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C/EBP α Dysregulation in AML and ALL

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Abstract

The transcription factor CCAAT/Enhancer Binding Protein α (C/EBP α) is a critical regulator of myeloid development, directing granulocyte and monocyte differentiation. As such, it is dysregulated in over half of patients with acute myeloid leukemia (AML). C/EBP α expression is suppressed as result of common leukemia-associated genetic and epigenetic alterations such as AML1-ETO, BCR-ABL, FLT3-ITD, or *CEBPA* promoter methylation. In addition, 10–15% of patients with AML with intermediate risk cytogenetics are characterized by mutations of the *CEBPA* gene. Two classes of mutations are described. N-terminal changes result in expression of a truncated dominant negative C/EBP α 30 isoform. C-terminal mutations are in-frame insertions or deletions resulting in alteration of the leucine zipper preventing dimerization and DNA binding. Often, patients carry both N- and C-terminal mutations each affecting a different allele, and a mouse model recapitulates the human phenotype. Patients with mutated *CEBPA* AML comprise a clinically distinct group with favorable outcome consistently seen in patients with biallelic mutations. In addition, C/EBP family members are aberrantly expressing from the immunoglobulin heavy chain locus in 2% of pre-B ALLs. This review summarizes the normal hematopoietic developmental pathways regulated by C/EBP α and discusses the molecular pathways involved in mutated *CEBPA* AML and ALL.

Keywords

leukemia; myeloid; differentiation; hematopoiesis

I. REGULATION OF NORMAL MYELOID DEVELOPMENT BY C/EBP α

A. The Biochemistry of C/EBP α

CCAAT/Enhancer Binding Protein α (C/EBP α) contains 358 amino acid residues, with the intronless human *CEBPA* gene located on chromosome 19q13.1. C/EBP α has an α -helical, 86 residue, C-terminal basic region-leucine zipper (BR-LZ or bZIP) DNA-binding domain.¹ The LZ contains a hydrophobic surface allowing homo-dimerization or hetero-dimerization with other bZIP proteins as a coiled-coil structure, thereby positioning the more N-terminal BR to enter the major groove and contact DNA (Figure 1).² Additional members of the C/EBP family of bZIP transcription factors include C/EBP β , C/EBP δ , and C/EBP ϵ . C/EBP as obligatory homo- or hetero-dimers bind the DNA motif 5'-T(T/G)NNGNAA(T/G). C/EBP proteins also hetero-dimerize with members of the CREB or AP-1 families of bZIP proteins to bind hybrid DNA elements.^{3,4} Once bound to DNA, C/EBP α activates transcription via its two N-terminal trans-activation domains.⁵

Full-length C/EBP α is 42 kd in molecular weight. Initiation of translation from an internal ATG located at amino acid 120 of the human protein leads to co-expression of a shorter, 30 kd isoform in a subset of normal tissues, though typically the p30 isoform is less abundant (Figure 1).⁶ C/EBP α p30 retains the ability to dimerize and bind DNA but lacks a potent trans-activation domain (TAD), allowing the p30 isoform to dominantly inhibit trans-activation by C/EBP α 42, at least for a subset of C/EBP target genes.

