

Reciprocal Roles for CCAAT/Enhancer Binding Protein (C/EBP) and PU.1 Transcription Factors in Langerhans Cell Commitment

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Abstract

Myeloid progenitor cells give rise to a variety of progenies including dendritic cells. However, the mechanism controlling the diversification of myeloid progenitors into each progeny is largely unknown. PU.1 and CCAAT/enhancer binding protein (C/EBP) family transcription factors have been characterized as key regulators for the development and function of the myeloid system. However, the roles of C/EBP transcription factors have not been fully identified because of functional redundancy among family members. Using high titer–retroviral infection, we demonstrate that a dominant-negative C/EBP completely blocked the granulocyte–macrophage commitment of human myeloid progenitors. Alternatively, Langerhans cell (LC) commitment was markedly facilitated in the absence of tumor necrosis factor (TNF) α , a strong inducer of LC development, whereas expression of wild-type C/EBP in myeloid progenitors promoted granulocytic differentiation, and completely inhibited TNF α -dependent LC development. On the other hand, expression of wild-type PU.1 in myeloid progenitors triggered LC development in the absence of TNF α , and its instructive effect was canceled by coexpressed C/EBP. Our findings establish reciprocal roles for C/EBP and PU.1 in LC development, and provide new insight into the molecular mechanism of LC development, which has not yet been well characterized.

Key words: myeloid differentiation • lineage commitment • dendritic cells • eosinophils • dominant-negative C/EBP

Introduction

The hematopoietic system is maintained by the continuous proliferation and differentiation of progenitor cells generated from the pluripotent hematopoietic stem cells (1). Among lineage-committed progenitor cells, myeloid progenitors give rise to a variety of progenies, such as granulocytes, monocytes/macrophages, osteoclasts, mast cells, and dendritic cells. However, the mechanism controlling the

diversification of myeloid progenitor cells into each myeloid progeny is largely unknown. Transcriptional regulation is a key step in this process and myeloid-specific transcriptional regulatory factors establish gene expression programs that are intrinsic to cell diversification (2). Several transcription factors have been implicated in this process, including PU.1, CCAAT/enhancer binding protein (C/EBP)* family (2), IFN consensus sequence binding protein (3), JunB (4), and

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*Abbreviations used in this paper: bZIP, basic region-leucine zipper; C/EBP, CCAAT/enhancer binding protein; DC, dendritic cells; EGFP, enhanced green fluorescent protein; Eo, eosinophil; EPO, eosinophil peroxidase; FBS, fetal bovine serum; IRES, internal ribosome entry site; LC, Langerhans cells; MSCV, murine stem cell virus; NGFR, nerve growth factor receptor; RT, reverse transcription; SCF, stem cell factor.

MafB (5). Among them, PU.1 and C/EBP family proteins have been characterized as key regulators for the development and function of the myeloid system (2).

PU.1 is a member of the Ets transcription family whose expression is confined to hematopoietic cells. PU.1 mRNA is detectable even in multipotent hematopoietic stem cells and is up-regulated during myeloid and B cell differentiation. A large number of PU.1 target genes have been identified in myeloid cells (2, 6), indicating that PU.1 plays important roles at various stages of myeloid differentiation. Loss of function experiments revealed that in general PU.1 is essential for the development of myeloid and B cells (7, 8).

C/EBP proteins comprise a family of transcription factors that have a basic region-leucine zipper (bZIP) structure consisting of a DNA-binding basic region and a leucine zipper dimerization domain (9). All of the members of this family (C/EBP α , C/EBP β , C/EBP γ , C/EBP δ , C/EBP ϵ , and C/EBP ζ) share the highly conserved dimerization domain, by which they homodimerize or heterodimerize each other and bind to the cognate C/EBP consensus sequences. Among them, C/EBP α , C/EBP β , C/EBP δ , and C/EBP ϵ have a transcriptional activation domain and cooperatively activate the transcription of a variety of myeloid-specific genes with PU.1. On the other hand, C/EBP γ lacks the transcriptional activation domain and attenuates transcriptional activation of target genes through heterodimerizing with other family members, suggesting its dominant-negative characteristics. C/EBP ζ also works in a dominant-negative fashion because its unique structure of DNA-binding region prevents heterodimer binding to the classic C/EBP consensus sequences. C/EBP proteins control the transcription of genes involved in a broad range of physiological processes, including the development and function of hematopoietic cells, adipocytes, and hepatocytes (9). In the hematopoietic system, C/EBP α , C/EBP β , C/EBP δ , and C/EBP ϵ are preferentially expressed in myeloid lineage, whereas the expression of C/EBP γ and C/EBP ζ is ubiquitous. The overlapping expression of C/EBP members suggests that myeloid development and functions are regulated by various combinations of C/EBP homodimers and heterodimers. In this process, it is believed that there is a functional redundancy among C/EBP α , C/EBP β , C/EBP δ , and C/EBP ϵ , and that they could functionally compensate each other (10). Recent work with mice that are genetically altered to abolish the expression of C/EBPs, underscore the role of these factors in the myeloid system. Knockout mice of the C/EBP α gene show a selective block in the differentiation of granulocytes, including both neutrophils and eosinophils (11). In contrast, C/EBP ϵ knockout mice show impaired terminal differentiation and defective function of granulocytes, both of which cause susceptibility to fatal infection (12). These results strongly suggest a critical role of C/EBP α and C/EBP ϵ in granulocytic differentiation. C/EBP β knockout mice, however, do not show any defects in myeloid differentiation; instead, the macrophage function is profoundly impaired (13). In C/EBP δ knockout mice, my-

eloid cells are virtually intact (14). So far, no information is available on myeloid cells in C/EBP γ and C/EBP ζ knockout mice, although NK cell function is affected in C/EBP γ knockout mice (15). Given the functional redundancy among C/EBP members whose expression is highly overlapping in myeloid cells, single-gene knockout mice do not unveil all the physiological roles of C/EBP family members. Functional redundancy has been partially canceled by making double knockout mice of C/EBP β and C/EBP δ genes in terms of adipocyte differentiation (14).

Dendritic cells (DC) are the most effective type of antigen-presenting cells and are characterized by an exceptional ability to stimulate naive T cell responses, as well as B cell responses. Presently, two distinct types of DC, myeloid and lymphoid, are known (16). In human bone marrow, immature DC originate from CD34⁺ hematopoietic progenitor cells and are seeded via the bloodstream to the tissues where they capture and process antigens. Displaying large amounts of MHC-peptide complexes at their surface, they migrate to lymphoid organs where they activate antigen-specific T cells. There are two discrete populations of myeloid DC: Langerhans cells (LC) and dermal or interstitial DC (16). Bone marrow CD34⁺ hematopoietic progenitor cells contain progenitors for both populations. The LC progenitors express cutaneous lymphocyte antigen, lack CD14, and cannot differentiate into macrophages. In contrast, the dermal DC progenitors lack cutaneous lymphocyte antigen, give rise to CD14⁺ monocytes, and differentiate into either macrophages in response to M-CSF, or DC in response to GM-CSF and IL-4, or TNF α (17, 18). LC are specialized DC that are present in the epidermis, bronchi, and mucosae. Mice that are deficient in TGF β show selective absence of epidermal LC (19). On the other hand, mice with a dominant-negative mutation of the Ikaros gene lack all DCs except for LC (20), whereas RelB knockout mice selectively lack CD8 α ⁻ myeloid DC, but retain LC (21). These findings indicate the specialized development of LC from bone marrow progenitor cells.

