Aus der Medizinischen Klinik und Poliklinik III am Klinikum Großhadern
der Universität München,
Vorstand: Prof. Dr. med. W. Hiddemann

The granulocytic inducer C/EBP inactivates the myeloid master regulator PU.1: possible role in lineage commitment decisions

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

Vorgelegt von
Venkateshwar A Reddy
Aus Kagaznagar/Indien

2002

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:	Prof. Dr. med. W. Hiddemann
Mitberichterstatter:	Prof. Dr. P.B. Becker Prof. Dr. M. Schliwa Prof. Dr. C. Haas
Mitbetreuung durch die promovierten Mitarbeiter:	Dr. G. Behre
Dekan:	Prof. Dr. med. H.C.K. Peter

Tag der mündlichen Prüfung: 30 Jan 2003

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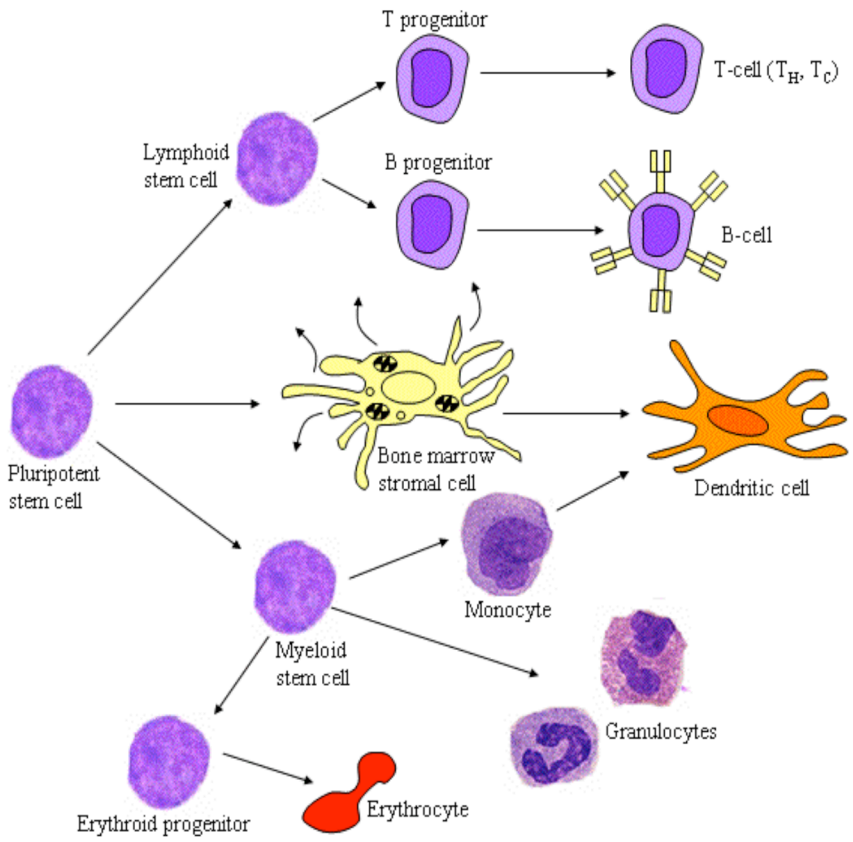
1 Introduction

1.1 Hematopoiesis

Hematopoiesis is the process by which mature blood cells of distinct lineages (e.g. red, white, and lymphoid cells) are produced from pluripotent hematopoietic stem cells (HSCs); [Scheme 1][1]. To sustain hematopoiesis through an individual's life time, HSCs must be capable of (i) maintenance in a non-cycling state, (ii) self-renewal to generate additional HSCs, and (iii) production of progenitor cells with more limited developmental potential.



Scheme 1. Hematopoiesis: Differentiation of specific lineages from a common pluripotent stem cell.

Progenitors commit to subsets of lineages and ultimately to single pathways with concomitant expression of the end stage markers representative of each cell type (Scheme 1). Hematopoiesis is dynamic both with respect to lineage decisions and location during development. Within the mammalian embryo the site of hematopoiesis changes from its initial position in the yolk sac blood glands (primitive hematopoiesis) to the fetal liver and then to the bone marrow (definitive hematopoiesis). Although it has been considered axiomatic that HSCs which populate the adult arise within the yolk sac and migrate to the fetal liver, recent evidence suggests an intraembryonic origin[1].

1.2 Transcription Factors involved in Hematopoiesis

Transcription factors are sequence specific DNA binding proteins with a variety of functions that include: (i) folding of the DNA molecule, (ii) the initiation of the DNA replication and (iii) control of gene transcription[2]. Transcription factors can be classified according to the three dimensional structure of their DNA binding motifs. Over 80% of all transcription factors are characterized by zinc fingers, helix-turn-helix, helix-loop-helix, leucine zipper and winged-helix motifs[2]. A number of transcription factors have been identified that play a role in the development of erythroid and myeloid lineages[3-7]. The central role of transcription factors in these processes has been highlighted by gene inactivation studies, promoter analysis and ectopic expression of lineage restricted factors[8-10]. Rather than being controlled by single master regulators, lineage-specific gene expression appears to depend on the combination of factors in overlapping expression domains[11]. Various transcription factors involved in hematopoiesis are outlined in Table 1.

Table 1. Transcription factors involved in hematopoiesis.

Transcription factor	Role in Hematopoiesis	References
PU.1	essential for the development of myeloid and lymphoid lineages. The defect in neutrophil differentiation is limited, as incomplete maturation although delayed is observed in PU.1$^{-/-}$ mice. In contrast, PU.1 down-regulation is necessary for erythroid differentiation	12-24
Ets-1	required for NK cell development as well as proper gene expression control in B and T lymphocytes	25-28
Fli-1	positive regulation of Fli-1 expression is linked to the (i) induction of proliferation, (ii) inhibition of apoptotic cell death, and (iii) inhibition of the terminal differentiation program in erythroid progenitors. In addition Fli-1 is associated with the induction of the megakaryocytic phenotypic, and maintenance of normal T cell numbers	29
C/EBPα	critical regulator of granulocyte differentiation. C/EBP$\alpha^{-/-}$ mice show defects in granulocyte and eosinophil development, other lineages including monocytes/macrophages, red blood cells. Platelets and lymphoid cells appear to develop normally	30-41
C/EBPβ	plays an important role in macrophage and B cell development. In macrophages it functions primarily as a regulator of cytokine gene expression during inflammatory responses. In addition, increased expression has been described during neutrophilic differentiation	42-47 {Doppler, Welte, et al. 1995 702 /id}
C/EBPδ	involved in B cell development and fetal liver hemopoiesis	48-50
C/EBPε	upregulated during myelopoiesis. Similarly to C/EBPβ, in macrophages it functions primarily as a regulator of cytokine gene expression during inflammatory responses	35
C/EBPγ	regulator of eosinophil, neutrophil, and macrophage terminal differentiation. C/EBP$\gamma^{-/-}$ mice show marked defects in granulocyte and eosinophil development. No C/EBPγ mRNA was detected in erythroid, megakaryocyte, basophil, B-lymphoid, or non-hematopoietic cell lines	51-58
AML1/PEBP2α	disruption of gene expression studies indicate that AML1/PEBP2α is necessary for normal development of all hematopoietic lineages	59;60
PLZF	down-regulation of PLZF may be a necessary step for the differentiation of early bone marrow progenitors	61
MZF-1	important role in the induction of granulopoiesis, possibly linked to the expansion of myeloid precursors prior to terminal differentiation	62-64
WT-1	maximal expression found in CD34^{+}/CD33^{-}/Lin^{-} early progenitor cells down-regulation of WT-1 expression is necessary for differentiation of myeloid progenitors	65-67
mFOG-1	plays a fundamental role in the development of erythroid and megakaryocytic lineages	68-70
HOXA10	its expression is associated with amplification of early hematopoietic precursors and megakaryocytes. Down-regulation of HOXA10 is important for myeloid and B-lymphoid differentiation	71-73
HOXA5	several lines of evidence indicate that HOXA5 gene expression	74

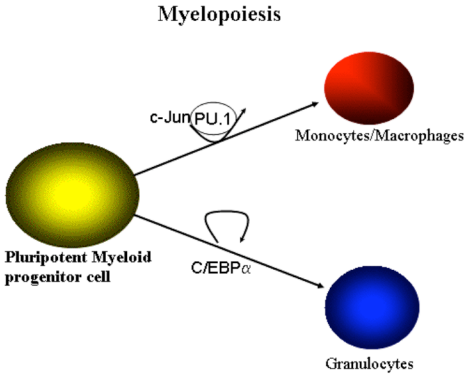
	positively regulates granulocytic/monocytic differentiation, whereas its down-regulation is necessary for progression of erythropoiesis	
HOXA9	important role in the development of myeloid, erythroid, and B cell progenitors	75;76
HOXB3	down regulation of HOXB3 expression, compared to $CD34^{+}/Lin^{-}$ bone marrow progenitors, is necessary for normal lymphoid (B and T cell) development. In contrast, HOXB3 over-expression preferentially upregulates myeloid development.	77
HOXB4	key role in the expansion of primitive bone marrow hemapoietic cells	78;79
HOXB8	role in positive regulation of macrophage development, and negative regulation of granulocytic development	77
other HOX genes	several other genes of the HOXB (B2, B6-B9), and HOXC (C6, C8), appear to play a role in the regulatory development of erythropoiesis	80
EGR-1	selective upregulation of EGR-1 gene expression is associated with differentiation along the macrophage lineage, where maximal expression is reached in mature cells. In contrast, EGR-1 negatively regulates granulocytic lineage development	81
c-Myb	expression of c-Myb is crucial for the survival and proliferation of early hemapoietic progenitors. In contrast, c-Myb down-regulation is required for terminal cell differentiation.	82-84
c-Myc	expression levels are high in proliferating myeloid progenitors and its down-regulation activates the terminal differentiation program. In contrast, forced c-Myc expression negatively regulates the terminal differentiation program.	85;86
AP-1 (Jun/Fos)	several lines of evidence suggest a role for AP-1 in the modulation of apoptotic pathways and the functional development of several hemopoietic lineages including the monocyte/macrophage, granulocyte, megakaryocyte, monocyte and erythroid lineages	87;88
GATA	GATA-1 and GATA-3 play key roles in the positive regulation of erythroid and megakaryocyte development, as well as the negative regulation of myeloid development. In the presence of C/EBP□, however GATA-1 induces eosinophil lineage development. GATA-3 expression is restricted to T- and NK-cells, and has been found to be required for T cell lineage commitment and differentiation	89;90
STAT1	central role in differentiation along the mononuclear phagocyte lineage, through the induction of functional genes, such as ICAM-1 and Fc□R1	91
STAT3	essential for gp130-mediated induction of proliferation, differentiation and survival signals in response to IL-6 signalling	92
STAT4	STAT4 expression, associated with early myeloid progenitors, is down regulated during erythroid and granulocytic differentiation	93
SCL	absolutely required for the development of embryonic, fetal and adult hemopoietic stem cells. SCL modulates cellular differentiation both positively in erythroid, myeloid, and megakaryocytic lineages and negatively in macrophage lineage	94;95
IRF-1	modulates gene expression in developing thymocytes, required for lineage commitment and selection of $CD8^{+}$ thymocytes	96
ICSBP	positive role in differentiation of bipotential myeloid progenitors	97;98

	cells towards the macrophage lineage, as well as repression of specific genes associated with terminal granulocytic differentiation	
MafB	specific-upregulation of MafB expression plays an important role in the induction of the mononuclear phagocyte differentiation program and repression of erythroid specific gene expression in myeloid cells	99;100
E2A	the earliest appearing gene thought to be responsible for commitment to the B cell lineage	101;102
BSAP/Pax5A	expressed immediately after commitment to the B cell lineage in the bone marrow, BSAP/Pax5A continues to be expressed throughout B cell development except in plasma cells, where it maintains commitment to the B cell lineage	102
EBF	function in the development of B cell progenitors following commitment to the B cell lineage	103

: Adapted from Barred et al., *Developmental and comparative immunology,* 2001

1.3 Myelopoiesis

The coordinated production of all blood cells from a common stem cell is a highly regulated process involving successive stages of commitment and differentiation. The myelopoietic system includes the hematopoietic cells derived from a common hematopoietic stem cell that includes erythroid, granulocytic, monocytic and megakaryocytic lineages. Myeloid cell differentiation (Scheme 2) has been investigated on many levels, from the cytokine signals required by each cell lineage to the