B. The Biology of C/EBP α During Normal Myelopoiesis

Within hematopoiesis, C/EBP α is specifically expressed in granulocytes, monocytes, and eosinophils,⁷ though it is also found in hepatocytes, adipocytes, and type II pneumocytes.⁸ Low level C/EBP α expression is detectable in the hematopoietic stem cell (HSC) population, and expression increases as these cells develop into the common myeloid progenitor (CMP) and subsequently into the granulocyte-monocyte progenitor (GMP); C/EBP α levels finally diminish as immature myeloid cells mature to neutrophils or monocytes.^{7, 9} Deletion of the C/EBP α gene leads to arrest at the CMP to GMP transition, with reduced formation of both granulocytes and monocytes.⁹ When expressed in 32Dcl3 cells, representative of granulocytic progenitors, exogenous C/EBP α directs granulopoiesis.¹⁰ However, transduction of marrow cells with C/EBP α leads to increased monopoiesis at the expense of granulopoiesis¹¹ – this may reflect formation of C/EBP:AP-1 hetero-dimers, as C/EBP α proteins containing artificial acid and basic LZs that force their homo-dimerization do not induce increased monopoiesis, whereas forced hetero-dimerization of C/EBP α with c-Jun or c-Fos strongly favors monocytic development.¹² Induction of AP-1 proteins in myeloid cell lines using phorbol ester or IL-6 allows formation of endogenous C/EBP:AP-1 complexes during monopoiesis, whereas C/EBP α homodimers are more abundant in cells undergoing granulopoiesis in response to G-CSF.³ Reduced levels or activity of C/EBP α may be sufficient for monopoiesis via interaction with AP-1 proteins but not for granulopoiesis, as suggested by the finding that absence of RUNX1 or NF- κ B p50 leads to about 25% of normal C/EBP α protein expression in marrow and diminished granulopoiesis.^{11, 13}

In addition to interacting with AP-1 proteins, C/EBP α may stimulate monopoiesis by inducing transcription of the *PU.1* gene via interaction with its promoter and -14 kb distal enhancer.^{14, 15} Notably, in contrast to C/EBP α reduced PU.1 protein levels due to gene or enhancer deletion favors granulopoiesis over monopoiesis,^{16–18} and exogenous C/EBP α induces granulopoiesis rather than monopoiesis in marrow cells lacking the *PU.1* distal enhancer.¹⁵ A summary of the transcriptional control of myeloid development by RUNX1, C/EBP α , PU.1 and cooperating factors is shown (Figure 2).

As a major regulator of differentiation C/EBP α has a strong anti-proliferative effect,^{19, 20} and in myeloid cells it inhibits the progression from G1 to S phase.¹⁰ This effect is independent of DNA binding²¹ and occurs at least in part through protein:protein interaction with E2F mediated by the basic region.²² As a result, C/EBP α down regulates pro-proliferative transcription factors such as c-Myc.²³ Inhibition of cell cycle is part of the differentiation program induced by C/EBP α but is not sufficient by itself to direct terminal differentiation.²¹

Cytokine signals cooperate with C/EBP α to regulate myeloid development. Study of individual GMP exposed to G-CSF or M-CSF demonstrates that these cytokines not only provide survival and proliferative signals but also contribute to lineage specification.²⁴ Comparison of M-CSF with G-CSF signaling in lineage-negative marrow cells demonstrates that M-CSF more potently activates ERK via PLC γ , whereas G-CSF more potently activates STAT3 and specifically induces SHP2 tyrosine phosphorylation.²⁵ Moreover, ERK inhibitors reduce formation of monocytic progenitor colonies whereas a SHP2 inhibitor

reduces formation of granulocytic colonies. shRNA-mediated knockdown of SHP2 similarly reduces granulopoiesis via inhibition of *CEBPA* gene transcription.²⁶ G-CSF activation of SHP2 may increase *CEBPA* mRNA expression due to the ability of SHP2 to increase the activity of RUNX1,²⁷ with RUNX1 then directly inducing *CEBPA* gene transcription.¹³ ERK phosphorylates C/EBP α serine 21, reducing the activity of C/EBP α , perhaps by weakening its N-terminal TAD.²⁸ Partial inactivation of C/EBP α by ERK downstream of M-CSF receptor would be expected to favor monoopoiesis – in particular, weakened C/EBP α might be incapable of directing granulopoiesis but remain able to hetero-dimerize with AP-1 proteins to direct monoopoiesis.

II. C/EBP α DYSREGULATION DURING MYELOID TRANSFORMATION

Two mechanisms affect C/EBP α function in AML: reduced expression as a downstream result of other AML-related mutation or mutation of the *CEBPA* gene.