To further understand the roles of C/EBP transcription factors in myeloid development, we tried to disrupt the functional redundancy among C/EBP family members by expressing a dominant-negative C/EBP that could antagonize all C/EBP members. The expression of a dominant-negative C/EBP completely blocked both granulocyte and monocyte/macrophage development from CD34⁺ hematopoietic progenitor cells, and instead facilitated LC development. Conversely, C/EBP transcription factors were shown to be inhibitory to LC development induced by cytokines, whereas another myeloid transcription factor, PU.1, positively regulated LC development. Our findings establish novel roles of C/EBP and PU.1 transcription factors in LC development.

Materials and Methods

Production of Retrovirus. The retroviral vector pGCSam, with an LTR derived from murine stem cell virus (MSCV), has intact splice-donor and splice-acceptor sequences for the generation of

subgenomic mRNA (22). A dominant-negative form of C/EBP (A-C/EBP), followed by IRES enhanced green fluorescent protein (EGFP) and mouse PU.1 cDNA, followed by ires nerve growth factor receptor (NGFR) truncated in the cytoplasmic domain, were subcloned into pGCSam. Human C/EBP α and C/EBP β followed by ires EGFP were subcloned into pMSCV, a retroviral vector with an LTR derived from MSCV. Human C/EBP α mutants, Δ bZIP (Δ 282–358) tagged with nuclear localization signals from SV40 large-T antigen, and Δ AD (Δ 1–174) were generated by PCR and tagged with a FLAG epitope at the NH₂ terminus. These mutants, followed by IRES EGFP, were similarly subcloned into pMSCV. To produce the recombinant retrovirus, plasmid DNA was transfected into 293 gp cells (293 cells containing the *gag* and *pol* genes but lacking an envelope gene), along with 10A1 *env*-expressed plasmid (pCL-10A1)(23) by CaPO₄ coprecipitation and supernatant from the transfected cells was collected to infect cells. 293 gp cells were supplied by Nikunj Somia (University of Minnesota, Minneapolis, MN).

Purification of Human Primary Cells. Human umbilical cord blood samples were obtained with informed consent from placentas of full term, normal newborn infants. Cord blood was supplied by Yoshihiro Shiina (Shiina Hospital, Ushiku, Ibaraki, Japan). Peripheral blood samples were obtained from normal healthy volunteers who had given informed consent. After the isolation of mononuclear cells by density-gradient centrifugation, CD34⁺ hematopoietic progenitors and CD14⁺ monocytes were obtained using magnetic bead separation (Miltenyi Biotec). In all experiments, ~95% of purified cells were positive for CD34 or CD14, as judged by specific antibodies (22).

Transduction of CD34⁺ Cells. CD34⁺ cells were prestimulated in IMDM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 50 ng/ml stem cell factor (SCF), 50 ng/ml thrombopoietin (provided by KIRIN), 50 ng/ml IL-6 (PeproTech), and 25 ng/ml Flt-3 ligand (PeproTech) for 20 h. After replating onto recombinant fibronectin fragment-coated culture dishes (Takara Shuzo Co.) containing virus supernatant and 5 μ g/ml protamine sulfate (Sigma-Aldrich), cells were centrifuged at 1,000 *g* for 30 min. Transduction was repeated three times with fresh virus supernatant every 12 h. 60 h after the first transduction, NGFR- or EGFP-positive cells were selected by cell sorting on a FACSVantage[®] (Becton Dickinson) and subjected to subsequent analyses. At this time point, ~85% of the cells were still positive for CD34 (22). To detect the expression of NGFR truncated in the cytoplasmic domain on the cell surface, cells were stained with mouse anti-human NGFR (CHEMICON) followed by PE-conjugated rabbit anti-mouse Igs (Dako).

Colony Assay and In Vitro Liquid Culture. CD34⁺ cells transduced with the indicated retrovirus were plated in methylcellulose medium (StemCell Technologies, Inc.) or cultured in IMDM with 10% FBS. Cytokines were supplemented to culture at the concentrations of 50 ng/ml SCF, G-CSF, GM-CSF, M-CSF, IL-3, and 5 U/ml erythropoietin. The culture dishes were incubated at 37°C in a 5% CO₂ atmosphere. Colony numbers were counted at day 14. To check the development of IL-5-responsive eosinophils, transduced cells were cultured in the presence of SCF, IL-3, and GM-CSF for the first 5 d. Then, cells were incubated in the presence of 50 ng/ml of IL-5 alone.

Generation of DC. CD34⁺ cells transduced with the indicated retrovirus were cultured in IMDM supplemented with 10% FBS, and 50 ng/ml SCF, GM-CSF, and TNF α (PeproTech) at 37°C in a 5% CO₂ atmosphere. To assess the frequency of DC colony formation, transduced cells were sorted into 96-well round-bottomed plates (Corning) at 10 cells per well and cultured under the

conditions described above. Peripheral blood CD14⁺ monocytes were cultured in IMDM supplemented with 10% FBS, and 50 ng/ml of GM-CSF and IL-4 (PeproTech) for 10 d. To activate monocyte-derived DCs, 50 ng/ml TNF α was added to the culture for the last 3 d.

Cell Surface Analysis. Expression of cell surface antigens was analyzed on a FACSVantage[®]. Cells were stained with APC-conjugated anti-human CD11b (BD Pharmingen) and CD14 (Immunotech); PE-conjugated anti-human CD1a, CD15 (Immunotech), CD14, CD40, and HLA-DR (BD Pharmingen); biotinylated anti-human CD80 and CD86 (BD Pharmingen), followed by PE-conjugated streptavidin (BD Pharmingen); and unconjugated anti-human Langerin (Immunotech) and E-cadherin (R&D Systems), followed by PE-conjugated rabbit anti-mouse Igs (Dako). Cells that became marked with propidium iodide were gated out as dead.

Reverse Transcription (RT)-PCR. After the isolation of mononuclear cells, cord blood CD34⁺ cells and peripheral blood CD14⁺ monocytes were purified by cell sorting on a FACSVantage[®]. DCs were generated from cord blood CD34⁺ cells and peripheral blood CD14⁺ monocytes as described above, and then CD1a⁺ DCs were purified by cell sorting. The total RNA was isolated from sorted cells using ISOGEN-LS solution (Nippon Gene), and reverse-transcribed using ThermoScript RT-PCR system (GIBCO BRL) and oligo-dT primer. The amount of cDNA was normalized by the quantitative PCR using TaqMan rodent GAPDH control reagent (PerkinElmer). Semiquantitative RT-PCR reactions were then performed for 35 cycles using normalized cDNAs and recombinant Taq DNA polymerase. The cycling parameters were denaturation at 94°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 30 s. PCR products were separated on agarose gel and visualized by ethidium bromide staining. The primer sequences were: C/EBP α sense primer 5'-AAGGTGCTGGAGCTGACCAG-3'; antisense primer 5'-AATCTCCTAGTCCTGGCTCG-3', C/EBP β sense primer 5'-ACAGCGACGAGTACAAGATCC-3'; antisense primer 5'-GCAGCTGCTTGAACAAGTTCC-3', C/EBP γ sense primer 5'-ATGGATCGAAACAGTGACGAG-3'; antisense primer 5'-TCAGTGCTAATGGACTGTACG-3', C/EBP δ sense primer 5'-ACATAGGAGCGCAAAGAAGC-3'; antisense primer 5'-GCTTTATTCTTACAAATACTG-3', C/EBP ϵ sense primer 5'-AGTCTGGGGAAGAGCAGCTTC-3'; antisense primer 5'-ACAGTGTGCCACTTGGTACTG-3', C/EBP ζ sense primer 5'-TACCTATGTTTCACCTCCTGG-3'; antisense primer 5'-ATTCTCTCAGCTAGCTGTGC-3', PU.1 sense primer 5'-ATGTGCCTCCAGTACCCATC-3'; and antisense primer 5'-TCTTCTGGTAGGTCATCTTC-3'.

