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Regular Article

HEMATOPOIESIS AND STEM CELLS

C/EBPα is required for development of dendritic cell progenitors

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Key Points

- C/EBPα is needed for transition from stem/progenitor cells to common dendritic cell progenitors.
- C/EBPα is dispensable in later stages of dendritic cell maturation.

Dendritic cells (DCs) are master regulators of the immune system, but molecular regulation of early DC differentiation has been poorly understood. Here, we report that the transcription factor C/EBPα coordinates the development of progenitor cells required for production of multiple categories of DCs. C/EBPα was needed for differentiation from stem/progenitor cells to common DC progenitors (CDPs), but not for transition of CDP to mature DCs. C/EBPα deletion in mature DCs did not affect their numbers or function, suggesting that this transcription factor is not needed for maintenance of DCs in lymphoid tissues. ChIP-seq and microarrays were used to identify candidate genes regulated by C/EBPα and required for DC formation. Genes previously shown to be critical for DC formation were bound by C/EBPα, and their expression was decreased in the earliest hematopoietic compartments in the absence of C/EBPα. These data indicate that C/EBPα is important for the earliest stages of steady-state DC differentiation. (*Blood.* 2013;121(20):4073-4081)

Introduction

Dendritic cells are bone marrow–derived antigen-presenting cells, and subsets differ with respect to location, phenotype, and function.[1-4] Murine conventional $CD11c^+$ $B220^-$ dendritic cells (cDC) regulate adaptive immune responses and are distinct from other phagocytic cells involved in innate immunity. $CD11c^+$ $B220^+$ plasmacytoid dendritic cells (pDC) represent another major category of these cells specialized to respond to viral infection and produce substantial amounts of type I interferons (IFN). Because dendritic cells in lymphoid organs have no self-renewing potential, they must be continually replenished from hematopoietic stem and progenitor cells.[5-9] However, the routes and transcriptional regulators of differentiation used for production of DCs are not completely understood.

Hematopoiesis is a continuous process in which stem cells differentiate into non–self-renewing progenitors that subsequently give rise to mature blood cells.[10] Some studies have considered DCs to be activated or more differentiated monocytes.[11] However, several categories of progenitors have been characterized to be committed to DC production, and at least the early stages of these precursors are marked by expression of the Flt3 receptor for Flt3 ligand.[12-14] The sequential differentiation from a relatively primitive $ckit^+$ $Sca\text{-}1^+$ lineage marker negative (KSL) category may give rise to common myeloid progenitors (CMP), Lin^- $cKit^+$ $Flt3^+$ $MCSFR^+$ macrophage/DC progenitors (MDP) and CDPs,[5-7,12] and there is substantial evidence that commitment to only a DC fate can occur as early as the KSL fraction.[7] These data have clearly shown that steady-state DC populations are not derived from monocytes but rather arise from a newly characterized set of DCPs and subsets, all which have unique and specialized functions.[6,7,9,15,16] It has also been suggested that some DC subsets are derived from common precursors, MDPs and CDPs, whose fate depends on cytokine signals or environmental cues. Although this likely represents a major differentiation sequence for producing cDC and pDC, several studies of DC differentiation have shown that DC subsets can be generated from both myeloid and lymphoid progenitors.[8,12,17,18] Common lymphoid progenitors (CLPs) can also produce DCs during steady state but have increased potential to do so during infections.[12,19,20] In summary, several recent studies indicate that steady-state DCs can be derived from a number of DC-committed progenitors, including KSL.

Production of specialized blood cells depends on the orchestrated actions of transcription factors, and many have been implicated in DC formation.[21] IRF4, IRF8, E2-2, Id2, Gfi1, and PU.1 may all contribute, whereas only PU.1 is required for all DC subsets.[13,22,23] This may function in part by controlling the expression of Flt3, although rescue of this surface receptor did not overcome the PU.1 deficiency.[13] Many of these other transcription factors have broader influence not only on DCs, but also on myeloid lineage cells.[1,21]

The CCAAT/enhancer binding protein (C/EBP) family of transcription factors has basic region leucine zipper structures with DNA-binding basic regions and leucine zipper dimerization domains.[24-26] In particular, C/EBPα is expressed by stem cells, and levels steadily increase with differentiation toward GMPs.[27] Mice lacking C/EBPα have cell surface–defined CMPs but lack GMPs and more differentiated granulocytic stages.[28,29] However, the importance of C/EBPα in DC formation has not been rigorously assessed. Recent

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reports suggest that cells in an early progenitor fraction of the thymus that are marked with a C/EBPα–Cre × EYFP reporter displayed enhanced potential to form DCs.[19] Here, we demonstrate that C/EBPα is critical for early DC differentiation, especially when these cells are derived from myeloid progenitors. In addition, although C/EBPα is required for the formation of primitive cells with DC potential, it is not needed for the maintenance of DC maturation. These novel findings demonstrate discrete C/EBPα-dependent steps in production of specialized DC subsets. That knowledge is crucial in understanding how these immune response regulators are controlled in health and disease, such as in leukemia.