A. Inhibition of C/EBP α in AML

Inhibition of C/EBP α expression or activity occurs via several mechanisms in different subsets of AML. As discussed above, deletion of the RUNX1 gene reduces *CEBPA* expression, and RUNX1 might directly activate *CEBPA* transcription. Related to these findings, mutation of RUNX1 leading to reduced RUNX1 levels or expression or fusion proteins that dominantly inhibit RUNX1 activity occurs in at least 30% of AML cases.²⁹ In particular, t(8;21) leads to expression of RUNX1-ETO, which binds DNA via RUNX1 *cis* elements to repress target expression; inv(16) leads to expression of CBF β -SMMHC, which interacts with RUNX1 to either sequester RUNX1 off chromatin or inhibit its activity on chromatin; t(3;21) expresses RUNX1-MDS1/EVI1 which also inhibits RUNX1 activity; and point mutations that inactivate RUNX1, the majority heterozygous, are present in a subset of patients.^{29, 30} RUNX1-ETO directly represses the *CEBPA* promoter, and blasts from patients with t(8;21)-associated AML indeed have reduced C/EBP α protein levels.³¹ Abnormal expression of EVI1 through translocations involving chromosome 3, is associated with high risk AML or MDS. EVI1 fusion protein expression is associated with translational suppression of C/EBP α expression.³²

Signaling pathways activated in AML can inhibit C/EBP α expression or activity. The activated receptor tyrosine kinase receptor mutant, FLT3-ITD, found in 30% of AML cases, reduces *CEBPA* transcription and leads to ERK modification of C/EBP α S21 to reduce the activity of C/EBP α .^{33, 34} BCR-ABL, an intracellular, constitutively active tyrosine kinase, inhibits translation of the *CEBPA* mRNA.³⁵ Trib2 induces C/EBP α proteosomal degradation, dependent upon interaction with COP1.³⁶

CEBPA promoter methylation is found in half of AML cases, often as the indirect consequence of several of the above mechanisms that down-modulate *CEBPA* transcription, and is most commonly associated with inv(16) and t(15;17).³⁷ In addition, a subgroup of AML whose gene expression profile aggregates with mutated *CEBPA* AML is characterized by *CEBPA* silencing through promoter hypermethylation and expression of T cell markers. The majority of these patients harbor activating *NOTCH1* mutations and have poor outcome.^{38, 39} Finally, the *CEBPA* gene open-reading frame itself is subject to mutation in approximately 10% of AML cases. Biologically and clinically this is a distinct subtype of AML as recognized in the 2008 World Health Organization classification of myeloid neoplasms⁴⁰ and elaborated on the next section.

B. *CEBPA* Gene Mutation in AML

As a key regulator of myeloid differentiation *CEBPA* is mutated in approximately 5–15% of patients with AML.^{41–45} Two categories *CEBPA* mutations are found in AML cases (Figure 1).^{42, 44} Interestingly, C/EBP α null mutations are rare, and the mutated proteins are expressed by the leukemic blasts, suggesting a selective pressure and an active role in leukemogenesis for the mutated C/EBP α proteins. N-terminal mutations typically lead to premature termination and translational reinitiation at methionine 120, leading to expression of the N-terminally-truncated C/EBP α 30, lacking a major TAD. C/EBP α 30 retains the capacity to bind DNA and to hetero-dimerize with C/EBP α 42, thereby dominantly inhibiting C/EBP α 42-mediated trans-activation. In addition, C/EBP α 30 induces expression of Ubc9, an E2 conjugating enzyme, which in turn sumoylates C/EBP α 42 on lysine 161. Sumoylated C/EBP α 42 has reduced capacity to activate transcription or slow proliferation.^{46, 47} C-terminal mutations typically occur in the vicinity of the first α -helix of the LZ, preventing dimerization and therefore preclude DNA-binding. Strikingly, these C/EBP α LZ variants are in-frame insertions or deletions, indicating that the resulting proteins contribute to leukemic transformation. Indeed, although they themselves cannot bind DNA, several C/EBP α LZ oncoproteins inhibit apoptosis via induction of bcl-2 or Flice inhibitory protein, dependent upon interaction of their BR with NF- κ B p50 bound to DNA in the promoter regions of these target genes.^{48, 49} Interaction of C/EBP α LZ oncoproteins with NF- κ B p50 displaces HDACs, inducing transcriptional derepression, with the C/EBP α TAD then directing transcriptional activation.⁵⁰

