6]. A dominant-inhibitor of C/EBP similarly prevented the formation of G-CSF, M-CSF, or GM-CSF induced human myeloid colonies [7]. Together, these findings indicate that C/EBPα is essential for the formation of both the granulocytic and monocytic lineages.

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PU.1(−/−) mice lack monocytes and have markedly diminished neutrophils, with reduced numbers of GMP [8–10]. Expression of low levels of PU.1 in PU.1(−/−) cells induces granulopoiesis whereas high levels induce monopoiesis [11,12]. Reduction of PU.1 expression allows retention of granulopoiesis but not monopoiesis: Lack of one PU.1 allele gives preference to neutrophil development [11]; deletion of the -14 kb PU.1 distal enhancer results in loss of monopoiesis with preservation of granulopoiesis [13]; and deletion of PU.1 in adult mice leads to loss of monocytes but not granulocytes [14]. Together, these studies indicate that increased PU.1 expression is required for monocyte but not granulocyte lineage development.

C/EBPα induces PU.1 gene transcription

C/EBPα-ER rapidly induces endogenous PU.1 mRNA in Ba/F3 or 32Dcl3 hematopoietic cell lines even in the presence of cycloheximide, but C/EBPα(L12V)-ER, which cannot heterodimerize or bind DNA due to mutation of the first two leucines of the leucine zipper (LZ) to valine, does not induce PU.1 mRNA [15,16]. C/EBPα binds the murine PU.1 promoter in a chromosomal immunoprecipitation (ChIP) assay, and binds the sequence TAGCGCAAG located at -68 in the murine PU.1 promoter and conserved in the human promoter. This site is similar to the C/EBP consensus site, T(T/G)NNGNAA(T/G). However, binding to this site was weak compared to interaction with the neutrophil elastase (NE) promoter, and mutation of the -68 site only reduced activity about 2-fold in 32D myeloid cells [16]. We have more recently investigated regulation of the proximal segment of the PU.1 distal enhancer by C/EBPα. C/EBPα binds this enhancer in a ChIP assay in two myeloid cell lines, and two strong binding sites were identified using the gel shift assay. Mutation of one of these sites reduced enhancer activity, when linked to the PU.1 promoter and luciferase, 2-fold, and mutation of the second site reduced activity 5-fold [17]. These findings strengthen the conclusion that C/EBPα directly regulates PU.1 transcription.

C/EBPα induces monocyte lineage commitment in cooperation with PU.1

To complement gene knockout loss-of-function studies, we have assessed the ability of exogenous C/EBPα to affect myeloid lineage commitment from murine marrow progenitors. As C/EBPα potently inhibits G1 to S cell cycle progression in myeloid cells via interaction with E2F1 and likely other mechanisms [18–21], we developed an assay that minimizes biases due to lineage-specific cell cycle inhibition. One feature of our approach is use of C/EBPα-ER fusion proteins that remain inactive in the absence of estradiol. The second feature is lineage-depletion to remove mature myeloid cells *after* the five days required for retroviral transduction and drug selection rather than the common approach of lineage-depletion prior to transduction. The resulting population consists mainly of immature myeloid cells, and the effect of C/EBPα-ER activation can be studied daily after estradiol addition. By 48 hrs the majority of the cells have regained the Mac-1 and Gr-1 surface markers, making this early time ideal for FACS analysis. For CFU assays, we found that exposure of lineage-depleted cells to estradiol for only one day, followed by plating in the absence of estradiol, was sufficient to see significant changes in lineage specification while minimizing colony suppression due to cell cycle inhibition. C/EBPα-ER induced monocyte formation in liquid culture and CFU-M compared to CFU-G by at least 2-fold, in either IL3/IL6/SCF or GM-CSF [22]. Q-RTPCR analysis of cultured cells demonstrated 2-fold induction of PU.1 mRNA and induction of several monocytic markers, with reduction of the MPO and NE granulocyte markers. PU.1-ER also induced monocytic development in this assay.

The conclusion that C/EBPα has the capacity to induce monopoiesis is consistent with findings from other groups. C/EBPα induces monocytic but not granulocytic development in B or T cell progenitors [23–25], and MEP or CLP isolated from mice expressing C/EBPα-ER(T) from the H2K promoter develop into macrophages upon exposure activation of the transgene [26].

In contrast, C/EBPα-ER induces granulopoiesis in 32Dcl3 myeloid cells [15], perhaps reflecting their commitment to the myeloid lineage.