Allogenic T Cell Proliferation (MLR). Allogenic CD4 T cells were purified from cord blood cells using magnetic bead separation (Miltenyi Biotec). In all experiments, 95–97% of purified cells were positive for CD4 as judged by a specific antibody. CD34⁺ cells, transduced with the indicated retrovirus, were cultured in the DC condition for 10 d and used as stimulator cells. A constant number of 10⁵ allogenic CD4 T cells (responder) were incubated with graded numbers of irradiated (3,000 rad, ¹³⁷Cs source) stimulators. The experiments were performed in 96-well flat-bottomed plates (Corning) in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS. The proliferation of T cells was monitored by measuring BrdU (5-bromo-2'-deoxyuridine) incorporation (Boehringer) on day 4 of culture. Cells were harvested for 16 h after the addition of BrdU. BrdU incorporation was assessed by absorbance at a wavelength of 370 nm using a multiwell ELISA reader.

Cytochemical Analysis. Cells were cytocentrifuged onto glass slides and were stained by May-Grünwald Giemsa staining. To detect eosinophil peroxidase (EPO) expression, permeabilized cytospun cells were stained with anti-human EPO (BD Pharmingen), followed by EnVision-labeled polymer/alkaline phosphatase (Dako).

Development of Cell Lines Expressing A-C/EBP. U937 cells were transfected with a linearized human methallothionein promoter (24), driving A-C/EBP expression by electroporation and selected by G418. We isolated several U937/A-C/EBP clones that induced a high level of A-C/EBP protein after treatment with 100 μ M ZnSO₄. Monocytic differentiation of U937 cells was induced with 50 ng/ml human GM-CSF.

Western Blotting. Cells were solubilized with lysis buffer (50 mM Tris [pH 8.0], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 150 mM sodium chloride, 50 μ g/ml aprotinin, and 1 mM PMSF), and then lysates were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was probed with biotinylated anti-FLAG antibody (Sigma-Aldrich), followed by streptavidin-HRP (Amersham Pharmacia Biotech).

Results

A Dominant-negative C/EBP Switches Myeloid Cell Fate from Granulocytes/Macrophages to LCs. To disrupt the functional redundancy among C/EBP family members in myeloid cells, we adopted a dominant-negative C/EBP that potentially antagonizes all C/EBP members. The dominant-negative C/EBP, designated as A-C/EBP, is a 102-amino acid protein consisting of an amino-terminal 9-amino acid FLAG epitope, a 13-amino acid linker, a 31-amino acid-designed acidic amphipathic helix, and a 49-amino acid leucine zipper domain of C/EBP α (25). The leucine zipper from C/EBP α specifically interacts with endogenous C/EBP leucine zippers, whereas the NH₂-terminal acidic extension forms a coiled coil with endogenous C/EBP basic regions. This heterodimeric coiled coil structure is much more stable than C/EBP α bound to DNA and causes the dominant-negative protein to abolish DNA binding of endogenous C/EBP family members.

By using a retrovirus-mediated gene transfer system, we expressed A-C/EBP in human cord blood CD34⁺ progenitor cells and analyzed its effects on myelopoiesis. We transduced cells with the retroviral vector GCsam-A-C/EBP-internal ribosome entry site (IRES)-EGFP (Fig. 1 A), which drives expression of both A-C/EBP and EGFP from a single bicistronic message. After transduction, EGFP-positive cells were selected by cell sorting and subjected to *in vitro* assay. Among various kinds of cytokines, CD34⁺ progenitors expressing A-C/EBP failed to respond to myeloid-specific cytokines, including G-CSF, M-CSF, and IL-5 (Fig. 1 B). On the other hand, they normally responded to erythropoietin, an erythroid-specific cytokine, and showed a distinct growth profile in response to GM-CSF as compared with the mock control (Fig. 1 B). GM-CSF is a cytokine that supports cell growth and the differentiation of all kinds of myeloid cells. In the presence of SCF and GM-CSF, the transduced cells showed comparable proliferation with the control at the beginning, but after 1 wk they

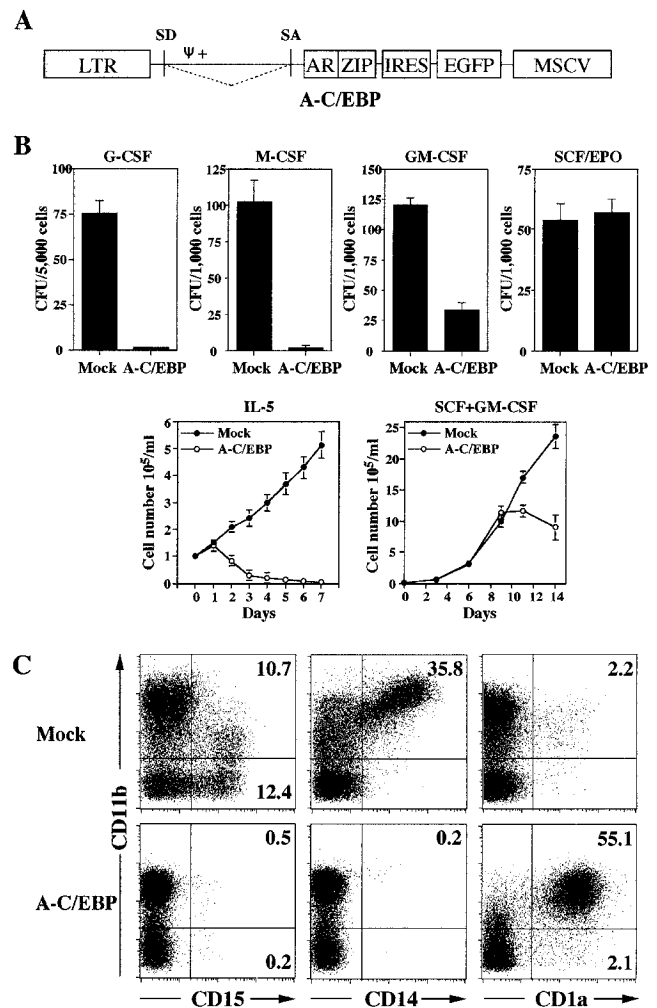


Figure 1. Altered differentiation of CD34⁺ progenitors expressing a dominant-negative C/EBP. (A) The schematic representation of the retroviral vector, GCsam-A-C/EBP-IRES-EGFP, encoding A-C/EBP, a dominant-negative C/EBP, linked by an IRES to a cDNA encoding EGFP. The 3' LTR of the vector is replaced with MSCV. ψ +, packaging signal; SD, splice donor; SA, splice acceptor. (B) The effect of A-C/EBP on the growth of transduced CD34⁺ cells. After transduction, EGFP-positive cells were selected. Then, cytokine-dependent cell growth was evaluated by CFU generated in the presence of indicated cytokines and by liquid culture in the presence of IL-5 or SCF+GM-CSF. To evaluate IL-5-dependent cell growth, cells were cultured in the presence of SCF, IL-3, and GM-CSF for the first 5 d to promote the development of eosinophil progenitors. Then, cytokines were replaced to IL-5 alone. Results are shown as mean \pm SD of three representative experiments (CFU assay), or of triplicate cultures (liquid culture). (C) Flow cytometric profiles of transduced cells cultured for 8 d in the presence of SCF and GM-CSF. Mock represents the cells transduced with empty vector. Results represent repeated experiments.

stopped growing. Flow cytometric analysis revealed that although the transduced cells failed to develop CD15⁺ granulocytes and CD14⁺ macrophages, they unexpectedly gave rise to CD1a⁺ CD11b⁺ myeloid DCs (Fig. 1 C).