In two-thirds of AML cases harboring *CEBPA* gene mutations, one allele harbors an N-terminal variant and the other allele a C-terminal variant. Of note, in a mouse model, C/EBP α 30 and a C/EBP α LZ oncoprotein synergistically contribute to AML formation.⁵¹ Patients with mutated *CEBPA* AML typically present with myeloblastic French-American-British types M1 or M2 morphology and associated with the following immunophenotype: HLA-DR(+), CD7(+), CD13(+), CD14(–), CD15(+), CD33(+), CD34(+).^{41–44, 52} *CEBPA* mutations occur almost exclusively in patients with intermediate-risk cytogenetics and predominantly in those with normal karyotype. Moreover, other Class II mutations such as Core Binding Factor (CBF) leukemia, mutated *NPM1*, or *MLL-PTD* only rarely overlap with mutated *CEBPA*.^{41, 43, 45, 53}

Multiple cooperative groups reported that patients with mut*CEBPA* have significantly improved outcome compared to wt*CEBPA*, in par with patients with favorable risk cytogenetics, such as CBF leukemia. Further analysis of several,^{53–57} but not all,^{43, 57} studies suggests that this benefit is restricted to patients with biallelic mutations. Further, patients with biallelic *CEBPA* mutations have a specific gene expression pattern, while monoallelic mutations do not aggregate in a specific pattern.^{56, 57} Of particular importance to risk stratification of normal karyotype AML is the interplay of several prognostic markers. For example, mutated *NPM1*/wt-FLT3 cases have a favorable outcome while wt-*NPM1*/FLT3-ITD is associated with a dismal prognosis. *NPM1* is rarely mutated in patients with biallelic mut*CEBPA*, and these patients have a 2–4-fold lower incidence of FLT3-ITD.^{41, 45, 53, 57} Interpretation of the combinatorial effect of FLT3-ITD and mut*CEBPA* is complicated by inconsistent analysis of mono vs. biallelic mutations and FLT3-ITD allelic ratio. However, in several reports mut*CEBPA* predicts favorable outcome independent of FLT3 status.^{41, 43, 57–59} The incidence of FLT3-ITD or mutated *NPM1* is similar in wt*CEBPA* and monoallelic mut*CEBPA*.^{53, 57}

Germline *CEBPA* N-terminal mutation were found in pedigrees with familial AML, and progression to AML is typically associated with acquisition of a somatic C-terminal mutation.^{57, 60, 61} Importantly, approximately 10% of patients with mut*CEBPA* AML harbor a germline mutation.^{57, 61}

MicroRNAs (miRNA) are small (20–22 bp) noncoding RNAs that play a key role in transcriptional regulation. miRNAs silence target genes by binding untranslated regions of mRNA resulting in translation inhibition or cleavage of coding mRNA. Several miRNAs are induced by C/EBP α in the course of normal myeloid differentiation.^{62–64} Dysregulation of miRNA expression is increasingly appreciated as a common feature of cancer. Global miRNA expression patterns accurately classify molecular subtypes of AML, including mutCEBPA leukemia that is characterized by down regulation of multiple miRNAs including the 181 family and miR-34a.^{59, 65} C/EBP α induces expression of miR-34a to silence E2F3 and suppress proliferation during normal granulopoiesis. Restoration of miR-34a expression in mutCEBPA AML blasts slows proliferation and induces differentiation.⁶⁴

In contrast to *RUNX1* and *CEBPA*, mutation of the PU.1 gene is rare in AML cases, despite the finding that deletion of the PU.1 –14 kb distal enhancer in mice leads to 20% of control PU.1 expression and highly penetrant AML.¹⁸ Nevertheless, as both RUNX1 and C/EBP α activate the PU.1 distal enhancer,^{15, 66} their diminished expression or activity would be expected to lead to reduced PU.1 levels, contributing to AML formation.