C/EBPα zippers with AP-1 proteins to favor monopoiesis

The initial suggestion that AP-1 proteins favor monocytic development comes from the finding that c-Jun or c-Fos can induce monocytic differentiation when expressed in myeloid cell lines and from the observation that phorbol esters rapidly induce AP-1 proteins and direct myeloid cell maturation to monocytes [27–29]. MafB can zipper with c-Fos, and c-Maf zippers with c-Jun, JunB, or c-Fos [30]. MafB is expressed predominantly in monocytic cells within hematopoiesis, and MafB or c-Maf also induce monocytic maturation [31–33].

C/EBPα binds c-Jun, but only if the LZ is present in both proteins [34]. To demonstrate that this interaction reflects zippering we employed two approaches. First, we swapped the LZ of c-Jun into C/EBPα and expressed this fusion protein with the shorter C/EBPα bZIP domain in 293T cells. Nuclear extracts from these cells bound a C/EBP consensus site in a gel shift assay, producing a band of intermediate size between full-length C/EBPα or the bZIP domain alone, which could only occur if the c-Jun and C/EBPα LZs zippered together, positioning the basic regions properly to contact the DNA major groove. Second, mutation of the first two leucines of the C/EBPα LZ prevented co-ip with c-Jun, Jun, or c-Fos. In addition, we confirmed the prior finding that endogenous C/EBPα and c-Jun co-ip from myeloid nuclear extracts [34], and we also found that C/EBPα co-ips with endogenous c-Fos or JunB [35]. JunB and c-Fos, but not MafB or c-Maf, also zippered with C/EBPα or C/EBPβ in the zipper swap:gel shift assay.

To assess the functional consequence of C/EBPα interaction with AP-1 proteins, we developed a procedure to direct specific heterodimerization by introduction of LZs that contain either negatively charged glutamic acid or positively charged lysine in the eight salt bridge positions (LZE or LZK). Combinations of ER fusion proteins were then transduced using pBabePuro and MIGR1 retroviral vectors, followed by puromycin selection and finally lineage-depletion. Gating on GFP^+ cells allowed FACS analysis of myeloid development in liquid culture, and flow cytometry for GFP^+ cells allowed CFU assays. Strikingly, forced heterodimerziation of C/EBPα with c-Jun or c-Fos markedly induced monopoiesis, the C/EBPα homodimer only mildly induced monopoiesis, and neither c-Fos:c-Jun nor c-Jun homodimers induced monocytic development [35]. In contrast to the inactivity of the c-JunLZE:c-JunLZK combination, wild-type c-Jun linked to ER potently induced monopoiesis, reminiscent of the activity of wild-type C/EBPα linked to ER. These findings suggest that C/EBPα-ER or c-Jun-ER heterodimerize with endogenous c-Jun or C/EBPα, respectively, to induce monopoiesis.

C/EBPα:AP-1 heterodimers may bind novel DNA elements to contribute to monocyte lineage development. Identifying such sites and the genes regulated by C/EBP:AP-1 heterodimers is likely more broadly relevant, given the widespread expression of these bZIP families. Using nuclear extracts from 293T cells expressing C/EBPαLZK and c-JunLZE, we carried out an oligonucleotide selection procedure, modifying the usual approach by alternating use of C/EBPα and c-Jun antiserum for each immunoprecipitation. In this manner, we selected a consensus site consisting of an AP-1 and C/EBP half-sites. Notably, the weak C/EBPα-binding site located in the PU.1 promoter is similar to this site and in fact binds C/EBPαLK:c-JunLZE in a gel shift assay. In addition, C/EBPαLZK:c-JunLZE transiently expressed in 293T cells activated the PU.1 promoter linked to luciferase, and the combination of C/EBPαLZK-ER and c-JunLZE-ER stably expressed in Ba/F3 cells bound the PU.1 promoter in the ChIP assay and induced endogenous PU.1 RNA expression (D.H. Cai, C. Yeamans, and A.D.F., unpublished). Activation of the PU.1 promoter by C/EBPα:c-Jun may contribute to induction of monocyte lineage specification by C/EBPα:AP-1 heterodimers. Microarray and ChIP on chip analysis

will likely identify additional genetic targets of relevance to myelopoiesis and other developmental and cellular processes.