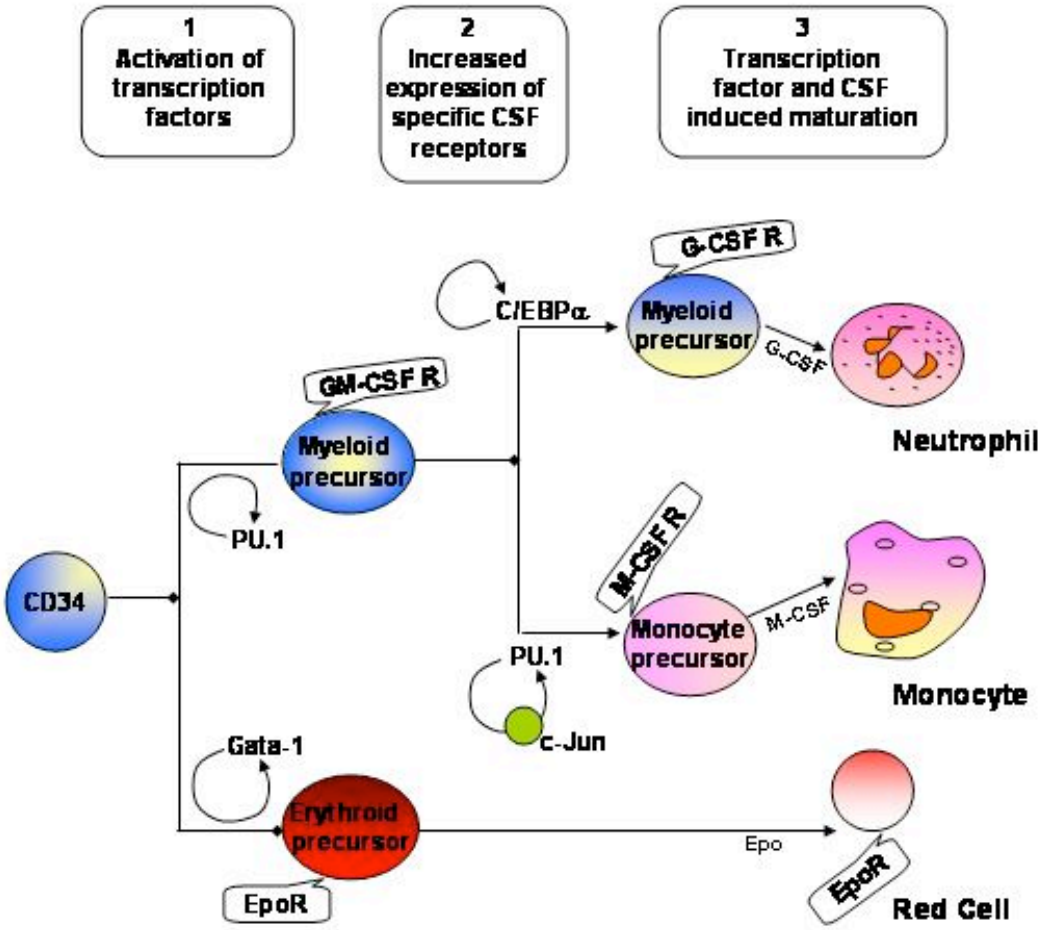


Scheme 2. Myeloid cell differentiation from a pluripotent myeloid progenitor cell into a single lineage monocyte or granulocyte.

scheduled expression of distinctive myeloid cell-specific genes and the programmed appearance of characteristic cell surface markers. By analogy to progress in other developmental systems, such as muscle and liver cell differentiation, it should be possible to establish a hierarchy of differentiation signals and transcriptional processes for developing myeloid cells. An attractive emerging concept implicates the programmed regulation of key transcription factors at different stages of development, coordinated by receptor-mediated signals from myeloid colony-stimulating factors[104]. The development of distinct lineages like monocytes-/macrophages, granulocytes and megakaryocytes from hematopoietic precursor cells is controlled by a myriad of transcription factors which regulate the expression of essential genes, including those encoding growth factors and their receptors, enzymes, adhesion molecules, and transcription factors themselves[105]. The various transcription factors which have been implicated in myelopoiesis are PU.1, C/EBP members (α, β, ε and δ), CBF, c-Myb, Ets, HOX, MZF-1, AML1/CBFα, AP-1 members etc. Various growth factors are responsible for myelopoiesis, the most important ones are G-CSF (granulocyte-colony stimulating factor), M-CSF (macrophage-colony stimulating factor) and GM-CSF (granulocyte-macrophage-colony stimulating factor).

1.4 Transcription factors PU.1, C/EBPα and c-Jun

Hematopoietic differentiation program involves activation of transcription factors like PU.1, C/EBPα, GATA-1 and c-Jun, followed by increased expression of colony stimulating growth factor receptors, and maturation of the committed cells induced by transcription factors and cytokines (Scheme 3).



Scheme 3. Model of induction of hematopoietic differentiation by specific transcription factors. These factors are expressed at low levels in CD34+ stem cells, as are specific growth factor receptors. Under direction of signals that are not defined yet, such as the influence of stromal interactions or growth factor signalling, specific transcription factors, such as GATA-1 or PU.1, are upregulated. Upregulation of specific transcription factors leads to their autoregulation and upregulation of specific growth factor receptors, resulting in increased proliferation, differentiation, and suppression of apoptosis of specific lineages. Downregulation of specific factors (such as Gata-1 during myeloid development) may also play an important role.

1.4.1 *PU.1*

PU.1 is a member of the Ets transcription family[12]. All Ets factors contain a characteristic DNA binding domain of approximately 80 amino acids[18,25,106]. PU.1 and the related Ets family member Spi-B form a distinct subfamily within the larger group of Ets proteins, with very distinct structure, patterns of expression, binding specificity, and functions that are non-overlapping with other members of the Ets family. The PU.1 protein consists of 272 amino acids, with the DNA binding domain located in the carboxyl terminal part of the protein, whereas the amino terminus contains an activation domain that has been implicated in inter-actions with other regulatory proteins[107-109]. The structure of the DNA binding domain has been recently determined and demonstrates a winged helix-turn-helix motif[110] (Figure 1).

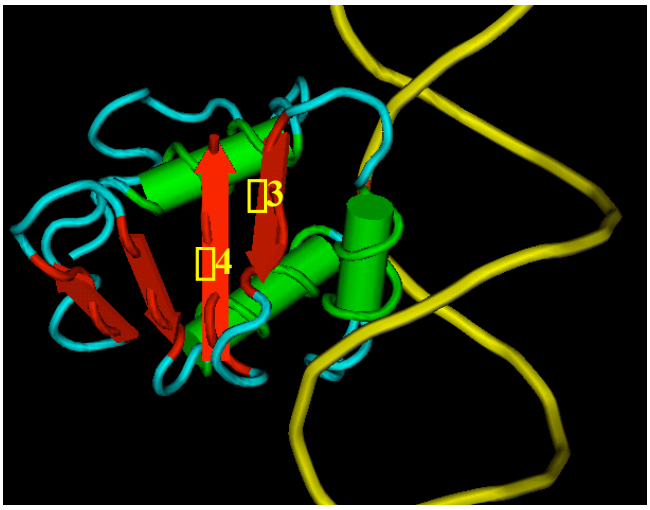


Figure 1. Crystal structure of PU.1 DNA binding domain, bound to DNA

PU.1 shows specific patterns of hematopoietic expression. PU.1 is expressed at highest levels in myeloid and B cells, but not in T cells[20,12]. During hematopoietic development, PU.1 mRNA is expressed at low levels in murine ES cells and human $CD34^+$ stem cells and is specifically upregulated during myeloid differentiation. The timing of this upregulation coincides with the first detection of early myeloid maturation, suggesting that this increase in expression may be an important process in myeloid development, and inhibition of PU.1 function at this juncture can block myeloid progenitor formation[111]. Studies in both primary $CD34^+$ cells and human leukemic cell line models suggest that PU.1 mRNA and DNA binding activity do not increase further with subsequent myeloid

maturation from the promyelocytic to more mature stages[111,20], but these studies do not preclude the possibility that the high levels of mRNA observed in human monocytes is a result of subsequent final maturation and/or activation. These expression studies suggest that regulation of PU.1 mRNA may play a significant role in the commitment of early multipotential progenitors to the myeloid lineages, as well as in the further differentiation and maturation of these cells.

Using retroviral transduction of PU.1 complementary DNA into mutant hematopoietic progenitors, it has been demonstrated that differing concentrations of the protein regulate the development of B lymphocytes as compared to macrophages. A low concentration of PU. 1 protein induces the B cell fate, whereas a high concentration promotes macrophage differentiation and blocks B cell development. Conversely, a transcriptionally weakened mutant protein (in the transactivation domain) preferentially induces B cell generation[112].

The important functional sites identified so far in the PU.1 promoter, an octamer site at bp -54, an Sp1 site at bp -39, and a site for PU.1 itself at bp +20, are conserved in both human and mouse[113]. In B cells, the octamer site plays a major role in PU.1 expression[114]. These findings suggested that PU.1 is auto-regulatory in its expression. PU.1, like other Ets factors, was first noted to bind to a sequence characterized by a purine-rich core (GGAA), hence its name[25]. However, the DNA binding specificity of PU.1 appears to be quite distinct from that of other Ets factors.

PU.1, like other Ets proteins, interacts with other transcriptional regulators. The various factors known, so far, to interact are PU.1 interacting protein (Pip), NF-IL6 (C/EBP□), c-Jun, TBP, RB, GATA1 and CBP[115-117,107].

Ets family members have been shown to play an important role in several signal transduction pathways[118-121]. PU.1 is phosphorylated *in vitro* by casein kinase II and JNK kinase, but not by ERK1 (MAP) kinase. A recent study has shown that lipopolysaccharides (LPS) can activate casein

kinase II and phosphorylation of PU.1 in LPS-stimulated macrophage lines[122]. The LPS stimulation of PU.1 transactivation of a reporter construct was abolished by a serine to alanine mutation of residue 148, suggesting that stimulation of macrophages with LPS leads to increased phosphorylation and activity of PU.1.

Several groups have addressed the function of PU.1 on murine development using targeted disruption of the PU.1 gene[17,24]. Singh et al., have reported that PU.1$^{-/-}$ embryos die in utero, usually at day E16[123]. These animals demonstrated variable anemia, but no production of any type of white blood cells, including monocytes, neutrophils, and B cells, as might be expected based on the expression of PU.1 and its known target genes. The concomitant failure to produce T cells in the (-/-) animals was surprising, given that PU.1 has not been known to be expressed in this lineage. These studies suggested that the defect was either due to a block of a very early multi-lineage progenitor cell or that T-cell development is dependent on the presence of macrophages and/or B cells. The PU.1 knockout mice Maki et al., yielded a phenotype with some distinct differences[124]. The PU.1$^{-/-}$ mice in this case were viable at birth and could be kept alive for days by housing them in a sterile environment with the administration of antibiotics. These animals also lacked monocytes and mature B cells, but were capable of producing B-cell progenitors. Several days after birth, T cells and cells resembling neutrophils were detected in the peripheral blood. In this case, the findings suggested a role for PU.1 in B-cell and monocytic development, which is more in keeping with its known pattern of expression and its known gene targets. Although neutrophilic cells were observed, they were Mac-1 negative and reduced in number, suggesting that targeting PU.1 results in a partial defect in neutrophil development. The former knockout model suggests that PU.1 plays an important role in multipotential cells; the latter knockout model suggests a more limited role in B-cell and myeloid development.

In vitro differentiation of PU.1$^{-/-}$ embryonic stem cells does not produce macrophages and points to the M-CSF receptor as a major target of PU.1 in understanding its effect on myeloid differentiation[125,126].

1.4.2 *C/EBP*

CCAAT/enhancer binding protein (C/EBP) transcription factors belong to the bZIP family of proteins, which contain a basic domain involved in DNA binding, and a leucine zipper motif involved in homo and heterodimerization. C/EBP the founding member of the C/EBP transcription factors is most abundantly expressed in adipose tissue, placenta, liver and is also detected in a variety of other organs such as lung, kidney, small intestine, brain and hematopoietic cells[127]. C/EBP has been shown to play a significant role in adipocyte differentiation, regulation of both liver and adipocyte specific genes, energy metabolism and cell proliferation[128,129]. C/EBP is also expressed in early myeloid cells[130,61].

A number of granulocytic specific genes including the granulocyte-colony stimulating factor (G-CSF) receptor, neutrophil elastase, and myeloperoxidase genes have been shown to be regulated by C/EBP[34,35,131]. Inhibition of C/EBP blocks differentiation and expression of C/EBP induced differentiation.

The C/EBP promoter is autoregulatory. Murine C/EBP promoter has a binding site for C/EBP[132]. The human C/EBP promoter does not conserve the C/EBP binding site found in the murine promoter, and the autoregulation is indirect and mediated by the action of C/EBP on a helix-loop-helix protein, the upstream stimulatory factor (USF)[133].

Northern blot analysis of human CD34^{+} cells shows that C/EBP expression is maintained during granulocytic differentiation but is markedly downregulated with monocytic or erythroid differentiation. In addition mature peripheral blood neutrophils show high levels of C/EBP

mRNA, which is completely undetectable in adherent peripheral blood monocytes.

As noted above, C/EBP was the first protein noted to have the leucine zipper motif[134]. The leucine zipper is a protein motif structure common to a new class of DNA binding proteins and is directly involved in homodimerization and heterodimerization. C/EBP, C/EBP, and C/EBP are very similar in their C-terminal basic region and leucine zipper domains and diverge in the N-terminal transactivation domain[135-138].

In addition to other members of the C/EBP family, the C/EBP proteins can interact with a number of transcription factors, including NF-B and Rel proteins[139,140], members of the CREB/ATF family[141,142], Sp1[143], RB and members of the fos/jun zipper family[144]. The amino terminal region of C/EBP has been shown to physically interact with TBP[145]. As noted above, C/EBP has been shown to interact with PU.1[146], and PU.1 can physically interact with C/EBP as well (P. Auron unpublished observation).

C/EBP knockout mice show an interesting phenotype: homo zygous mice die within the first few hours after birth of impaired glucose metabolism; their viability can be extended to about 1 day with injections of glucose. Analysis of the hematopoietic system of the knockout embryonic and newborn mice demonstrated a significant defect in production of granulocytic cells[147]. Newborn knockout (-/-) animals do not produce any mature neutrophils, which comprise 90% of the peripheral white blood cells of newborn heterozygote (+/-) or wild-type (+/+) mice. Eosinophils are also not observed, but all of the other lineages, including peripheral blood monocytes and peritoneal macrophages, red blood cells, platelets, and lymphoid cells, appear quantitatively unaffected. Myeloid markers (Mac-1 and Gr-1) were greatly reduced, with normal B- and T-cell subsets. Expression of the G-CSF receptor mRNA was profoundly and selectively reduced, whereas that of M-CSF receptor and GM-CSF receptor , $_c$, and IL3 were all comparable to wild-type.