To evaluate the effect of A-C/EBP on myelopoiesis, we took advantage of well-characterized cell lines. We first transduced cell lines with GCsam-A-C/EBP-IRES-

EGFP, and then EGFP-positive cells were selected by cell sorting and subjected to in vitro liquid culture. Interestingly, A-C/EBP dramatically inhibited cell proliferation of myeloid cell lines, U937 (promonocytes), and HL60 (promyelocytes) (Fig. 2 A). In contrast, A-C/EBP did not at all affect the growth of nonmyeloid cell lines, Jurkat (T cells), and K562 (erythroid cells) (Fig. 2 A).

Next, we generated U937 cells, stably transfected with the A-C/EBP cDNA where expression is under the control of the zinc-inducible human metallothionein promoter, and analyzed the effect of A-C/EBP on U937 cell differentiation. After treatment with GM-CSF, CD14⁻ U937 cells differentiate into CD14⁺ monocytes/macrophages (Fig. 2 B). However, the expression of A-C/EBP in U937 cells markedly inhibited monocytic differentia-

tion and promoted an alternative pathway, CD1a⁺ DCs (Fig. 2 B). CD1a⁺ cells were also positive for CD11b and CD11c (unpublished data). These data indicate that C/EBP transcription factors are essential for the development of both granulocytes and macrophages, but not for DC development.

A Dominant-negative C/EBP Promotes LC Development. DC differentiation from human CD34⁺ hematopoietic progenitor cells can be triggered in vitro by a combination of cytokines consisting of SCF, GM-CSF, and TNF α . With these cytokines, hematopoietic progenitors give rise to two DC progenitors: CD1a⁺CD14⁻ progenitor that differentiates into LCs, and CD1a⁻CD14⁺ monocyte that differentiates into non-LC DCs (17). As shown in Fig. 3 A, CD34⁺ hematopoietic progenitor cells transduced with empty retroviral vector gave rise to DCs differentiated through both pathways. In contrast, cells expressing A-C/EBP barely took the pathway through CD14⁺ monocytes, but showed enhanced DC differentiation from CD1a⁺CD14⁻ progenitors, even in the absence of TNF α (Fig. 3 A). Although there were some variations in DC population ranging at ~15%, the characteristic profiles of DCs differentiated from control and A-C/EBP-expressing cells were always reproducible. There was no significant difference in DC morphology with and without A-C/EBP expression (Fig. 3 A). CD14⁺ monocyte-derived DCs and LCs could be distinguished by the expression of well-characterized cell surface antigens. E-cadherin is a molecule that is highly expressed on LCs and is involved in interactions between keratinocytes and LCs in the epidermis (26). Langerin, a lectin that is specific to LCs, is an endocytic receptor involved in the induction of Birbeck granule formation (27). DCs derived from transduced CD34⁺ cells with A-C/EBP were highly positive for both E-cadherin and Langerin when compared with control cells (Fig. 3 B), which confirms the predominant development of LCs with a dominant-negative C/EBP expression. As is shown in Fig. 3 C, CD34⁺ cells transduced with A-C/EBP, generated a significantly higher number of CD1a⁺ DCs than the mock control in the absence of TNF α . Moreover, even though A-C/EBP has only additive effects on the total DC number in the presence of TNF α (Fig. 3 C), the absolute LC number is much higher than the mock control, as judged by the higher percentages of E-cadherin- and Langerin-positive LC (Fig. 3 B).

To evaluate the effect of A-C/EBP on LC commitment, we assessed the frequency of DC colony formation. Transduced CD34⁺ cells were sorted into 96-well plates at 10 cells per well. At day 10, each well was examined individually by microscopy for the presence of DC colonies consisting of cells with long spiny processes. Under the condition supplemented with TNF α , control cells and A-C/EBP-expressing cells efficiently formed DC colonies at comparable frequencies (Fig. 3 D). Significantly, A-C/EBP-expressing cells exclusively gave rise to DC colonies in 74% of the wells in the absence of TNF α , the condition under which control cells preferentially formed granulocyte/macrophage colonies, but very few DC colonies (Fig. 3 D).

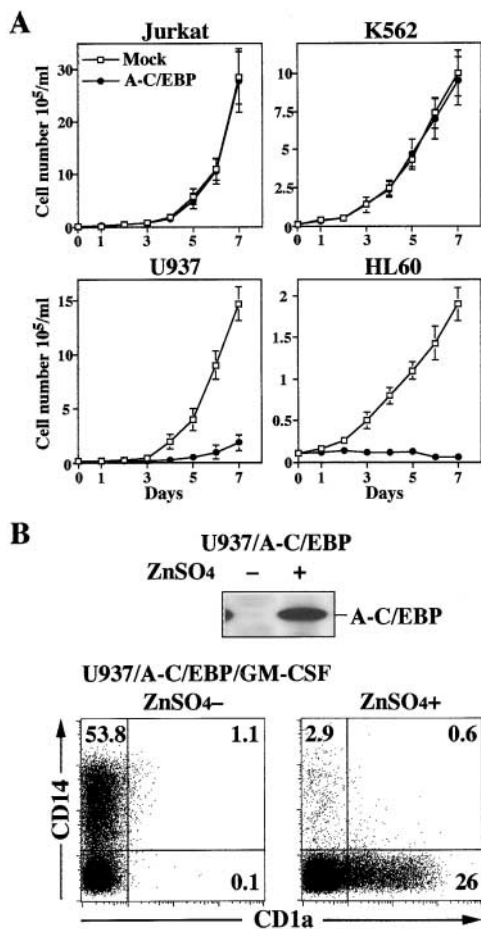


Figure 2. Growth and differentiation of human myeloid cell lines expressing a dominant-negative C/EBP. (A) The effect of A-C/EBP on the growth of human cell lines. After transduction, EGFP-positive cells were selected by cell sorting, and then subjected to in vitro liquid culture. Results are shown as mean \pm SD of triplicate cultures. (B) The effect of A-C/EBP on the differentiation of a myeloid cell line. Lysates from 2×10^5 U937/A-C/EBP cells, either untreated or treated with 100 μ M ZnSO₄ for 24 h, were separated by SDS-PAGE and immunoblotted with anti-FLAG antibody (upper panel). U937/A-C/EBP cells were treated with GM-CSF in the absence or presence of 100 μ M ZnSO₄ for 10 d to induce differentiation, and then analyzed by flow cytometry (lower panel). Results represent two independent clones of transfectants.