Besides stimulating myeloid differentiation and inhibiting apoptosis in cooperation with NF- κ B p50, C/EBP α inhibits G1 to S cell cycle transition via direct interaction with E2F in myeloid progenitors, and this effect requires integrity of the C/EBP α N-terminus via an unclear mechanism.^{20–22} Thus, reduced C/EBP α expression or its N-terminal mutation might contribute to the myeloid transformation in part via removal of this cell cycle inhibitory effect.

III. C/EBP α OVEREXPRESSION IN ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

Within hematopoiesis, C/EBP α is restricted to the myeloid lineages and it is not expressed in lymphocytes or their progenitors or in the erythroid/megakaryocytic lineages⁷. Moreover, ectopic expression of C/EBP α leads to suppression of Pax5, a central transcription factor in B-cell development, and redirects the fate of B cells into macrophages⁶⁷. Overexpression of wild type C/EBP α occurs in B precursor ALL carrying the t(14;19)(q32;q13) translocation which juxtaposes *CEBPA* and the immunoglobulin heavy chain enhancer locus.⁶⁸ The leukemic blasts from patients with pre-B ALL carrying this translocation express high levels of wild type C/EBP α mRNA and protein.⁶⁸ Importantly, no mutations were found in *CEBPA*, and the AML-associated p30 isoform⁴⁴ was not excessively expressed. Akasaka et al extended these findings and demonstrated involvement of other C/EBP family members in translocations with the IgH locus in pre-B ALL patients, and consequently overexpression of wild type C/EBP α , C/EBP β , C/EBP δ , C/EBP ϵ , or C/EBP γ in approximately 2% of patients with pre B ALL.⁶⁹ The similar phenotype resulting from translocations of the various C/EBPs suggests an important role for the bZIP domain that is highly conserved among the different family members. Of note, this region mediates protein:protein interaction with NF- κ B p50 and consequent induction of bcl-2 and FLIP and protection from apoptosis.^{22, 48, 49}

IV. SUMMARY

C/EBP α is a key mediator of normal myeloid differentiation, contributing to both granulopoiesis and monopoiesis. C/EBP α also inhibits cell cycle progression and stimulates cell survival in cooperation with NF- κ B p50. Alterations in the *CEBPA* gene or in pathways that down-modulate C/EBP α expression at the transcriptional, translational, or post-translational levels likely contribute to myeloid transformation by inhibiting myeloid

differentiation while favoring myeloid progenitor cell cycle progression. In addition, C/EBP α 30, C/EBP α LZ variants, or residual wild-type C/EBP α may also contribute to myeloid transformation by inhibiting apoptosis. Restoration of C/EBP α expression in AML might provide a means to induce cell differentiation and slow cell proliferation to contribute to AML therapy.

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Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
BR-LZ or bZIP	basic region-leucine zipper
C/EBPα	CCAAT/Enhancer Binding Protein α
CMP	common myeloid progenitor
GMP	granulocyte-monocyte progenitor
HSC	hematopoietic stem cell
miRNA	microRNA
TAD	trans-activation domain

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Figure 1. Diagram depicting C/EBP α p42, truncated C/EBP α p30, and the location of C/EBP α LZ in-frame insertions and deletions. BR, basic region; LZ, leucine zipper; TAD, trans-activation domain.

Figure 2.

Model for the transcriptional control of myeloid development by RUNX1, C/EBPα, and PU. 1. RUNX1 stimulates transcription of the genes encoding C/EBPα and PU.1, and C/EBPα also activates the PU.1 gene. C/EBPα then hetero-dimerizes with AP-1 proteins and cooperates with PU.1 to favor monopoiesis, whereas C/EBPα homo-dimers cooperate with NF-κB p50 to favor granulopoiesis.