These findings suggested that much of the phenotype may be due to decreased or absent G-CSF signaling due to markedly reduced receptor levels. However, G-CSF receptor knockout animals produce mature granulocytes, in contrast to C/EBP knockouts, strongly suggesting that there must be important C/EBP target genes in myeloid progenitors in addition to the G-CSF receptor[148].

In summary, the C/EBP proteins are differentially expressed and can interact with themselves and other transcription factors. These studies showed that C/EBP (and C/EBP) may be involved in activation of cytokines in mature cells, whereas C/EBP has a major role in granulocytic maturation through regulation of the G-CSF receptor and other as yet not identified target genes.

1.4.3 *c-Jun*

AP-1 transcription factors play a major role in myelopoiesis. Proto-oncogenes c-Jun, junB, and junD are stably induced suggesting their role in the initiation, progression, and maintenance of the myelopoietic differentiation program.

c-Jun belongs to the bZIP group of DNA binding proteins and is a component of the AP-1 transcription factor complex[149]. c-Jun forms homodimers or can heterodimerize with other Jun family members or with other bZIP proteins including members of the fos and ATF/cAMP response element-binding protein (CREB) families[88].

AP-1 members have been shown to be involved in many cellular processes including proliferation, apoptosis and stress responses. In particular, there is evidence that c-Jun plays a role in monocytic differentiation. c-Jun mRNA is upregulated upon monocytic differentiation of bipotential myeloid cell lines. While stable expression of c-Jun into myeloid cell lines results in partial differentiation[150,151]. For several years it was unclear if c-Jun plays any role in the upregulation of M-CSF receptor (M-CSFr) expression, though there are AP-1 binding sites

in M-CSFr promoter. TPA treatment of U937 cells induces c-Jun expression showing the role of c-Jun in myeloid differentiation.

Lately it was shown that c-Jun enhances the ability of PU.1 to transactivate the human monocyte-specific M-CSF receptor promoter, and it was also shown that c-Jun is a JNK independent co-activator of the PU.1 transcription factor[117]. C/EBP kockout mice show high level of c-Jun expression. This points to the fact that C/EBP might down-regulate c-Jun expression to drive the cells to granulocytic lineage.

1.5 Aim of the Study

The aim of this study is to elucidate the molecular mechanisms that decide whether a common precursor will develop and differentiate along the granulocyte or monocyte lineage.

In particular we set out to examine how the transcription factors PU.1 and C/EBP□ are regulating the development of pluripotent myeloid progenitor cells.

2 Materials

2.1 Reagents

Acrylamide-Bisacrylamide	Biorad GmbH, Munich, Germany
Agar	Life Technologies, Paisley, Scotland
Agarose	Life Technologies, Paisley, Scotland
APS	Fluka, Buchs, Switzerland
Acetic acid	Merck, Darmstadt, Germany
Bromphenolblue	Sigma, St. Louis, U.S.A
Coomassie-Blue R-250	Life Technologies, Paisley, Scotland
DMEM	PAN Biotech, Aidenbach, Germany
DTT	Sigma, St. Louis, U.S.A
Ethanol	Merck, Darmstadt, Germany
Ethidium bromide	Sigma, St. Louis, U.S.A
Fetal bovine serum	Life Technologies, Paisley, Scotland
Formaldehyde	Sigma, St. Louis, U.S.A
Formamide	Sigma, St. Louis, U.S.A
l-Glutamine	Life Technologies, Paisley, Scotland
Glycerin	Merck, Darmstadt, Germany
HEPES	Sigma, St. Louis, U.S.A
IPTG	Sigma, St. Louis, U.S.A
Isopropanol	Merck, Darmstadt, Germany
□-Mercaptoethanol	Sigma, St. Louis, U.S.A
Methanol	Merck, Darmstadt, Germany
MOPS	Sigma, St. Louis, U.S.A
PBS	PAN Biotech, Aidenbach, Germany
Penicillin/Streptomycin	Life Technologies, Paisley, Scotland
RPMI	PAN Biotech, Aidenbach, Germany
Sodium chloride	Merck, Darmstadt, Germany
SDS	Serva, Heidelberg, Germany

TEMED	Biorad, Munich, Germany
Tris-HCl	Merck, Darmstadt, Germany
Trichostatin A	Sigma, St. Louis, U.S.A
Trypsin/EDTA	PAN Biotech, Aidenbach, Germany
Xylencyanol	Sigma, St. Louis, U.S.A
Zinc sulfate	Sigma, St. Louis, U.S.A

2.2 Radioactive Substances

□-[^{32}P]-dCTP	Amersham, Braunschweig, Germany
L-^{35}S-Methionine	Amersham, Braunschweig, Germany

2.3 Enzymes

Restriction enzymes	New England Bio-labs, Frankfurt, Germany
	GIBCOBRL, Paisley, Scotland
RNasein	Promega, Mannheim, Germany

2.4 Reagent Kits

MACS (CD34 positive cell isolation kit)	Miltenyi Biotech, Gladbach, Germany
Lymphoprep	Nycomed, Oslo, Norway
Plasmid Isolation kit	Qiagen, Hilden, Germany
Glutathione Sepharose	Pharmacia, Freiburg, Germany
TNT-Reticulocyte-lysate-system	Promega, Mannheim, Germany
LipofectAMINE plus	GIBCOBRL, Paisley, Scotland
Effectene	Qiagen, Hilden, Germany
Biorad-protein estimation kit	Biorad, Munich, Germany
Luciferase assay kit	Promega, Mannheim, Germany
Fast Start DNA SYBR	Roche Diagnostics, Mannheim,

green I-Kit	Germany

2.5 Standards, Markers and Ladders

Protein marker	Biorad, Munich, Germany
DNA marker	NEB, Frankfurt, Germany
1-kb ladder	NEB, Frankfurt, Germany
100bp ladder	NEB, Frankfurt, Germany

2.6 Miscellaneous

Blotting paper	Schleicher and Schüll, Stuttgart, Germany
Membrane (Nitrocellulose)	Amersham, Millipore
Pipet tips	Star Labs (K&K labordarf), Munich, Germany
Plastic material (tubes etc)	Eppendorf, Greiner, Falcon, Munich, Germany
Quartz Cuvette	Hellma
Reaction tubes	Eppendorf
X-ray film	Kodak (Biomax)
Filters	Millipore
Ultra centrifuge tubes	Beckman
Cell culture material	CoStar, Cellstar, Nunc and Greiner

2.7 Biological material

2.7.1 *Bacteria*

Escherichia coli DH5 alpha

Escherichia coli BL-21

2.7.2 *Mammalian cell lines*

293T (human kidney fibroblasts)

F9 (mouse embryonal carcinoma)

CV1
U937 (human myeloid cell line)
U937 (Zn^{2+} inducible C/EBP□)

2.8 Plasmids

p(PU.1)4TK
p(mut.PU.1)4TK
p(C/EBP)2TK
pTK
pGL2
pXP2
pRL-null
pCMV5
pECE PU.1
pcDNA3.1 C/EBP□
pMSCV C/EBP□ mBR
pMSCV C/EBP□ □LZ
pMSCV C/EBP□ (rat)
pGEX2TK-PU.1
pGEX-C/EBP□ DNA binding domain
pS3H-c-Jun

2.9 Buffers

Buffers provided with the kits were used in the case of Plasmid isolation, protein expression, RNA isolation, and Real time Polymerase chain reaction. The other buffers used are listed below.

Binding buffer (GST-Pull down)	
(NETN)	150 mM $NaCl_2$
(with protease inhibitors)	0.5 mM EDTA
	50 mM Tris

	1% NP40 5% Glycerol
Electrophoresis buffer (SDS-PAGE)	25 mM Tris, pH 8.3 250 mM Glycine 0.1 % SDS
Destaining solution	30 % Methanol 10 % Acetic acid
Staining solution	30 % Methanol 10 % Acetic acid 0.25 % Coomassie-Blue R-250
Electrophoresis buffer (Tris-Glycine) (pH 8.3)	250 mM Tris 1.9 M Glycine 10mM EDTA
Tris-Borate buffer	0.89 M Tris 0.89 M Boric acid 0.5 M EDTA
EMSA binding buffer	20 mM HEPES 60 mM KCl 24 % Glycerol 0.5 mM DTT 0.4 mM PMSF 10 mM $MgCl_2$ 0.2 mM EDTA
Agarose gel electrophoresis loading dye (6x)	40 % Saccharose

	0.25 %Bromophenolblue 0.25 % Xylenecyanol
SDS-PAGE gel loading dye (2x) pH (6.8)	125 mM Tris/HCl, 4 % SDS 10 % β-Mercaptoethanol 30 % Glycerol 0.004 % Bromophenolblue
Western Stripping solution	0.1 M β-Mercaptoethanol 2 % SDS 1M Tris (pH 6.8)
TE buffer	50 mM Tris/HCl, pH 8.0 1 mM EDTA
Trypsin/EDTA solution	0.05 % Trypsin 0.02 % EDTA in PBS

2.10 Growth media

2.10.1 *Bacteria*

LB (*luria bertani*) Medium	lb medium with respective antibiotics

2.10.11 *Mammalian cell culture*

293T, CV1	DMEM 10 % FBS 1 % L-Glutamine 1% Penicillin-Streptamycin
F9	DMEM (4,5 g/l Glucose)

	10 % FBS 1 % L-Glutamine 1% Penicillin-Streptamycin
U937	RPMI 10 % FBS 1 % L-Glutamine 1% Penicillin-Streptamycin

3 Methods

3.1 Transient transfection of adherent eukaryotic cells by LipofectAMINE

The day before transfection, cells were trypsinized, counted and plated so that they were 50-80% confluent the day of transfection. Antibiotics were avoided at the day of transfection and during plating to help cell growth and increased transfection efficiency. DNA was pre-complexed to PLUS reagent: DNA was diluted in serum free medium. PLUS reagent was mixed before adding it to the diluted medium. The complete mixture was incubated at room temperature for 15 min.

LipofectAMINE reagent was diluted and mixed in a serum free medium in a second tube. Pre-complexed DNA and diluted LipofectAMINE reagent were mixed and incubated for 15 min at room temperature. During this incubation period, cells were rinsed with serum free medium to enable higher transfection activity. DNA-PLUS-LipofectAMINE reagent complexes were added to each well containing fresh medium. The complexes were gently mixed with the medium; incubated at 37°C at 5% CO_2 for 3hrs. After 3hrs of incubation medium volume was increased to normal volume by adding medium containing 20% fetal bovine serum.

24hrs after the transfection assay cell extracts were obtained for reporter gene activity.

Amounts of reagents used for transfection:

	DNA (μg)	PLUS reagent (μl)	LipofectAMINE reagent (μl)	Transfection volume (ml)
24 well	0.4	4	1	0.25
12 well	0.7	5	2	0.5
6 well	1	6	4	1.0
60 mm	2	8	12	2.5
100 mm	4	20	30	6.5

Cell number: 1×10^4 – 24 well
1×10^5 – 6 well
1×10^5 – 60 mm
2×10^5 – 100 mm

3.2 Transient transfection of eukaryotic cells (adherent and suspension) by Effectene

Cells are plated to the density stated above the day before trans fection. On the day of tranfection DNA was diluted in DNA condensation buffer, buffer EC, to a total volume of 150 μl. 8 μl of enhancer were added and vortexed for 1s. The complex is incubated at room temperature for 2-5 min and spun down shortly. 25 μl of effectene transfection reagent was added to the DNA-enhancer mixture. The complex was vortexed for 10s, and incubated at room temperature for 5-10 min to allow transfection-complex formation. During this period the growth medium was gently aspirated from the plate and cells were washed with 4 ml of PBS. 4 ml of fresh medium with FBS and antibiotics was added (RPMI for suspension cells and DMEM for adherent cells). Fresh medium was added to the transfection complexes and added on to the cells. The dish was gently

swirled to ensure the uniform distribution of the transfection complexes. Cells were incubated in normal conditions for an appropriate time for expression of the transfected gene.

Amount of reagents used for transfection:

Culture format	DNA (μg)	Enhancer (μl)	Final volume of DNA in buffer EC (μl)	Effectene Reagent (μl)	Volume of medium to cells (μl)	Volume of Medium to complexes (μl)
24 – well	0.2	1.6	60	5	350	350
6 – well	0.4	3.2	100	10	1600	600
60 mm	1.0	8.0	150	25	4000	1000
100 mm	2.0	16.0	300	60	7000	3000

Cell number:

24-well	2-8 x 10^4
6-well	0.9-4 x 10^5
60 mm dish	2-8 x 10^5
100 mm dish	0.5-2.5 x 10^6

3.3 *in vitro* Transcription

PU.1, C/EBPα and c-Jun were in vitro transcribed and translated, using the TNT Reticulocyte Lysate System and with labeled [^{35}S] methionine. For a 50 μl reaction the following volumes were used:

TNT rabbit reticulocyte lysate	50 μl
TNT reaction buffer	2 μl
Enzyme(c-Jun SP6; C/EBPα T7; PU.1 T3)	1 μl
Amino acids (-methionine)	1 μl
^{35}S - methioine	4 μl

RNAsin 1 µl
plasmid DNA (~ 1µg)
dH_2O
to make the final volume to 50 µl
The reagents were mixed and incubated at 30°C for 90 minutes.