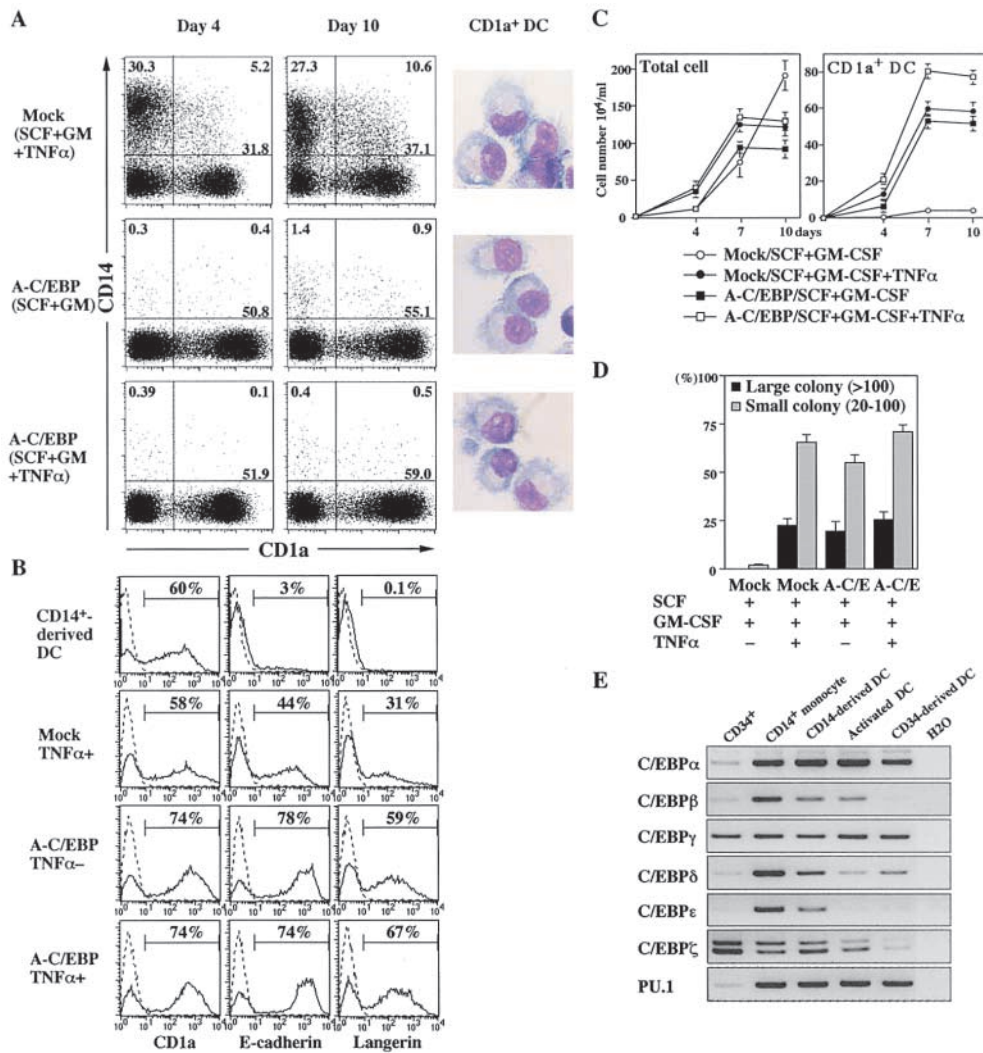


Figure 3. Promoted LC commitment in CD34⁺ progenitors expressing a dominant-negative C/EBP. (A) DC differentiation of CD34⁺ progenitors transduced with A-C/EBP. After transduction, EGFP-positive cells were selected and cultured for the indicated days either with or without TNF α , in addition to basic cytokines, SCF, and GM-CSF. Expression of CD1a and CD14 cell surface antigens were analyzed by flow cytometry. At day 10, CD1a⁺ cells were collected by cell sorting and processed for May-Griinwald Giemsa staining. (B) Flow cytometric profiles of transduced CD34⁺ progenitors cultured for 10 d either with or without TNF α , in addition to SCF and GM-CSF. CD14⁺ monocytes were cultured for 10 d in the presence of GM-CSF and IL-4, and treated with TNF α for the last 3 d, and then similarly processed as a control. (C) The effect of A-C/EBP on DC development from transduced CD34⁺ cells. After transduction, EGFP-positive cells were selected and cultured in the presence of indicated cytokines. The absolute cell number of CD1a⁺ DCs (right panel) was calculated from the percentage of CD1a⁺ cells detected by FACS[®] analysis. Results are shown as mean \pm SD of triplicate cultures. (D) The plating efficiency of transduced CD34⁺ cells to give rise to DC colonies. Transduced CD34⁺ cells were sorted into 96-well

plates at 10 cells per well and cultured for 10 d with the indicated cytokines. DC colonies consisting of more than 100 cells and of 20–100 cells were defined as large and small colonies, respectively. Results are shown as mean \pm SD of triplicate cultures. A-C/E represents A-C/EBP (E) The expression of human C/EBP family. RT-PCR was performed on normalized cDNA templates. PCR products were electrophoresed on agarose gels and visualized by ethidium bromide staining. Cord blood CD34⁺ cells (CD34⁺) and peripheral blood CD14⁺ monocytes (CD14⁺ monocytes) were collected by cell sorting. Peripheral blood CD14⁺ monocytes were cultured for 10 d in the presence of GM-CSF and IL-4, either without (CD14-derived DC) or with TNF α , for the last 3 d (activated DC), and then CD1a⁺ DCs were collected by cell sorting. Cord blood CD34⁺ cells were cultured in the presence of SCF, GM-CSF, and TNF α for 14 d, and then CD1a⁺ DCs were collected by cell sorting (CD34-derived DC). Lane H₂O represents the negative control without template.

These data suggest that CEBP transcription factors are inhibitory to LC commitment.

The expression of C/EBP family members in granulocytes and macrophages has been characterized in detail (2). However, their expression in DCs has not yet been analyzed. By using RT-PCR, we analyzed mRNA expression of C/EBP family members (Fig. 3 E). C/EBP members were expressed weakly in freshly isolated CD34⁺ cells, with the exception of C/EBP γ and C/EBP ζ . However, their mRNA expression was up-regulated in CD14⁺ monocytes, and maintained during DC differentiation induced by GM-CSF and IL-4, although some of them were down-regulated in activated DCs. In contrast, only C/EBP α and C/EBP δ were up-regulated during the DC

differentiation from CD34⁺ progenitors. Expression of another myeloid regulator, PU.1, was similarly up-regulated in both monocytes and DCs.

Function of DCs Expressing a Dominant-negative C/EBP. Even after the DC commitment, MSCV-driven A-C/EBP expression is maintained throughout differentiation. Thus, we analyzed the effects of A-C/EBP on DC maturation and function. We first checked the expression of functional molecules, including costimulatory molecules CD40, CD80, and CD86, an activated marker molecule CD83, and an MHC class II molecule, HLA-DR (Fig. 4 A). DCs with A-C/EBP expressed significant levels of functional molecules even in the absence of TNF α , and expressed comparable or even higher levels of functional molecules

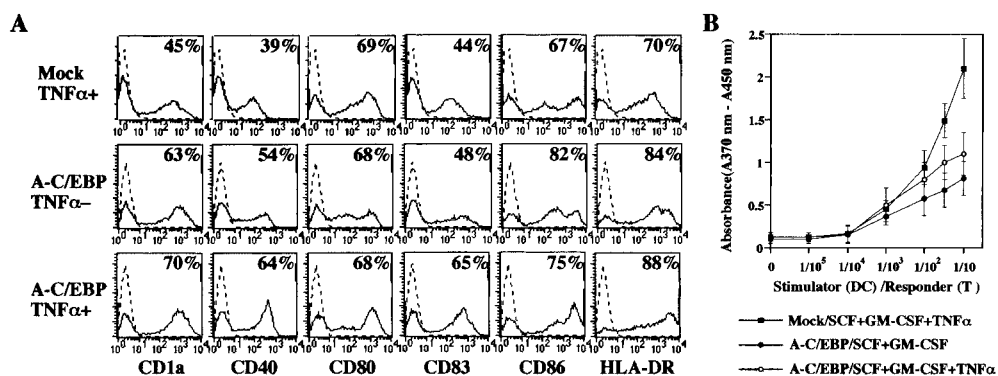


Figure 4. Maturation and function of DCs derived from transduced CD34⁺ cells with A-C/EBP. (A) Flow cytometric profiles of transduced CD34⁺ progenitors cultured for 10 d either with or without TNF α , in addition to SCF and GM-CSF. (B) Allogenic T cell proliferation stimulated by cells generated from transduced CD34⁺ cells. CD34⁺ cells transduced with indicated retrovirus were cultured with indicated cytokines for 10 d (stimulator). A constant number of 10⁵ allogenic CD4 T cells (re-

sponder) were incubated with graded numbers of irradiated (3,000 rad, ¹³⁷Cs source) stimulators. Proliferation of T cells was monitored by measuring bromodeoxyuridine incorporation after 4 d of culture. Results are shown as mean \pm SD of triplicate cultures.