C/EBPα interacts with NF-κB to regulate inflammation, survival, and potentially myelopoiesis

C/EBPs and NF-κB cooperate to induce the IL-6, IL-8, G-CSF, serum amyloid, ICAM-1, superoxide dismutase, and Mediterranean fever promoters during the inflammatory response [36–40]. The bZIP domain of C/EBPα or C/EBPβ interacts with NF-κB p50 or p65 [41,42]. C/EBPα or C/EBPβ cooperates specifically with NF-κB p50 to activate the bcl-2 P2 promoter, leading to inhibition of apoptosis in myeloid or lymphoid cells. Endogenous C/EBPα preferentially interacts with endogenous NF-κB p50 compared with p65 in extracts from the HL-60 or U937 myeloid cell lines. This increased affinity may result from contact with the outer, non-DNA contact surface of the C/EBPα basic region, as mutation of this domain prevents interaction with NF-κB p50 but not p65 [43]. Of note, the C/EBPα basic region that contacts NF-κB p50 is identical in C/EBPβ and C/EBPδ, and their leucine zipper domains, which may also contribute to interaction with NF-κB p50 and p65, are also highly related. In contrast, the N-terminal trans-activation regions of these C/EBPs are more divergent. The interaction of NF-κB with the most conserved region of C/EBPα and C/EBPβ suggests the importance of this interaction for the evolutionarily ancient innate inflammatory immune response of myeloid cells. Infectious challenges not only activate existing inflammatory cells but also stimulate production of new myeloid cells. We therefore speculate that activated NF-κB cooperates with C/EBPα and C/EBPβ to direct marrow progenitors to produce additional neutrophils and monocytes. Although myeloid defects were not observed in p50(−/−) mice, other NF-κB family members may compensate [44].

Summary and future directions

Onset of C/EBPα expression in multipotent stem/progenitors cells, such as the lymphoid-myeloid progenitor (LMMP) or CMP may direct these cells to the GMP state, initiating myeloid development (Fig. 1). In this model, induction of PU.1 in cooperation with AP-1 and monocyte-specific cytokine signals directs monopoiesis, and C/EBPα and reduced PU.1 cooperate with other transcription factors to d9rect granulopoiesis. Many key questions remain. It will be of interest to identify monocytic genes dependent upon increased PU.1 for expression and to determine if there are common features to their regulatory regions. Similarly, we have initiated efforts to identify genes activated cooperatively by C/EBPα and c-Jun or by C/EBPα and NF-κB. In addition, to C/EBPα, PU.1, and AP-1 proteins, key regulators of myeloid maturation include Gfi-1, RARα, Vitamin D Receptor, HoxA10, Egr-1 and Egr-2, IRF-8, c-Maf and MafB, C/EBPε, and CDP [2]. It will be of interest to determine which of these factors acts at the earliest stages of myeloid lineage commitment together with C/EBPα and PU.1 and which act downstream. Ras was recently found to induce monocyte lineage commitment dependent upon PKC activation in human marrow cells [45]. Determining whether monocyte-inducing cytokines such as M-CSF provide signals different from granulocyte-inducing cytokines such as G-CSF that impact on myeloid transcription factors to direct lineage commitment is also an important area for future investigation.

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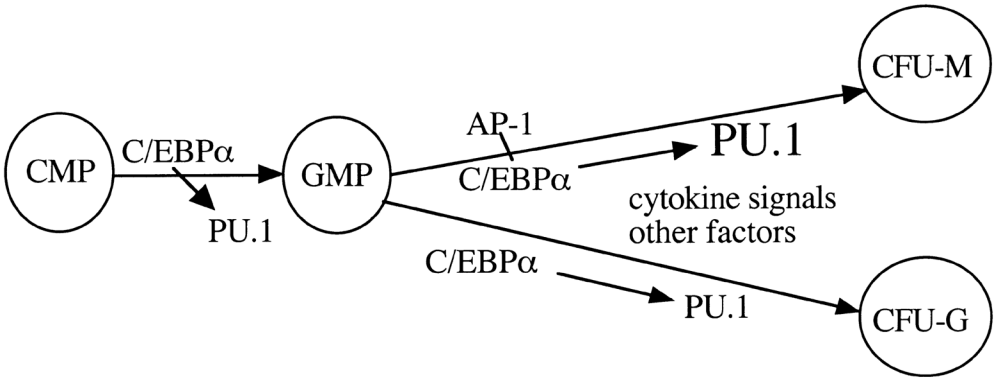

Figure 1.
Model for the role of C/EBPα in myeloid lineage commitment. In this model, C/EBPα induces PU.1 in the CMP to specify the GMP. Further induction of PU.1 in cooperation with AP-1, monocytic cytokine signals and potentially additional transcription factors specifies the monocyte lineage. In the presence of C/EBPα and alternative cytokine signals and transcription factors, PU.1 is not further induced and the GMP is directed to granulocytic development.