3.4 GST expression of proteins fused to GST

[e.g., PU.1-GST, C/EBPα-GST, PU.1 (α3-α4)-GST, DNA binding domain C/EBPα-GST]

DH5α bacteria carrying the PU.1-GST fusion constructs, and the BL-21 cells carrying the C/EBPα-GST constructs were grown in Luria bertani with the respective antibiotics. Starter cultures of 10 ml were grown overnight and transferred to 100 ml fresh media and the bacteria was grown at 37°C till the OD_{600} was 0.6. The culture was then induced with 0.5 mM isopropyl b-D-thiogalactoside (IPTG) for 2-3hrs at 30°C. The bacteria were pelleted at 4,000 rpm. The bacteria were suspended and lysed in NETN buffer with the respective protease inhibitors. The cell membrane was opened by sonication (duty cycles 40-50 / amplitude 20%, 5 times with interval of 5 min). The lysates were spun down for 30 min at 4°C at maximum speed (14,000 rpm). The supernatant was saved and preserved at -20°C.

Meanwhile Glutathione sepharose beads were washed with PBS two times and spun at 2.000 rpm at 4°C for 5 min. Finally the beads were suspended in NETN buffer with low salt concentration (100mM NaCl). The supernant was bound to the glutathione sepharose beads at 4°C over night on a rocking platform. The protein bound beads were then washed 4 times with the wash buffer to eliminate non-specifically bound protein. The protein bound beads were centrifuged at 2,000 rpm at 4°C for 5 min everytime. The purified protein recovery was roughly estimated by SDS polyacrylamide gel electrophoresis, with known concentration of BSA loaded in the same gel.

3.5 Co-immunoprecipitation

293T cells were transfected at a density of $1x10^6$ in 100 mm plates. The cells were transfected with Effectene with respective DNA´s (eg., PU.1, C/EBP□, C/EBP□ mBR and C/EBP□ □LZ) and the empty vector pCMV5 in different combinations. 24 hours post transfection cells were scrapped with a cell scraper. Cells were washed with PBS and then subjected to lysis for whole cell lysate with Co-IP buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1mM EDTA, 5 % Glycerol, 1 % NP40 and protease inhibitors).

Sample preparation – lysates were diluted in 1 ml buffer, the cells were mixed well, and kept on ice for 30 min. It was vortexed every 10 minutes. The lysates were spun at 14,000 rpm for 30 min at 4°C. The supernatant were preserved at -20°C.

Preparation of Protein A sepharose beads – 50 ml of 75 % protein A beads were diluted in 5 ml of PBS. Spun at 300 rpm for 5 min. The buffer was removed and fresh buffer added. Respective antibodies were added to the protein A sepharose beads. The antibody bound to the beads was recovered after washing with the wash buffer.

Immunoprecipitation & western blot for coimmunoprecipitation– The lysate is added to the protein A agarose beads bound to the antibody and incubated at 4°C rotating for 120 min. Western blot analysis was performed to find the proteins interacting with immunoprecipitated protein.

3.6 Transductions

3.6.1 *Production of retrovirus*

Mouse PU.1 cDNA followed by IRES nerve growth factor receptor truncated in the cytoplasmic domain (tNGFR) and human C/EBP□ cDNA followed by IRES EGFP were subcloned into a retroviral vector, pMSCV, with an LTR derived from MSCV (pMSCV-PU.1-IRES-tNGFR and pMSCV- C/EBP□-IRES-EGFP, respectively). To produce virus, plasmid

DNA was transfected into 293gp cells (293 cells containing the *gag* and *pol* genes but lacking an envelope gene) along with 10A1 *env* expression plasmid (pCL-10A1) by $CaPO_4^-$ co-precipitation and supernatant from the transfected cells was collected to transduce cells.

3.6.2 *Transduction of $CD34^+$ cells*

Human umbilical cord blood samples were obtained, with informed consent of the parents, from placentas of full-term normal newborn infants. After isolation of mononuclear cells from cord blood by density gradient centrifugation, $CD34^+$ cells were obtained using magnetic bead separation according to the manufacturer's instructions. $CD34^+$ cells were prestimulated in IMDM supplemented with 10% FBS, 50 ng/ml stem cell factor, 50 ng/ml thrombopoietin (kindly provided by KIRIN, Tokyo, Japan), 50 ng/ml IL-6 (Peprotech, Rocky Hill, NJ), and 50 ng/ml Flt-3L (Peprotech) for 20hrs. After replating onto recombinant fibronectin fragment coated culture dishes (Takara Shuzo, Otsu, Japan) containing virus supernatant and 5 □g/ml protamine sulfate (Sigma), cells were centrifuged at 1,000x g for 30 min. Transduction was repeated with fresh virus supernatant every 12hrs three times. At 60hr after the first transduction, NGFR- and/or EGFP-positive cells were selected by cell sorting on a FACS Vantage (Becton Dickinson, San Jose, CA) and subjected to subsequent analyses. To detect the expression of tNGFR on the cell surface, cells were stained with mouse anti-human NGFR (CHEMICON, Temecula, CA) followed by phycoerythrin (PE)-conjugated rabbit anti-mouse immunoglobulins (DAKO A/S, Glostrup, Denmark).

3.6.3 *In vitro liquid culture*

$CD34^+$ cells transduced with either PU.1 or C/EBP□ retroviruses, or co-transduced with PU.1 and C/EBP□ retrovirus were cultured in IMDM supplemented with 10% heat-inactivated FBS and 50 ng/ml stem cell factor, 50 ng/ml G-CSF, 50 ng/ml GM-CSF, and 50 ng/ml IL-3 (KIRIN) at

37°C in 5% CO_2. On day 10 of culture, expression of cell surface antigens were analyzed on a FACS Vantage using PE-conjugated anti-human CD1a (Immunotech, Marseille, France). Cells were also cytocentrifuged onto glass slides and were stained with May-Gruenwald's solution (Merck, Darmstadt, Germany) followed by Giemsa's solution (Kanto Chemical Co., Inc. Tokyo, Japan).

3.7 Immunolocalization

U937 cells were cytocentrifuged onto glass slides, cells were fixed with ice-cold acetone for 2 min, dried and rehydrated in PBS. Slides were blocked in 10% FCS for 30 min at RT, washed with PBS and incubated overnight at 4^{o}C with primary antibodies. Cytospins were washed with PBS and then incubated with secondary antibodies Texas red and Cy3 for 45 min. Slides were washed with PBS and mounted with anti-fade solution.

3.8 Real time polymerase chain reaction

Quantitative Realtime PCR using the LightCycler™-Systems (LC) offers real time monitoring of PCR product formation. PCR cycles, in which the PCR product increases logarithmically can be identified and the starting concentration of the target DNA determined. We used the Fast Start DNA SYBR Green I-Kit (Roche Diagnostics, Mannheim, Germany) as a mastermix. SYBR Green I Dye is a fluorescence dye, which binds to double-stranded DNA. The fluorescence signal was recorded at the end of each elongation phase and the increasing amounts of PCR product can be monitored from cycle to cycle. We quantified the expression of the transcription factor PU.1 in U937 cell line with zinc inducible expression of C/EBP□. For each sample, we also measured the concentration of the mRNA of the housekeeping gene G6PD to control for variances in the cDNA synthesis step. Thus, we performed relative quantification of the

target gene by comparing it to the concentration of the housekeeping gene G6PD. The concentrations of the target genes and the housekeeping gene G6PD were calculated by reference to a standard curve. For that we serially diluted a G6PD plasmid: pGdBBX, kindly provided by A. Hochhaus[32], to 10,000fg, 1,000fg and 100fg. We formed ratios between the target gene and the housekeeping gene G6PD (target gene/ G6PD) and compared them to each other. Downregulation of PU.1 upon C/EBP induction or the downregulation of PU.1 upon zinc addition in empty vector carrying U937 cells were shown as PU.1/G6PD ratio values directly compared to each other at various time points from 0hrs to 8hrs after C/EBP induction.

PCR for PU.1 was performed according to the manufacturer's protocol from Search LC-Heidelberg, Germany. Amplification occurred in a three-step cycle procedure initiated by a 10 min denaturation at 95°C to activate the polymerase: 95°C, 0s; annealing 68°C, 10s; and extension 72°C, 16s for 35 cycles. Fluorescence of SYBR Green I was measured after each extension step at 530 nm.

PCR for G6PD was performed using 2 l mastermix (Light Cycler FastStart DNA Master SYBR Green I, Roche Diagnostics, Mannheim, Germany), 2 l cDNA (from U937 Zn C/EBP and U937 pPC18) 4mM $MgCl_2$, .75 M of each primer, and water to a final volume of 20 l. Amplification occurred in a three-step cycle procedure initiated by a 10 min denaturation at 95°C to activate the polymerase: 95°C, 0s; annealing 64°C, 10s; and extension 72°C, 25s for 35 cycles. Fluorescence of SYBR Green I was measured after each extension step at 530 nm. For amplification of G6PD, primers were used according to Emig *et al*[32]. The final PCR cycle is followed by a melting curve analysis to confirm the PCR product identity and differentiate it from non-specific, e.g. primer-dimer products. For that, the products were denatured at 95°C, annealed at 58°C, and then slowly heated up to 95°C with fluorescence measurement at 0.2°C increments. Amplification products performed in the Light Cycler were checked by electrophoresis on 1% ethidium bromide stained agarose

gels. The estimated size of the amplified fragments matched the calculated size: for PU.1: 150 bp and G6PD: 343bp.

4 Results

4.1 C/EBPα blocks the transcriptional activity of PU.1 on a minimal TK promoter containing PU.1 DNA binding sites only

Within the myeloid compartment, the same committed precursor can give rise to monocytic or granulocytic cells. This raises a question: What are the molecular mechanisms that dictate the fate of the common precursor to one or the other of these two diverse myeloid lineages? Since PU.1 and C/EBPα are both expressed in myeloid progenitor cells we asked the questions, How do cells differentiate into a specific lineage? and Is there a direct interaction or cross talk between these two major transcription factor?

To address this question we used a minimal TK promoter with multimerized PU.1 binding sites [p(PU.1)4TK] only. This minimal promoter was transactivated six fold upon transfection of 293T cells with an expression plasmid of PU.1. The reporter gene expression was determined 24 hrs after transfection. Cotransfection of a C/EBPα express ion plasmid in the same experiment resulted in a four fold decrease of PU.1´s transactivation capacity (Fig. 2**A**). As a control, co-transfection of PU.1 and C/EBPα did not affect the activity of a minimal TK promoter with mutated PU.1 binding sites. In further control experiments we transfected 293T cells with a reporter construct of TK promoter with multimerized C/EBP binding sites and a C/EBPα expression plasmid, transactivating the promoter by 11 fold. There was no change in protein expression of PU.1 upon co-transfection with the C/EBPα expression plasmid as observed by western blot for PU.1 from transfected 293T cells, ensuring that both the proteins expressed in transfected 293T cells. To check if C/EBPα downregulates a transcription factor in a non-specific fashion, we transfected 293T cells with expression plasmids of Gal4-

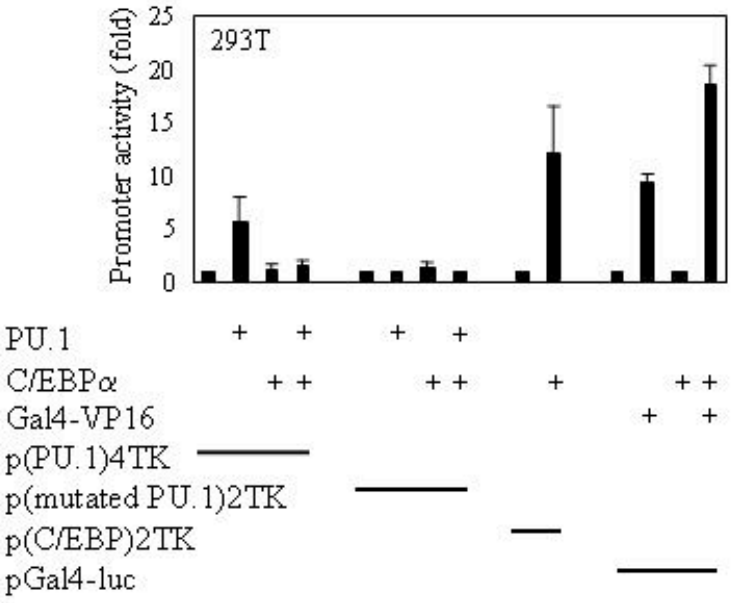


Figure 2A. C/EBPα blocks the transcriptional activity of PU.1 on a minimal TK promoter containing PU.1 DNA binding sites only

Transient transfection in 293T cells with a reporter construct of TK promoter with minimal PU.1 binding sites only (p(PU.1)4TK) or mutated PU.1 DNA binding sites only p(mutated PU.1)4TK, and expression plasmids of PU.1, C/EBPα and Gal4-VP16. As controls TK promoter with C/EBP binding sites (p(C/EBP)2TK), and Gal4-luc reporter constructs are used. The promoter activity was measured as luciferase activity 24 hrs after transfection.

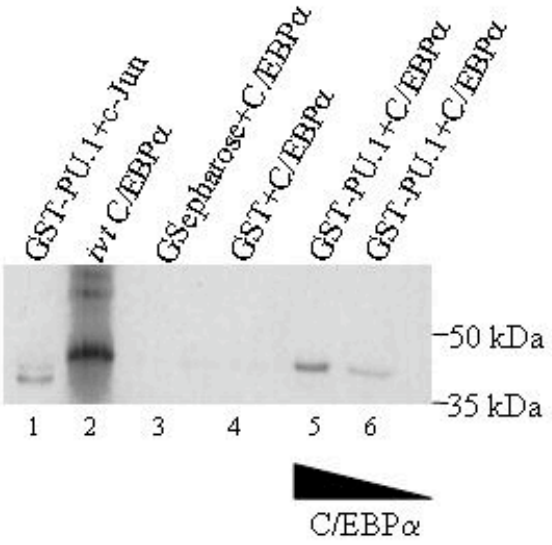


Figure 2B. C/EBPα binds to PU.1 *in vitro*

For the protein interaction assay (^{35}S)Met-labelled *in vitro* translated C/EBPα (lane 1) was incubated with 1mg of bacterially expressed GST-PU.1 (lanes 4,5). Equivalent amounts of GST protein or the glutathione sepharose beads (lanes 2,3) were incubated with *in vitro* translated C/EBPα.