than control cells. We then studied the T cell stimulatory capacity of DCs with A-C/EBP in the allogenic MLR (Fig. 4 B). Considering the advanced maturation status of the A-C/EBP-transduced DCs, we expected that they behave normally in function. However, they showed a limited capacity in stimulating T cell proliferation. Although the addition of TNF α in culture increased their stimulatory capacity, their capacity was always lower than the controls. During the MLR, DCs with A-C/EBP were prone to die from apoptosis earlier than the control cells (unpublished data).

C/EBP Facilitates Granulocytic Differentiation. The effects of C/EBP family members on myeloid differentiation are evident with C/EBP α , C/EBP β , and C/EBP ϵ . Knockout mice of C/EBP α and C/EBP ϵ genes show a selective block in the differentiation of granulocytes (11) and the impaired terminal differentiation of neutrophils (12), respectively. In addition, the forced expression of either

C/EBP α or C/EBP β induces eosinophilic differentiation of a multipotent chicken progenitor cell line, MEF (28), and of C/EBP α it induces a neutrophilic differentiation of a bipotential human myeloid cell line, U937 (29). However, no evidence has been reported that demonstrates the instructive effects of C/EBP transcription factors on the differentiation of primary hematopoietic cells.

To confirm their effects on myeloid differentiation, we transduced CD34⁺ progenitor cells with either C/EBP α or C/EBP β and then cultured cells in the presence of SCF and GM-CSF (Fig. 5 A). As expected, transduced cells showed enhanced development of CD15⁺ granulocytes. Among granulocytes, eosinophils preferentially developed, which were positive for EPO (57% and 52% of total cells with C/EBP α and C/EBP β , respectively) and showed IL-5-dependent cell growth (Fig. 5 B). In addition, neutrophil maturation was markedly promoted as judged by the nuclear segmentation, although the neutrophil population was

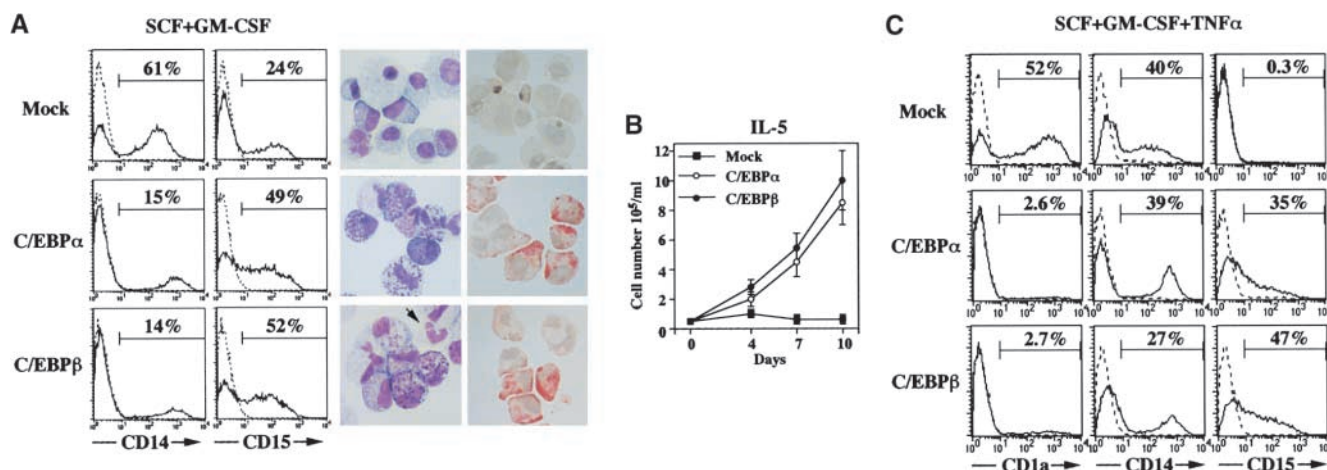


Figure 5. C/EBP promotes granulocytic differentiation and inhibits DC differentiation. (A) Promoted granulocytic differentiation of CD34⁺ cells transduced with wild-type C/EBP. CD34⁺ progenitors transduced with either C/EBP α or C/EBP β were cultured for 10 d in the presence of SCF and GM-CSF, and then analyzed by flow cytometry, May-Grünwald Giemsa staining, and immunostaining for intracytoplasmic EPO. Arrows indicate neutrophils with advanced differentiation. (B) The effect of C/EBP expression on the IL-5-dependent cell growth. After transduction, cells were cultured in the presence of SCF and GM-CSF for 3 d. Then, cytokines were replaced to IL-5 alone. Results are shown as mean \pm SD of triplicate cultures. (C) The inhibited DC differentiation of CD34⁺ progenitors transduced with wild-type C/EBP. CD34⁺ progenitors transduced with either C/EBP α or C/EBP β were cultured for 10 d in the presence of SCF, GM-CSF, and TNF α , and then analyzed by flow cytometry.

much lower than eosinophils. In contrast, monocytic differentiation was partially suppressed, but a significant number of CD14⁺ monocytes/macrophages still developed.

Next, we analyzed the function of C/EBP α and C/EBP β with respect to their ability to inhibit DC differentiation. Given the strong promoting effect of a dominant-negative C/EBP on LC differentiation, it is easy to speculate that C/EBP transcription factors function as negative regulators in this process. Enforced expression of C/EBP α or C/EBP β in CD34⁺ progenitor cells completely inhibited TNF α -induced DC differentiation of both LC and DC derived from CD14⁺ monocytes (Fig. 5 C), and instead induced differentiation of CD15⁺ granulocytes, leaving a significant number of CD14⁺ monocytes/macrophages still developed. These findings confirm the inhibitory effect of C/EBP transcription factors in LC development, as well as raise the possibility that C/EBP inhibits the differentiation of monocytes into DCs.

C/EBP Cancels DC Differentiation Induced by PU.1. In search of transcription factors that positively regulate DC differentiation, we identified that PU.1 facilitates DC differentiation. CD34⁺ cells transduced with PU.1 preferentially differentiated into DCs without TNF α (Fig. 6 A). The majority of DCs induced by PU.1 were positive for E-cadherin and Langerin, suggesting that PU.1 promotes the development of LC-type DC. In addition, PU.1 showed

comparable capacity to A-C/EBP in terms of DC commitment in the absence of TNF α (Fig. 6 B). These data establish PU.1 and C/EBP as positive and negative regulators for LC commitment, respectively.