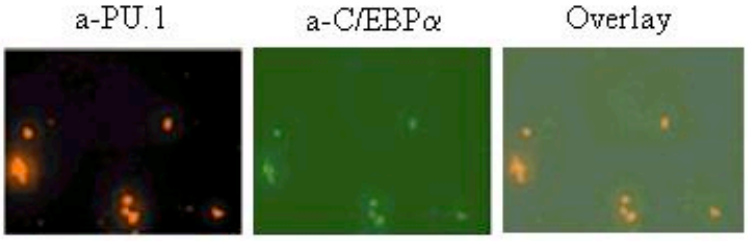


Figure 3. Co-localization of PU.1 and C/EBPα in U937 cells

U937 cells were incubated with mouse anti-PU.1 antibody and rabbit anti-C/EBPα antibody. Cy3-conjugated (green) anti-rabbit IgG and Texas red anti-mouse IgG were used as secondary antibodies. Co-localization is demonstrated by the *yellow* signal, generated by the overlay of the *red* and *green* signals.

VP16, C/EBP□ and the reporter construct Gal4-luc. Gal4-VP16 transactivates Gal4-luc by 10 folds. C/EBP□ does not downregulate the transcriptional activation of Gal4-VP16 in a non-specific fashion under the same conditions.

4.2 C/EBP□ physically interacts with PU.1

To find out whether there is a direct protein-protein interaction between C/EBP□ and PU.1, we used GST purified GST-PU.1 and incubated it with *in vitro* translated C/EBP□. An interaction between PU.1 and C/EBP□ was observed. This interaction was resistant to the effect of chaotropic agents like DTT and a change in ionic strength during the incubation reaction (Fig. 2**B**). Even in the lowest concentration of C/EBP□, we observed an interaction of C/EBP□ and PU.1. C/EBP□ did not bind to GST or the beads alone. Given the observed interaction between C/EBP□ and PU.1, the intranuclear location of these proteins was examined. U937 cells were cytocentrifuged and labeled with PU.1 and/or C/EBP□ antibodies, respectively. The secondary antibodies were Texas red for PU.1 and Cy-3 (green) for C/EBP□. A diffuse nuclear staining was observed. The overlay shows that both proteins co-localize in the nucleus (yellow) (Fig. 3). Immuno-localization studies point to a strong interaction between PU.1 and C/EBP□.

4.3 C/EBP□ inhibits c-Jun co-activation of PU.1

To understand the molecular significance of the interaction between PU.1 and C/EBP□, and in addition, to understand if the co-activator of PU.1, c-Jun, has any role in the interaction of PU.1 and C/EBP□, we used F9 cells and performed transient transfections with expression plasmids of PU.1, C/EBP□ and c-Jun. Using a minimal promoter TK with multimerized PU.1 binding sites only [p(PU.1)4TK], we observed that

PU.1 transactivated p(PU.1)4TK by six fold in F9 cells (Fig. 4**A**). F9 cells do not express c-Jun (c-Jun expression was checked by Real time polymerase chain reaction). When cotransfected with the expression plasmids of c-Jun and PU.1 a strong synergistic transactivation of around 40 fold as described before was observed[152]. Co-transfection of C/EBP in the same experiment totally blocked PU.1´s transactivation capacity of the p(PU.1)4TK promoter and also the co-activation of PU.1 by c-Jun (Fig. 4**A**). As a control, F9 cells were transfected with expression plasmids of Gal4-VP16, C/EBP, and reporter construct Gal4-luciferase, similar to the previously stated experiment. C/EBP does not downregulate the transactivation of Gal4-VP16 in a non-specific fashion under the same conditions.

4.4 C/EBP displaces c-Jun from binding to PU.1 *in vitro*

As shown above C/EBP inhibits c-Jun co-activation of PU.1, c-jun has been shown to interact strongly with PU.1. We observed C/EBP´s interaction with PU.1. To answer the question: What is the role of c-Jun in the context of the protein-protein interaction between C/EBP and PU.1 complex, we performed GST pull down experiments; ^{35}S-labelled *in vitro* translated c-Jun and C/EBP were incubated with GST-PU.1. c-Jun strongly binds to PU.1 and C/EBP also binds to PU.1 strongly, but when both factors were incubated with PU.1, C/EBP competed with c-Jun for the binding to PU.1 (Fig. 4**B**). This result suggest that C/EBP and c-Jun interact with PU.1 through the same domain. It was shown earlier[152] that c-Jun binds to the 3-4 region of PU.1, therefore we checked if C/EBP also interacted with PU.1 in the 3-4 region of the DNA binding domain. This was indeed the case (Fig. 4**C**). C/EBP has been shown to interact with PU.1 via its DNA binding domain. Due to a very high sequence similarity of C/EBP and C/EBP, we would expect that DNA binding domain of C/EBP interacts with PU.1 (Fig. 4**D**).. Indeed, incubation of

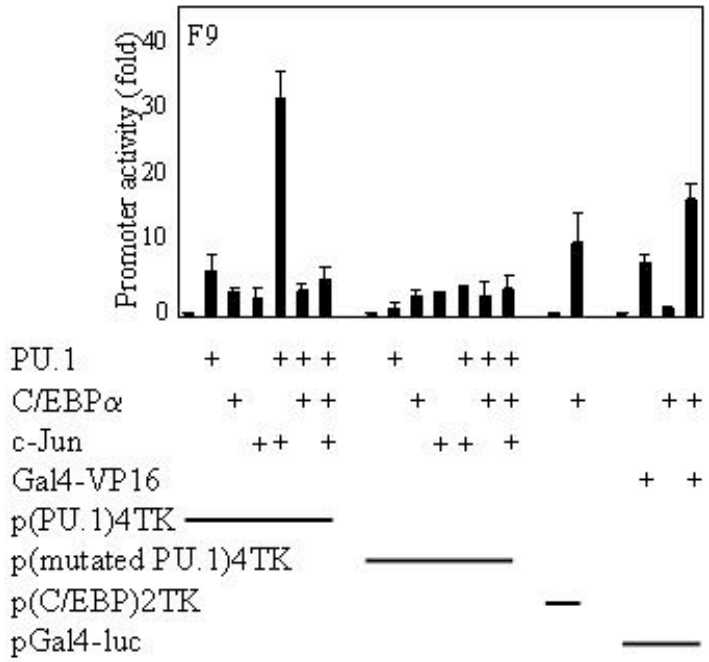


Figure 4A. C/EBPα blocks the co-activation of PU.1 by c-Jun

Transient transfection in F9 cells with a reporter construct of p(PU.1)4TK, p(mutated PU.1)4TK and expression plasmids of PU.1, C/EBPα and c-Jun. p(C/EBP)2TK is the control reporter for C/EBPα. Gal4-VP16 and Gal4-luc reporter construct was used as a control. Luciferase activities were measured 24 hrs after transfection.

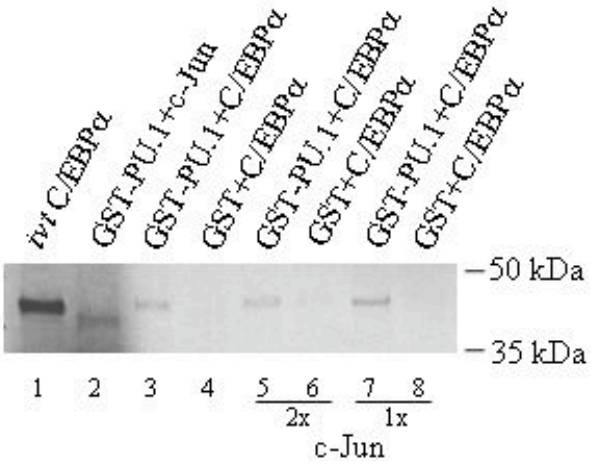


Figure 4B. C/EBPα displaces c-Jun from binding to PU.1

(^{35}S) Met-labelled *in vitro* translated C/EBPα and c-Jun were incubated with GST-PU.1 (lanes 2,3) and respectively with GST protein (lanes 4,6 and 8). Both proteins were incubated with GST-PU.1 (lanes 5,7). *In vitro* translated C/EBPα was run alone in lane 1.

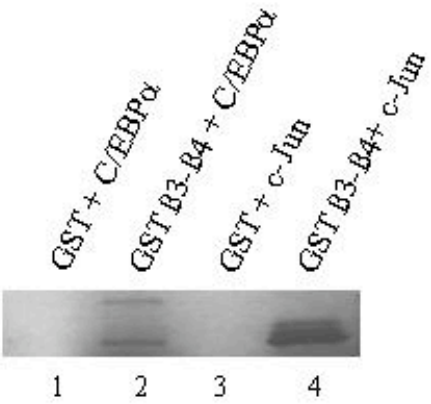
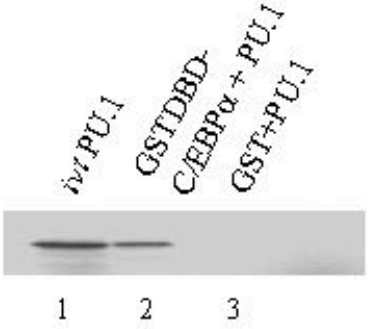


Figure 4C. C/EBPα binds to the β3-β4 region of the PU.1 DNA binding domain

(^{35}S) Met-labelled *in vitro* translated C/EBPα and c-Jun were incubated with GST- β3-β4 PU.1 (lanes 2,4 respectively) and with GST alone (lanes 1,3).



FFigure 4D. The DNA binding domain of C/EBPα interacts with PU.1

(^{35}S) Met-labelled *in vitro* translated PU.1 (lane 1) was incubated with GST-DNA binding domain of C/EBPα (lane 2) or GST alone (lane 3).

^{35}S-labelled *in vitro* translated PU.1 with the GST-DNA binding domain of C/EBP showed that C/EBP interacts with PU.1 via its DNA binding domain (Fig. 4**D**).

4.5 C/EBP interacts with PU.1 via its leucine zipper in the DNA binding domain

To answer the question if both the proteins interact *in vivo*, we transfected 293T cells with expression plasmids of PU.1, C/EBP, C/EBP LZ and C/EBP mBR using lipofectamine. The use of mutants of C/EBP in the DNA binding domain would further narrow down the region in the DNA binding domain responsible for interaction with PU.1. Whole cell lysates were immunoprecipitated with either rabbit-IgG or rabbit anti-PU.1 polyclonal antibody. C/EBP was detected by western blotting with C/EBP rabbit polyclonal antibody only in PU.1 immunoprecipitates (Fig. 5**A**). In the control IgG immunoprecipitate no C/EBP was detected. The blot was stripped and blotted for PU.1 expression (Fig. 5**B**). Expression of C/EBP and its mutants were checked by western blotting for C/EBP (Fig. 5**C**). C/EBP could not interact with PU.1 when the leucine zipper in its DNA binding domain was deleted, suggesting that C/EBP interacts with PU.1 via its leucine zipper in the DNA binding domain. This interaction of C/EBP with PU.1 is similar to that of C/EBP, because C/EBP also interacts with PU.1 via its leucine zipper in the DNA binding domain. This shows the conservation of interaction domains between different transcription factors of the same family, in this case C/EBP and C/EBP of the C/EBP family.

To address the question of whether C/EBP can interact with the activation domain of PU.1, 293T cells were transfected with a Gal4-luciferase reporter construct containing a minimal promoter with Gal4 DNA binding sites only, and expression plasmids of C/EBP and PU.1 activation domain fused to the DNA binding domain of Gal4 (PU.1-Gal4).

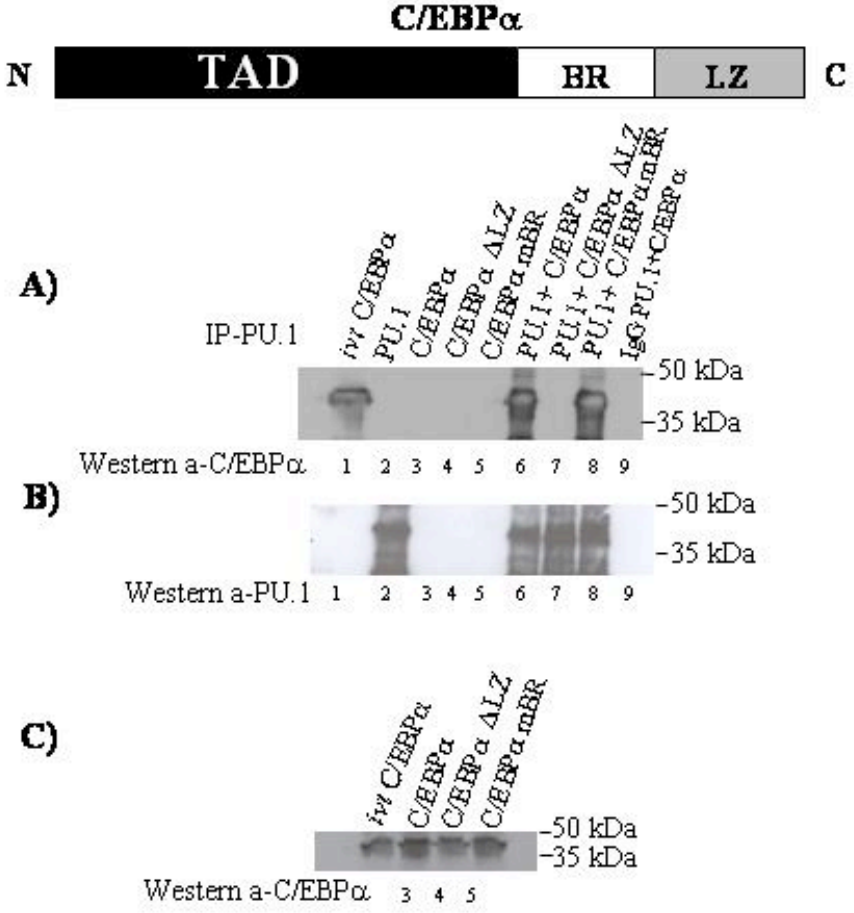


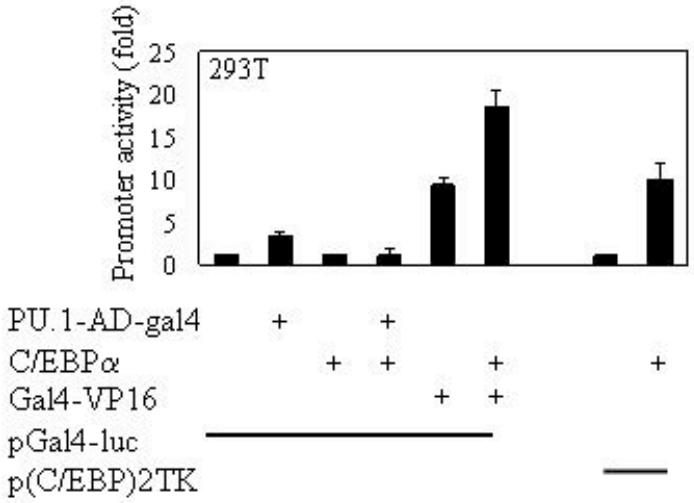
Figure 5. Coimmunoprecipitation of PU.1 and C/EBPα

A) Coimmunoprecipitation of PU.1 and C/EBPα from whole-cell lysates of transfected 293T cells with anti-PU.1 antibody or normal IgG. Western blot analysis was performed by using anti-C/EBPα antibody.

B) Coimmunoprecipitation of PU.1 and C/EBPα from whole-cell lysates of transfected 293T cells with anti-PU.1 antibody or normal IgG. Western Blot analysis was performed by using anti-PU.1 antibody.

C) Western blot analysis of the whole-cell lysates of the cells transfected with C/EBPα, C/EBPαΔLZ, and C/EBPαmBR was performed by anti-C/EBPα antibody.

- TAD- transactivation domain
- BR; LZ- Basic Region; Leucine Zipper DNA binding domain



PU.1-AD-gal4: Transactivation domain of PU.1 fused to Gal4DNA binding domain

Figure 6. C/EBPα interacts functionally with the activation domain of PU.1
Transient transfection in 293T cells with expression plasmids of C/EBPα activation domain of PU.1 fused to the DNA binding domain of Gal4 and Gal4-VP16. A minimal promoter with Gal4 DNA binding sites only (Gal4-luc) was used as reporter construct. p(C/EBP)2TK was used as a control reporter for C/EBPα. Luciferase activities were measured 24 hrs after transfection.

PU.1-Gal4 transactivates the Gal4-luciferase reporter 4.5 fold and this transactivation was blocked by C/EBP, C/EBP does not non-specifically inhibit the transactivation of Gal4-VP16 in transactivating Gal4-luc reporter construct. These results indicate atleast more than one functional interaction of C/EBP with PU.1; further investigations are required to define the interaction of C/EBP with the activation domain of PU.1. However, there are alternative interpretations for the above data e.g., C/EBP could quench more of the co-activators (CBP/p300) recruited by PU.1 activation domain and thereby a decrease in the transactivation capacity of PU.1 when co-transfected with C/EBP (Fig. 6) without physically interacting with PU.1.

4.6 C/EBP does not recruit TSA-sensitive co-repressors in downregulating PU.1´s transcriptional activity

To investigate whether C/EBP is recruiting co-repressors to down-regulate PU.1´s transactivation capacity, we transfected F9 cells with the TK promoter containing PU.1 binding sites and expression plasmids of PU.1, and C/EBP. We found that C/EBP blocks the activity of PU.1 transactivating the minimal TK promoter with PU.1 binding sites. Trichostatin A (TSA) was shown to be an inhibitor of a class of co-repressors. The transcription factor ETV6 (Tel) recruits these co-repressors and represses the promoter activity of Gal4-luciferase. Addition of TSA releases this repression as it is seen in the transfection of 293T cells with Gal4-Tel. In a similar experiment where the transactivation block of PU.1 by C/EBP is seen, addition of TSA did not release the repression. These data suggest that repression of PU.1´s activity by C/EBP is not mediated by the recruitment of TSA-sensitive co-repressors (Fig. 7).

4.7 C/EBP downregulates PU.1 expression in myeloid U937 cells

We investigated if C/EBP blocks the expression of PU.1 target genes. As PU.1 is autoregulatory in its expression[113], PU.1 itself is a target gene of PU.1. We therefore performed quantitative real time PCR using Real time Light Cycler technology (Roche) to determine the expression of PU.1 gene in the U937 cell line with Zn-inducible expression of C/EBP[153]. Ectopic expression of C/EBP in U937 bipotential myeloid cells resulted in differentiation of cells towards the neutrophil lineage. Induction of C/EBP expression upon stimulation with Zn was observed by western blot analysis for C/EBP. To correct for the variances in the cDNA synthesis step, PU.1 expression was set in relation to the G6PD housekeeping gene by calculating the ratios for PU.1/G6PD. C/EBP was expressed maximally after 6 hrs of Zn induction, and five time points of zinc induction were included to check for PU.1 expression. PU.1 expression was downregulated four fold after 8 hrs. In the control there was only a minimal change in PU.1 expression upon induction with zinc in U937 cells carrying the empty vector pPC18 (Fig. 8**A**). The data are consistent with the model that expression of PU.1 is downregulated after blocking of PU.1 function by C/EBP. We extrapolated this observation to promoter studies. 293T cells were transfected with human PU.1 promoter with luciferase activity and expression plasmids of PU.1 and C/EBP. PU.1 transactivated the promoter by about 5 folds. C/EBP alone transactivated the promoter by 2 folds. pGL2 was used as a control. C/EBP blocked the transactivation of PU.1 promoter by PU.1 (Fig. 8**B**).

4.8 C/EBP inhibits PU.1 induced dendritic cell development

It was previously shown that the enforced expression of C/EBP in a human bipotential myeloid progenitor cell line induces granulocytic

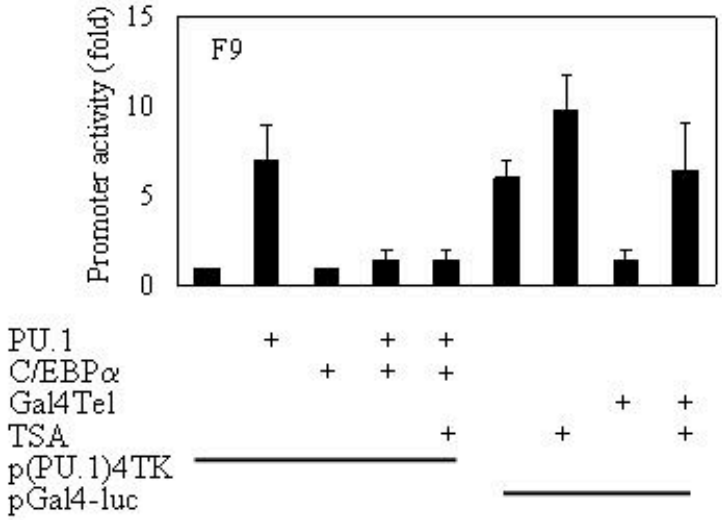


Figure 7. C/EBPα do not recruit TSA sensitive co-repressors to downregulate PU.1's activity

Transient transfection in F9 cells with reporter constructs of p(PU.1)4TK, p(mutated PU.1)4TK and expression plasmids of PU.1, C/EBPα and c-Jun. p(C/EBP)2TK is the control reporter for C/EBPα. Luciferase activities were measured 24 hrs post transfection.

differentiation and blocks monocytic differentiation[153]. On the other hand, PU.1 has been demonstrated to instruct transformed chicken multipotent hematopoietic progenitors to differentiate along the myeloid lineage[8]. In human $CD34^+$ hematopoietic progenitor cells, however, enforced expression of PU.1 promotes dendritic cell differentiation with characteristics of Langerhans cells, specified dendritic cells that reside in the epidermis[154]. To investigate biological significance of function blocking of PU.1 by C/EBP, we retrovirally expressed PU.1 and C/EBP in human $CD34^+$ hematopoietic progenitor cells. In contrast to mock control in which both granulocytic and monocytic cells differentiated (Fig. 9**A**), single transduction of PU.1 and C/EBP predominantly promoted differentiation of $CD1a^+$ dendritic cells (Figs. 9**B**,**E** and **F**) and granulocytes (Fig. 9**C**), respectively. PU.1 transduced cells were positive for CD1a, HLA-DR, CD80 and CD86 (Fig. 9**F**) suggesting that PU.1 specifically enhanced the dendritic cell expression. In the latter case of C/EBP transduction, terminal differentiation of neutrophils was markedly enhanced compared to mock control. Lineage specific differentiation markers were observed by FACS analysis of the surface antigens CD14 and CD15. Then, we coexpressed PU.1 and C/EBP in $CD34^+$ hematopoietic progenitor cells. In accordance with our mechanistic data of C/EBP blocking PU.1´s transcriptional activity, C/EBP blocked dendritic cell differentiation by PU.1, and instead induced granulocytic differentiation (Fig. 9**D** and **E**).

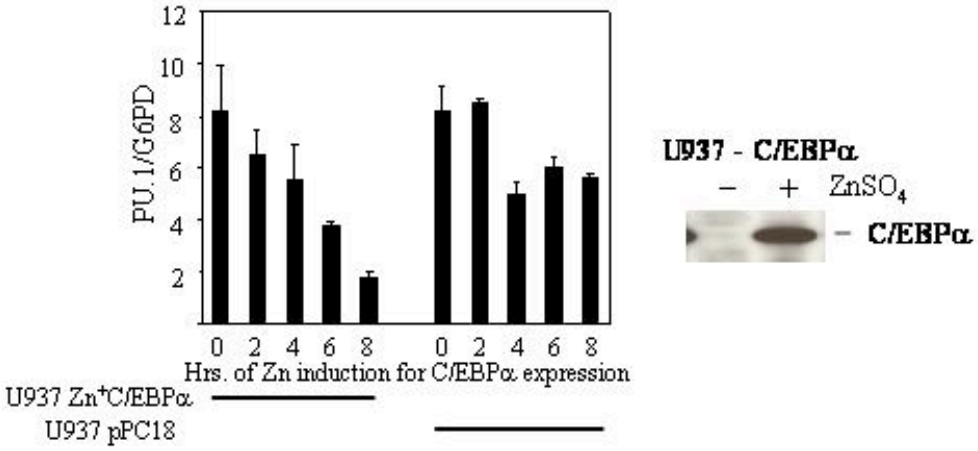


Figure 8A. Real Time PCR of PU.1 in ind. C/EBPα U937 cells

PU.1 expression was measured upon induction of C/EBPa with ZnSO4(right panel) by real time PCR. PU.1 expression is represented as ratio of PU.1 to house keeping gene G6PD. The same was done in the control cell line carrying the empty vector pPC18.

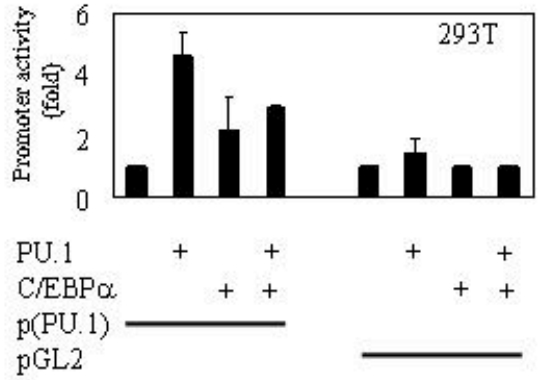
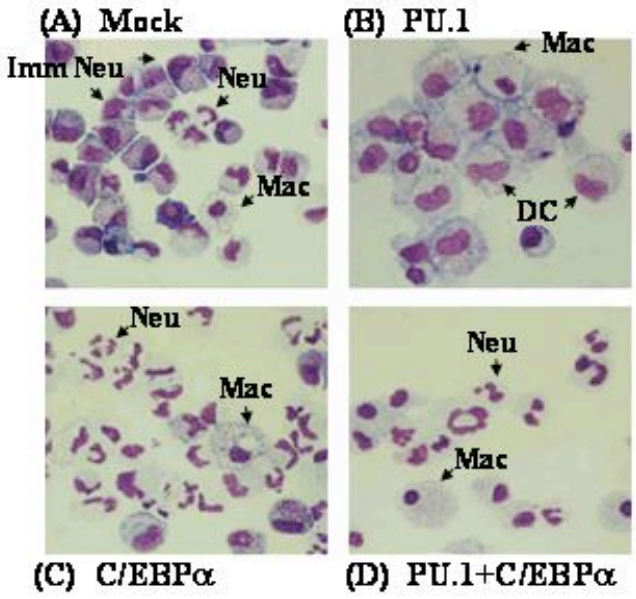


Figure 8B. C/EBPα blocks the transcriptional activity of PU.1 on PU.1 promoter

Transient transfection in 293T cells with a reporter construct of human PU.1 promoter (luciferase) and expression plasmids of PU.1 and C/EBPα. pGL2 was used as empty vector control. Luciferase activities were measured 24 hrs after transfection.