To analyze the functional interaction between the two factors, we cotransduced CD34⁺ cells with both PU.1 and C/EBP and then cultured cells in the absence of TNF α . As was the case with TNF α , both C/EBP α and C/EBP β completely inhibited DC differentiation induced by PU.1 (Fig. 7 A). Next, to localize the C/EBP domain responsible for the inhibition of DC development, we generated C/EBP α mutants that lack either basic leucine zipper domain or NH₂-terminal transactivation domain. Although the mutants were expressed at the same levels as wild-type C/EBP α (unpublished data), they failed to inhibit DC differentiation induced by both PU.1 and TNF α (Fig. 7 B), indicating that both domains are essential to inhibit DC development. We further analyzed the mRNA expression of C/EBPs and PU.1 in transduced cells by RT-PCR (Fig. 7 C). At the mRNA levels, however, we could not detect any direct effects on endogenous C/EBP and PU.1 expression by exogenous gene expression.

Discussion

Pluripotent myeloid progenitor cells give rise to progenitors for granulocytes/macrophages (CFU-GM/eosinophil [Eo]) and LC-type DCs (Fig. 8). In this study, we showed that myeloid progenitor cells expressing a dominant-negative C/EBP differentiate exclusively into LCs (Figs. 1 and 3). Even the U937 cell line, which has never been known to differentiate into DCs, showed DC differentiation when the A-C/EBP expression was induced (Fig. 2 B). As is clearly shown in Fig. 3 D, A-C/EBP strongly affected the lineage commitment of myeloid progenitor cells, and switched the myeloid progenitor cell fate from granulocytes/macrophages into LCs. Its effect on the lineage commitment is comparable with that of TNF α , a strong DC inducer. Conversely, the expression of wild-type C/EBP inhibited LC commitment induced by TNF α , and promoted granulocyte/macrophage differentiation (Fig. 5 C). These data indicate that C/EBP family is essential to commit myeloid progenitor cells to granulocyte/macrophage lineage. On the contrary, C/EBP family acts as a negative regulator for the commitment of LCs.

In search of transcription factors that positively regulate LC development, we identified that PU.1 facilitates LC development. PU.1 promoted LC commitment of CD34⁺ progenitor cells (Fig. 6). Moreover, LC commitment induced by PU.1 was completely inhibited by coexpressed wild-type C/EBP (Fig. 7 A). These data not only suggest that PU.1 is one of the positive regulators that drive LC commitment, but also support our finding that C/EBP family acts as a negative regulator for this process. These findings contrast with the requirement of both PU.1 and C/EBP in the development of granulocytes and macrophages, and suggest that dosage balance between the two regulators is the crucial determinant for LC commitment in

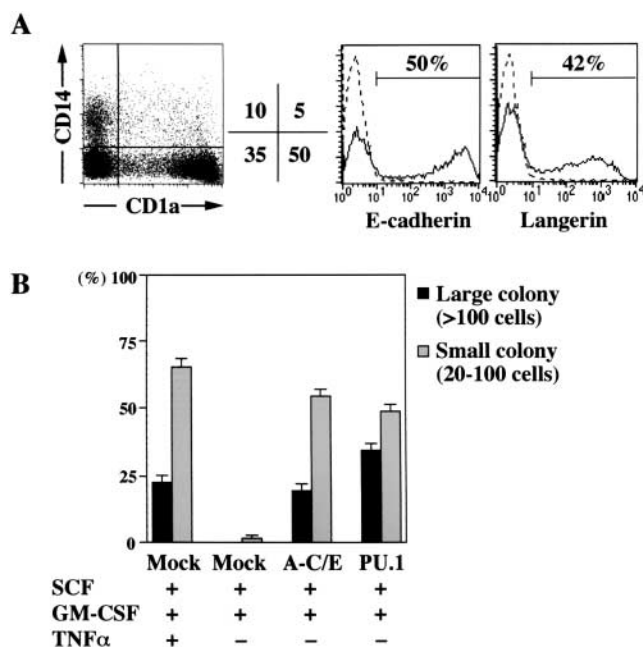


Figure 6. PU.1 promotes LC commitment in CD34⁺ progenitors. (A) PU.1 promotes LC differentiation. CD34⁺ progenitors transduced with PU.1 were cultured for 10 d in the presence of SCF and GM-CSF, and then analyzed by flow cytometry for cell surface antigen expression. (B) Plating efficiency of transduced CD34⁺ cells to give rise to DC colonies. Transduced CD34⁺ cells with the indicated retrovirus were sorted into 96-well plates at 10 cells per well and cultured for 10 d with the indicated cytokines. DC colonies consisting of more than 100 cells and of 20–100 cells were defined as large and small colonies, respectively. Results are shown as mean \pm SD of triplicate cultures. A-C/E represents A-C/EBP.

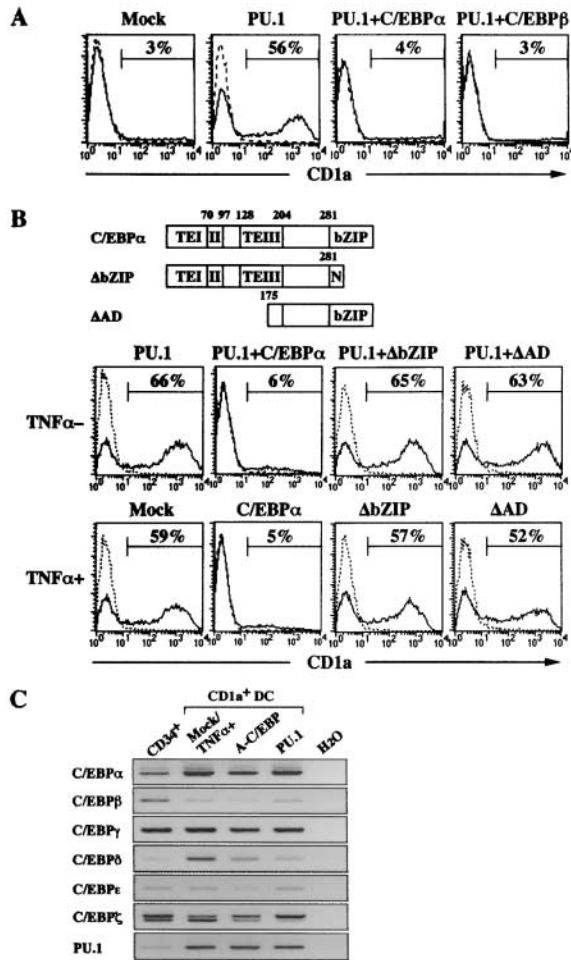


Figure 7. Reciprocal roles for C/EBP and PU.1 in the development of LCs. (A) The inhibition of PU.1-dependent LC differentiation by cotransduced wild-type C/EBP. CD34⁺ progenitors cotransduced with PU.1 and either C/EBP α or C/EBP β were cultured for 10 d in the presence of SCF and GM-CSF. Then, CD1a expression was analyzed by flow cytometry. (B) The effects of C/EBP α mutants on DC differentiation. Schematic representation of C/EBP α mutants (upper panel). TE, transactivation element; N, nuclear localization signal. CD34⁺ progenitors cotransduced with PU.1 and C/EBP α mutants were cultured for 10 d in the presence of SCF and GM-CSF only (middle panel). CD34⁺ progenitors that only transduced with C/EBP α mutants were similarly processed in the presence of SCF, GM-CSF, and TNF α (lower panel). Then, CD1a expression was analyzed by flow cytometry. (C) RNA expression of endogenous C/EBP and PU.1 in transduced cells by RT-PCR. Cord blood CD34⁺ cells (CD34⁺) were collected by cell sorting. Cord blood CD34⁺ cells transduced with empty vector (mock) were cultured for 10 d in the presence of SCF, GM-CSF, and TNF α . Cord blood CD34⁺ cells transduced with either with A-C/EBP or PU.1 were similarly processed in the presence of SCF and GM-CSF only. Then, CD1a⁺ DCs were collected by cell sorting. Lane H₂O represents the negative control without template.