	DC	Mac	Gra
Mock	4.0	31.0	65.0
PU.1	61.2	16.8	32.0
C/EBPα	0.4	25.2	74.4
PU.1+C/EBPα	4.0	30.8	65.3

DC:dendritic cells, Mac:macrophage,
Gra:granulocyte (neutrophil and eosinophils),
Neu: neutrophil
Imm Neu: immature neutrophil

Figure 9A-D. C/EBPα inhibits PU.1 induced dendritic cell development from human CD34+ pluripotent myeloid progenitor cells.

Human CD34+ cord blood cells were retrovirally transduced with either PU.1 or C/EBPα, or co-transduced with both PU.1 and C/EBPα. Cells transduced with retrovirus containing empty vector were prepared as negative control cells (Mock) and were processed in parallel. Cells were cultured in the presence of mixed cytokines that facilitate myeloid differentiation (SCF, IL-3, GM-CSF, and G-CSF). After 10 days cell morphology was evaluated by Giemsa staining (A-D)

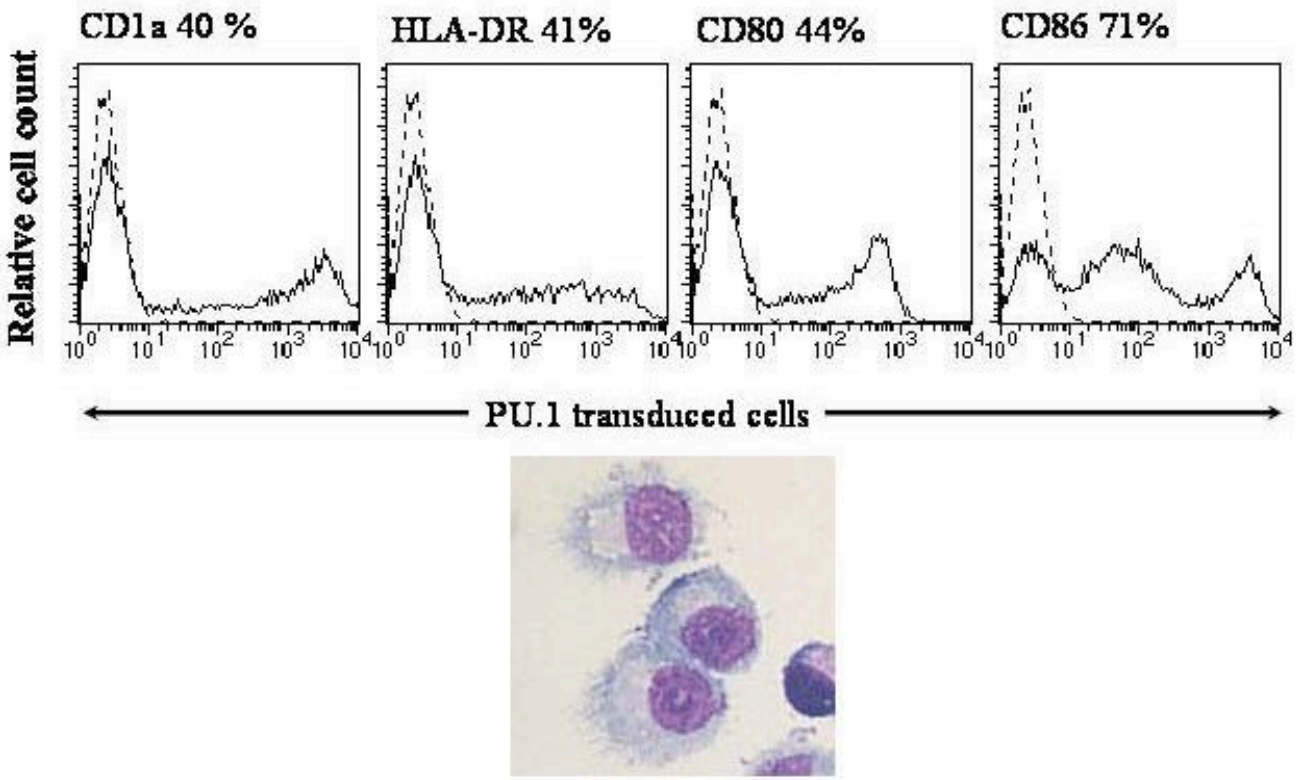


Figure 9E. Dendritic cells derived from PU.1 transduced CD34⁺ Cells

Expression of dendritic cell surface antigens (CD1a, HLA-DR, CD80, CD86) were analyzed by flow cytometry

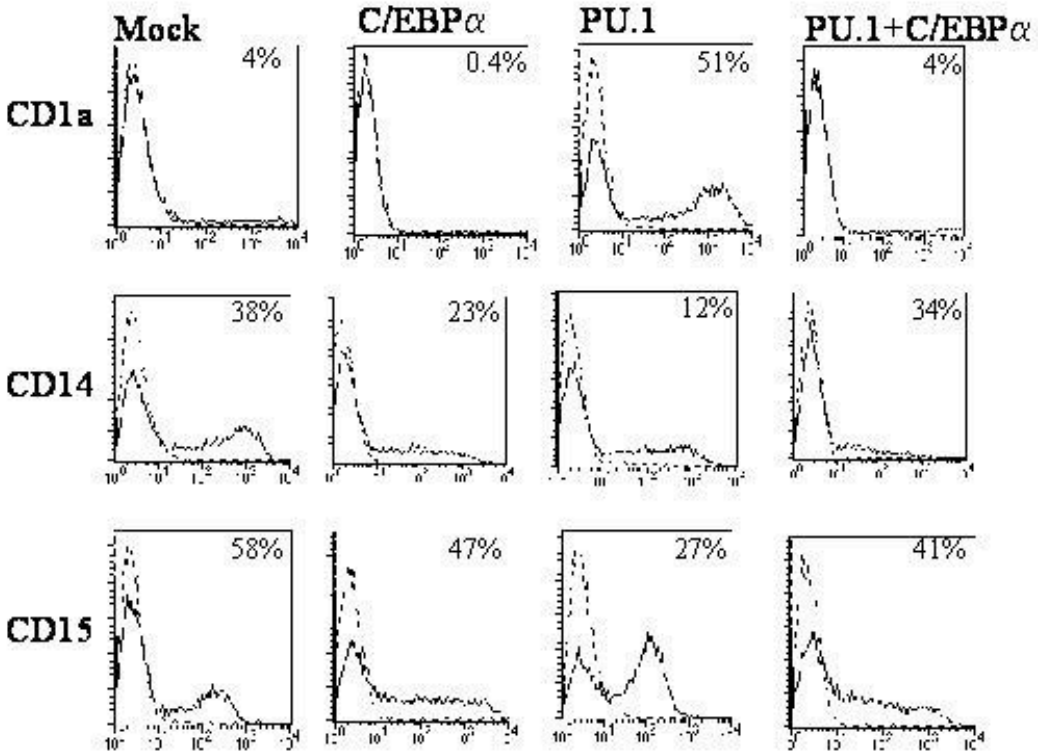


Figure 9F. Expression of cell specific surface antigens analyzed by Flow cytometry

Human CD34+ cord blood cells were retrovirally transduced with either PU.1 or C/EBPα, or co-transduced with both PU.1 and C/EBPα. Expression of cell surface specific antigen CD14, CD15 and CD1a expression was analyzed by flow cytometry analysis.

5 DISCUSSION

According to the current view of hematopoiesis, all blood cell types derive from a common pluripotent stem cell. In the adult organism the stem cells are found in the bone marrow where they divide to produce more stem cells (self renewal) and various progenitor cells committed to a single lineage which terminally differentiate to morphologically and functionally distinct erythroid, myeloid, or lymphoid cells. Within the myeloid compartment the same committed precursor can give rise to monocytic or granulocytic cells. This raises a question: What are the molecular mechanisms that dictate the fate of the common precursor to one or the other of these two diverse myeloid lineages?

The two transcription factors PU.1 and C/EBP play a major role in myelopoiesis. Both factors have been shown to synergize on various promoters including M-CSF receptor promoter and neutrophil elastase promoter. Both factors are expressed in bipotential myeloid cell, and therefore we were interested in knowing if there is any protein-protein interaction between these transcription factors and consequently if there is any functional significance of this interaction in lineage commitment. Although it was shown earlier that PU.1 and C/EBP synergize on promoters with both PU.1 and C/EBP binding sites like the M-CSF, G-CSF and GM-CSF receptor promoters, it had not been reported that C/EBP can block or downregulate the promoter activity of PU.1. The present work shows that the transcription factor C/EBP is capable of functionally blocking PU.1 ability to transactivate a promoter with PU.1 binding sites. C/EBP blocked PU.1 ability to transactivate the M-CSF receptor promoter with PU.1 binding sites alone. We also investigated if there are any protein-protein interaction between PU.1 and C/EBP. Our results show that there is indeed an interaction between the two proteins and the interaction sites responsible for this interaction were mapped: the leucine zipper in the DNA binding domain of C/EBP interacts with the

α3-α4 region of the DNA binding domain of PU.1. Various studies have reported that the transcription factors GATA-1, C/EBPα, c-Jun and AML-1 interact with this α3-α4 domain of PU.1. It has previously been shown that the transcription factor c-Jun is a JNK independent co-activator of PU.1, physically interacting with PU.1 in the α3-α4 region resulting in an increased M-CSF receptor expression. We show that as a consequence of the interaction of C/EBPα with PU.1 in the α3-α4 region the co-activator c-Jun is displaced from binding to PU.1 and PU.1 function is inhibited.

Investigating further the role of C/EBPα as a regulatory switch in hematopoietic cells, we used the bipotential U937 cells where it was shown that enforced expression of C/EBPα differentiates the cells along the granulocytic lineage[153]. Furthermore the induction of the granulocytic pathway results in up-regulation of C/EBPα. We investigated the expression of the monocytic specific factor PU.1 upon induction of U937 cells towards granulocytic lineage i.e., we looked for PU.1 expression in U937 cells with conditional ectopic expression of C/EBPα. PU.1 is auto-regulatory in its expression in myeloid cells. We observed that C/EBPα down-regulates PU.1 expression upon conditional ectopic induction of C/EBPα. It was further shown that C/EBPα blocks PU.1 transactivation of the human PU.1 promoter. Our data suggest that C/EBPα might block PU.1 transactivation capacity and thereby the ability of PU.1 to increase its own expression. It is interesting to note that a similar phenomenon of inhibiting PU.1 activity was observed with the transcription factor GATA-1. GATA-1 is capable of suppressing the myeloid phenotype without interfering with PU.1 gene expression, but instead was capable of inhibiting the activity of the PU.1 protein in a dose-dependent manner[155]. This inhibition was independent of the ability of GATA-1 to bind DNA, suggesting that it is mediated by protein-protein interaction. PU.1 interacts with GATA via PU.1 ETS domain. A similar interaction has been shown with a C/EBP family member C/EBPε: C/EBPε interacts with PU.1 via the leucine zipper of the DNA binding domain. This region of C/EBP interacting with PU.1 might be of great importance for the commitment of

cells to granulocytes. It should further be noted that mutations have been detected in the DNA binding domain of C/EBP in acute myeloid leukemia separately by two different groups[40], suggesting its importance in lineage commitment to granulocytes and in leukemia.

Repression of transcriptional activity by transcription factors has been attributed to the recruitment of co-repressors. Transcriptional repressors interacting with transcription factors reduce or repress the transactivation capacity. To rule out the possibility of such a phenomenon; i.e. C/EBP recruiting co-repressors to PU.1 and blocking its function, we used a repressor inhibitor to release the repression by C/EBP. Trichostatin A (TSA) is a HDAC inhibitor and it releases repression induced by HDAC's. In transient transfection assays C/EBP blocked PU.1´s activity even in the presence of TSA, suggesting that there are no co-repressors involved in blocking PU.1´s function by C/EBP.

It has previously been shown that PU.1 is important and essential for the development of monocytes. DeKoter *et al*. have also shown that the activation domain of PU.1 is essential to drive the cells to monocytes. We observed that C/EBP is functionally interacting with the activation domain of PU.1. Further investigation on this interaction and its possible role in lineage commitment is required. Interaction of C/EBP with the activation domain of PU.1 might disrupt possible protein-protein interactions, important for the PU.1 induced differentiation program. One of the candidates is CBP/p300, a co-activator of PU.1, which binds to the transactivation domain of PU.1. Inhibition of such an interaction by C/EBP would further block PU.1´s activity to induce monocytes/dendritic cells and enhance C/EBP´s capacity to induce granulocytes. One could then speculate that C/EBP might compete with the co-activator p300/CBP for binding to PU.1 thereby reducing the transactivation capacity of PU.1.

With regard to the instructive ability of C/EBP transcription factors to drive granulocytic differentiation, most of the evidence has been based on the experiments using cell lines. It has been shown earlier that the

C/EBP gene is activated at the stage of myeloid commitment and is specifically expressed in granulocytic cells. Increased levels of C/EBP expressed from an inducible promoter construct directed differentiation along the granulocytic pathway, as determined by morphological criteria. These data suggest the role of C/EBP as a molecular switch during early hematopoietic developmental events that direct cells to the granulocytic pathway. C/EBP blocked monocytic differentiation of these human bipotential myeloid progenitor cells. During TPA stimulation of monocytic development endogenous C/EBP was down-regulated and this down-regulation is required for differentiation along the monocytic pathway[153]. Ectopic expression of C/EBP represses PU.1 expression in bi-potential U937 myeloid cells.