pluripotent myeloid progenitor cells. We have recently found that C/EBP α physically interacts with PU.1 through its bZIP region, and displaces c-Jun, a coactivator of PU.1 (30) from binding to PU.1, resulting in the suppression of PU.1 transactivation (unpublished data). We expected that this kind of interaction might happen in the commitment of pluripotent myeloid progenitor cells. However, C/EBP α Δ AD mutant that retains bZIP domain failed to

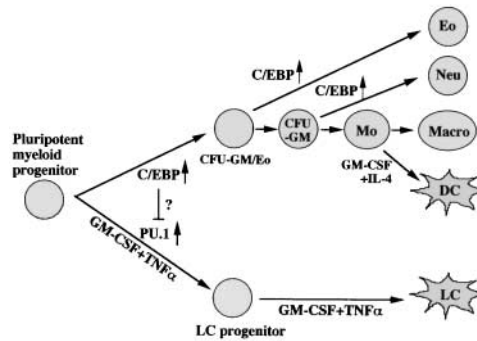


Figure 8. Model for the role of C/EBP and PU.1 in lineage commitment. As explained in the text, C/EBP activity is essential for the commitment of pluripotent myeloid progenitors into granulocyte/macrophage lineage. With impaired C/EBP function, pluripotent myeloid progenitors take an alternative differentiation pathway of LC. On the other hand, the enforced expression of PU.1 in pluripotent myeloid progenitor cells promotes LC commitment, whereas that of C/EBP promotes granulocytic differentiation and inhibits PU.1-induced LC commitment. These findings suggest that the lineage commitment of pluripotent myeloid progenitor cells is mediated by functional balance between PU.1 and C/EBP transcription factors. Eo, eosinophils; Neu, neutrophils; Macro, macrophages.

inhibit LC differentiation induced by PU.1 and TNF α (Fig. 7 B). Δ bZIP mutant also did not inhibit. These data indicate that the intact form of C/EBP is essential to inhibit LC differentiation. Moreover, we could not detect any direct effects on endogenous mRNA expression of C/EBP and PU.1 by exogenous A-C/EBP or PU.1 (Fig. 7 C). Nevertheless, these data do not necessarily deny the possible cross talk between C/EBP and PU.1. Detailed analysis is needed to precisely understand the possible interplay between the two regulators in LC commitment. Additionally, it is also possible that either the C/EBP and PU.1 are direct downstream targets of TNF α , or they have functional cross talks with downstream targets of TNF α , such as NF κ B. The roles of C/EBP and PU.1 in TNF α -signaling cascade also remain to be resolved. Reciprocal interactions of the two transcription factors have been reported to determine erythroid versus myeloid cells (GATA-1 vs. PU.1) (31, 32) and distinct pituitary cell types (Pit-1 vs. GATA-2) (33). Such a mechanism may also operate in a number of other cell types. Further analysis will help our understanding of the regulatory mechanism of lineage commitment involving multiple transcription factors.

A-C/EBP not only promoted DC commitment, but also DC maturation of transduced CD34⁺ progenitor cells. Transduced cells rapidly differentiated into mature DCs (Fig. 3 A) and the expression levels of functional molecules were mostly higher in cells with A-C/EBP rather than in the control cells (Fig. 4 A). These findings suggest that C/EBP transcription factors are not necessary for DC maturation and function. However, DCs with A-C/EBP showed limited capacity of T cell stimulation in the allogenic MLR. Given the mRNA expression of several C/EBP members in DCs (Fig. 3 E), C/EBP transcription factors seem to have implications in DC function. In many tissues and cells, C/EBP β and C/EBP δ are strongly up-regulated

at the transcriptional level by inflammatory stimuli such as bacterial lipopolysaccharide, and by cytokines such as IL-6, IL-1, and TNF α (34). It is possible that C/EBP members are up-regulated in activated DCs and play a role in regulating DC function, as C/EBP β has indispensable roles in the regulation of macrophage function (13). Further analysis is needed to determine the role of C/EBP transcription factors in DC function.

C/EBP transcription factors are essential for granulocytic and monocytic development. Pluripotent myeloid progenitor cells diverse into a number of myeloid progenies with distinct functions. PU.1 is expressed throughout myeloid lineage and is a master regulator involved in the development and function of all myeloid progenies (2). On the other hand, C/EBP family transcription factors have been shown to cooperate with PU.1 in transactivating promoters that are specific to granulocytes/macrophages (2). Impaired granulocytic differentiation is evident in mice deficient in the C/EBP α and C/EBP ϵ gene, which suggests the indispensable function of these two family members in supporting granulocytic differentiation. In contrast, single-gene knockout mice of C/EBP family genes have not shown impaired macrophage differentiation (9), suggesting a functional redundancy among C/EBP members in supporting macrophage differentiation. By using a dominant-negative C/EBP, we clearly demonstrated the essential role of C/EBP transcription factors in the development of macrophages and granulocytes. As shown in Fig. 1, A-C/EBP inhibited granulocyte/macrophage development from transduced CD34⁺ progenitor cells. In addition, A-C/EBP inhibited monocytic differentiation of an immature myeloid cell line, U937 (Fig. 2 B). In these experiments, however, A-C/EBP caused lineage switch into DCs rather than complete differentiation block. Thus, these data underscore the critical role of C/EBP transcription factors in the commitment of pluripotent myeloid progenitor cells into granulocyte/macrophage lineage (Fig. 8). Their roles in later stages of monocytic differentiation remain to be determined.

With regard to the instructive ability of C/EBP transcription factors to drive granulocytic differentiation, most of the evidence has been based on the use of cell lines in the experiments. We have presented the first evidence of C/EBP transcription factors driving granulocytic differentiation of primary hematopoietic cells (Fig. 5). CD34⁺ cells transduced either with C/EBP α or C/EBP β , preferentially developed into eosinophils. C/EBP α and C/EBP β have been implicated in the development of eosinophils in cooperation with GATA-1 (35, 36). Our data suggest that C/EBP overload is enough to drive eosinophilic commitment in myeloid progenitor cells. Eosinophils develop from the committed myeloid progenitor cells (CFU-GM/Eo) along with CFU-GM (Fig. 8). The expression level of C/EBP in combination with GATA-1 could be the critical determinant for lineage commitment in the committed myeloid progenitor cells. Moreover, although the neutrophil population was much lower than eosinophils, neutrophil differentiation and maturation were markedly acceler-

ated with wild-type C/EBP (Fig. 5). This finding confirms our previous observation that the enforced expression of C/EBP α in a bipotential human myeloid cell line promotes neutrophilic differentiation (29). Taken together, our findings suggest that C/EBP transcription factors play a role in each cell commitment throughout myeloid development, and regulate diversification of the myeloid system (Fig. 8).

Transcriptional regulation of myeloid DC development is largely unknown. In mice, CD8 α ⁻ myeloid DCs are missing in mice with a dominant-negative mutation of Ikaros gene, and in RelB knockout mice (20, 21). However, these mutant mice still retain LC development, supporting specialized development of LCs from bone marrow progenitor cells. In this study, we presented the first evidence of transcriptional regulation governing LC development. The positive regulator, PU.1, is a potential downstream player for TNF α , a strong inducer of DC development. Several cytokines other than TNF α are known to support DC development including GM-CSF, TGF β , and Flt-3 ligand (37, 38). We expect to further understand transcriptional regulation of DC development by identifying downstream transcription factors of these cytokines. 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.

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