To prove the biological meanings of the functional inhibition of PU.1 by C/EBP, $CD34^+$ hematopoietic progenitor cells were transduced with PU.1 and/or C/EBP. We present here the first evidence that the C/EBP transcription factor drives the granulocytic differentiation of primary hematopoietic cells. $CD34^+$ cells transduced with C/EBP preferentially developed eosinophills. C/EBP transcription factors have been implicated in the development of eosinophills in cooperation with GATA-1. Our data suggest that C/EBP overload is enough to drive eosinophilic commitment of myeloid progenitor cells. Neutrophilic differentiation and maturation is markedly enhanced with wild type C/EBP. This finding confirms the previous observation that enforced expression of C/EBP in a bipotential human myeloid cell line promotes neutrophilic differentiation. PU.1 is expressed throughout the myeloid lineage and is a master regulator involved in the development and function of all myeloid progenies. On the other hand, C/EBP family transcription factors have been shown to cooperate with PU.1 in transactivating promoters specific to granulocytes and/or macrophages. $CD34^+$ cells transduced with PU.1 promoted dendritic cell (DC) development. Mice with targeted disruption of the PU.1 gene reportedly lack the major population of myeloid DCs. DC development induced by PU.1 imitates

that induced by TNF. TNF activates c-Jun N-terminal kinase which then phosphorylates and activates c-Jun, a PU.1 co-activator. Thus, it is possible that the downstream target of TNF is PU.1 in terms of induction of DC development. In this context, C/EBP might act as a negative regulator by displacing c-Jun from binding to PU.1. Another possibility is that NFB activated by TNF cooperates with PU.1.

PU.1 and C/EBP binding sites are located in close proximity on most of the promoters specific to granulocytes and/or macrophages and these two factors synergistically transactivate these promoters. On the other hand, we show here that C/EBP physically interacts with PU.1 on the promoter with PU.1 binding site only, and displaces c-Jun, a co-activator of PU.1 from binding to PU.1, resulting in the suppression of PU.1 transactivation. We expect this kind of interaction to happen during commitment of pluripotent myeloid progenitor cells. PU.1 drives dendritic cell development while C/EBP direct cells towards granulocytes. In accordance with our model, when the cells were co-transduced with PU.1 and C/EBP, C/EBP inhibited PU.1-induced dendritic cell lineage commitment. Myeloid progenitors in the $CD34^{+}$ cell fraction are mostly pluripotent in their differentiation potential and can give rise to bipotential granulocyte/macrophage progenitors, eosinophils and Langerhans cells. Our biological data demonstrate that C/EBP blocks PU.1-induced dendritic cell commitment in pluripotent myeloid progenitors. Altogether, our data indicate that C/EBP drives granulocytic differentiation of myeloid progenitors by suppressing myeloid derived dendritic cell differentiation by blocking PU.1 function. DCs are major target cells for therapeutic approaches to allergy, autoimmune disease, infectious disease, and cancer. We propose that modulating transcription factors in myeloid progenitor cells would be a new approach to manipulate DC development and effector functions *in vivo*. Our approach will provide useful information to therapeutic manipulation of the immune system (Figure 10).

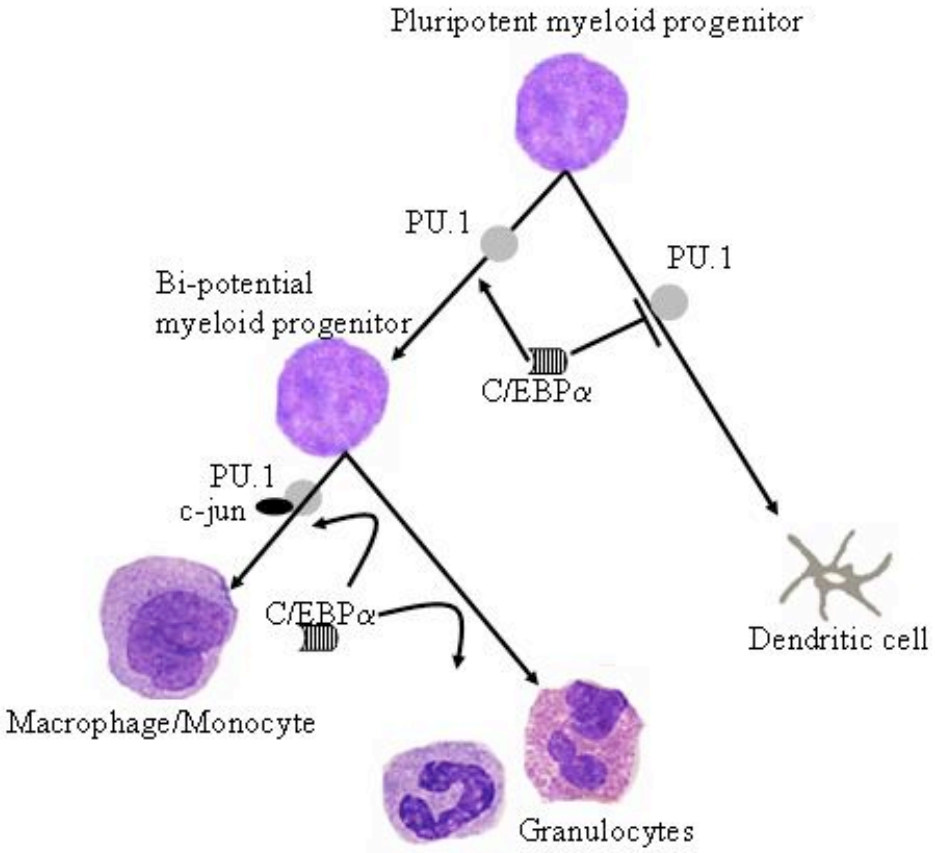


Figure 10. C/EBPα blocks PU.1 induced dendritic cell differentiation

C/EBPα interacts with PU.1, this interaction is between the β3-β4 region of the DNA binding domain of PU.1 and the leucine zipper of the DNA binding domain of C/EBPα. C/EBPα displaces the co-activator c-Jun from binding to PU.1, blocks PU.1´s function and downregulates its target genes. C/EBPα blocks PU.1 induced dendritic cell differentiation, and drives the cells to granulocytes.

In conclusion these results demonstrate a novel mechanism by which function of a lineage-specific transcription factor is inhibited by another lineage-restricted factor through direct protein-protein interactions. These findings not only contribute to understand the mechanisms involved in lineage commitment but also throw light on how protein-protein interactions participate in hematopoietic differentiation and leukemogenesis.

6 Summary

Several transcription factors have been shown to play a role in myelopoiesis. PU.1, an ets-family transcription factor, is required for the development of both myeloid and lymphoid lineages while the transcription factor CCAAT/enhancer binding protein family member C/EBP is essential for granulocytic development. We present here the first evidence that C/EBP blocks the function of PU.1. PU.1 and C/EBP interact physically and co-localize in myeloid cells. As a consequence of this interaction C/EBP can inhibit the function of PU.1 to activate a minimal promoter containing only PU.1 DNA binding sites. We further demonstrate that the leucine zipper in the DNA binding domain of C/EBP interacts with the 3/4 region in the DNA binding domain of PU.1, and as a result displaces the PU.1 co-activator c-Jun. Finally, C/EBP blocks PU.1 induced dendritic cell development from $CD34^+$ human cord blood cells. The functional blocking of PU.1 by C/EBP could be the mechanism by which C/EBP inhibits the cell fates specified by PU.1, and directs cell development to the granulocytic lineage.

Zussamenfassung

Verschiedene Transkriptionsfaktoren spielen eine Rolle in der Entwicklung myeloischer Zellen. PU.1, ein Transkriptionsfaktor aus der ETS-Familie, ist sowohl für die Entwicklung lymphatischer als auch für die Entwicklung myeloischer Zellen von Bedeutung. Der Transkriptions faktor C/EBP, ein an den CCAAT-Enhancer bindendes Protein, ist hingegen wesentlich verantwortlich für die Entwicklung von Granulozyten. Wir stellen hier den ersten Nachweis dafür vor, dass C/EBP die Funktion von PU.1 blockiert. PU.1 und C/EBP können einander binden und sind in myeloischen Zellen kolokalisiert. Wenn C/EBP PU.1 bindet, kann PU.1 einen minimalen Promotor mit Bindungsstelle für PU.1 nicht mehr aktivieren. Wir zeigen, dass der Leuzin-Zipper in der DNA-bindenden Domäne von C/EBP mit der 3/4-Region in der DNA-bindenden Domäne von PU.1 interagieren kann. Dadurch wird der Koaktivator von PU.1, c-jun, aus seiner Bindung mit PU.1 verdrängt. C/EBP hemmt PU.1 nicht, indem es Korepressoren rekrutiert. Vielmehr vermindert C/EBP die Expression von PU.1 in U-937-Zellen mit induzierbarem C/EBP, indem es den autoregulatorischen Effekt PU.1 auf den PU.1-Promotor hemmt.

Ausserdem blockiert C/EBP die durch PU.1 bedingte Entwicklung dendritischer Zellen aus $CD34^+$ menschlichen Nabel blutzellen. Diese funktionelle Blockade von PU.1 durch C/EBP könnte einer der Mechanismen sein, mit denen C/EBP den durch PU.1 determinierten Weg der Zelldifferenzierung hemmt und sich Zellen unter dem Einfluss von C/EBP zu Granulozyten entwickeln.

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Lebenslauf

Persönliche Daten

Name:	Reddy, Venkateshwar A
Geburtsdatum:	06.04.1975
Geburtsort:	Kagaznagar/Indien
Staatsangehörigkeit:	Indien
Familienstand:	ledig

Schulausbildung

1979-1989	FCHS Kagaznagar, India
1990-1992	DG&GA Junior College, Vijayawada, India

Studium

1992-1995	BSc (Applied Microbiology), Nizam College, Osmania University, Hyderabad, India
1995-1997	MSc (Biotechnology), Jawaharlal Nehru University, New Delhi, India (Masters thesis: Understanding protein folding, using Ribonuclease A as a model protein to isolate molten globule state during folding, and studying the folding kinetics by Circular Dichroism and isothermal titration colorimetry unter der Anleitung von Prof. Dr. Kunal B Roy)
1998-1999	Forschungs assistant bei Novartis/Universität Basel, Basel Schweiz, unter Anleitung von Prof. Dr. Ueli Aebi und Dr. Tudor Arvinte
1999-2002	Doktorarbeit unter Anleitung von Prof. Dr. med. W.Hiddemann und Dr. Gerhard Behre in den

	medizinischen klinik und poliklinik III am klinikum großhadern, München, Germany
Okt-2000	Promotions-Vorprüfung

Vorträge und abstracts

Vorträge:

Role of C/EBP in downregulating PU.1 trancriptional activity and its role in Lineage commitment of Multipotential progenitor cells, at **2nd Wissenschaftliches Symposium der MedizinischenKlinik III, Klinikum der universitäat München, Großhadern. Venue:WILBADKREUTH 13th july-15th july 2000.**

Poster Präsentation

1.Functional proteomics of PML-RAR alpha; Target proteins in t(15;17)-AML. Venkateshwar Reddy et al., **43rd American Society of Hematology annual meeting Orlando, USA. 7-11.12, 2001.**

2.The Granulocytic inducer C/EBP inactivates the monocytic factor PU.1, possible role in Lineage commitment decisions. **Deutschen und Osterreichischen Gesellschaften für Hämatologie und Onkologie (DGHO) Mannheim 30.9.2001-3.10.2001**

3.The Granulocytic inducer C/EBP inactivates the monocytic factor PU.1, possible role in Lineage commitment decisions. *Venkateshwar A Reddy et,al* . **42nd Annual Society of Hematology annual meeting San-Francisco, USA. 1.-5.12, 2000.**

4.The Granulocytic inducer C/EBP inactivates the monocytic factor PU.1, possible role in Lineage commitment decisions. **Biolog2000 2nd München Kongress for BioWissenschaften 18.11.2000**

5.Transcription factor C/EBP inactivates PU.1 during lineage commitment of the multipotential progenitor cells. Reddy, V.A. et al., **DGHO- Graz 2000**

6.Visualization of repetitively arranged Capsid protein gp23 by Electron Microscopy, high antibody titre of repetitively arranged gp23 at a distance of 10 A^o compared to purified protein gp23. Reddy,V.A. et al., **10th International Immunology Congress Sep 1998, New Delhi India.**

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1.Taschner N, Muller SA, **Reddy VA**, Goldie KN, Drake AF, Aebi U, Arvinte T. Modulation of antigenicity related to changes in antibody flexibility upon lyophilization. J Mol Biol. 2001 Jun 29;310(1):169-79.

2.Elsaesser A, Franzen M, **Reddy VA**, Tenen DG, Hiddemann W, Behre G. Leukemic fusion protien AML1-ETO positively regulates c-Jun protooncogene expression. Blood, 2000 96(11).

3.**Reddy VA** et al., The granulocytic inducer C/EBPa inactivates the myeloid master regulator PU.1: possible role in lineage commitment decisions. Blood 2001 Jul 15:100(2):483-490.

4.Behre G, **Reddy VA**, Peerzada A, Singh SM. Proteomic analysis of transcription factor interactions in myeloid stem cell development and leukemia. Expert. Opin. Ther. Targets 2002: 6(4).

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6.Elsässer A, Franzen M, Kohlmann A, Weisser M, Schnittger S, Schoch C, **Reddy VA**, Burel S, Zhang DE, Ueffing M, Tenen DG, Hiddemann W, Behre G. The fusion protein AML1-ETO in acute myeloid leukemia with translocation t(8;21) induces c-jun protein expression via the proximal AP-1 site of the c-jun promoter in an indirect, JNK-dependent manner. Oncogene. 2003 Aug 28;22(36):5646-57.

7.**Reddy VA**, Peerzada A, Pellicci P, Tenen DG, Behre G. Functional proteomics of PML-RARa target proteins in t(15;17)-AML (submitted to oncogene).