Materials and methods

Mice

Mice were housed in a sterile barrier facility approved by the IUCAC at the Beth Israel Deaconess Medical Center. MxCre C/EBPα and PU.1 conditional knockout mice have been described previously.[28,30] CD11c-Cre mice were purchased from Jackson Laboratory.[31]

Flow cytometry

Single-cell suspensions were analyzed by flow cytometry using the following conjugated antibodies obtained from BD Pharmingen (BD), BioLegend (San Diego, CA), or eBioscience (San Diego, CA). Cells were analyzed using an LSRII flow cytometer or sorted by FACSAria (BD Biosciences, San Jose, CA). FlowJo (Tree Star) was used for data analysis.

Western blot

75 000 cells were sort-purified into phosphate-buffered saline (PBS). Trichloroacetic acid (TCA) was added to a final concentration of 10% TCA. TCA-treated samples were incubated on ice for 15 minutes and spun down for 10 minutes at 13 200 rpm at 4°C. Pellets were solubilized by adding 4× loading buffer with β-mercaptoethanol and boiled for 10 minutes at 95°C. Proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred and blotted on polyvinylidene fluoride membranes (Millipore). Primary antibodies used were rabbit anti-C/EBPα (SC-61), anti-C/EBPβ (SC-150), anti-PU.1 (SC-352), goat anti–Gfi-1 (SC-8558, all from Santa Cruz), or rabbit anti–IRF-8 (#5628), anti-RelB (#4954), or anti-KLF4(#4964; Cell Signaling).

Immunofluorescence C/EBPα staining

Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature and blocked for 15 minutes with 5% fetal bovine serum in PBS containing 0.1% Triton-X. After incubation with primary antibodies in 1% fetal bovine serum in PBS containing 0.1% Triton-X, cells were washed in PBS and incubated for one additional hour with fluorophore-labeled secondary antibodies. Specimens were analyzed on an Axiovert 200M fluorescence microscope, and images were acquired with a Zeiss Axiocam camera.

T-cell proliferation assay

Sorted $CD11c^+$, $CD19^-$, and $CD3^-$ DCs from spleen were mixed with splenic $CD4^+$ T-lineage cells that were purified from the same mice. Increasing numbers of DCs were added to 96-well plates with 10^5 $CD4^+$ cells to each well. After 72 hours of coculture, tritiated thymidine was added and uptake into proliferating T cells was measured 16 hours later.

Reverse transcriptase–polymerase chain reaction

RNA was purified with an RNeasy Micro Kit (Qiagen). Cells were isolated by flow cytometry and directly resorted into an Eppendorf tube containing 350 uL of RLT buffer provided by the manufacturer (Qiagen). Samples were DNase-treated to remove any trace DNA. RNA was used directly in Taqman reverse transcriptase–polymerase chain reaction (RT-PCR) or was reverse-transcribed and subsequently amplified with a Rotor-Gene 6000 (Corbett). Cytokine expression patterns were produced from splenic control or C/EBPα-deleted DCs. The level of cytokine transcripts was assessed in steady-state DCs or after activation by lipopolysaccharide (LPS) (0.5 μg/mL). Total RNA was extracted from $CD11c^+$ fluorescence-activated cell sorting (FACS)-sorted DCs or those sorted DC incubated with LPS for 10 hours and analyzed for cytokines IL1α, IL1β, IL6, IL12, IL15, IFNγ, TNFα, and TGFβ1.

Cell lines

The stem cell factor (SCF)-dependent EML cell line was maintained in Iscove's modified Dulbecco medium supplemented with 20% horse serum, and 8% conditioned medium from BHK/MKL cells containing mSCF. EML cells cultured with mSCF (100 ng/mL) were infected with C/EBPα-ER retrovirus and selected with puromycin. Induction of nuclear localization of C/EBPα-ER fusion protein was achieved by the addition of 50 nM 4-hydroxytamoxifen (Sigma-Aldrich, St. Louis, MO) into the culture medium.

Normalization and statistical analysis of gene expression data

Data from wild-type multipotent progenitors (MPPs) and C/EBPαfl/fl MPPs were obtained from the gene expression repository at the Harvard Stem Cell Institutes (bloodprogram.hsci.harvard.edu) or from the Gene Expression Omnibus archive (GSE22432) for CDP. Original .CEL files were obtained and were processed with dChip.[32] After normalization using the smoothing spline algorithm, expression values were calculated by applying the perfect match–mismatch difference model algorithm. To identify C/EBPα-related changes, we looked for genes that are commonly expressed between wild-type MPPs and wild-type CDPs, as well as C/EBPα fl/fl MPPs and wild-type MPPs, and at the same time are differentially expressed between C/EBPα fl/fl MPPs and wild-type CDPs.

ChIP-Seq and transcription factor binding site analysis

ChIP was performed as described previously.[33] The transcription factors binding site analysis was perform on the C/EPBα peaks using Centdist[34] with both Jaspar and Transfac vertebrate databases.[35] Identification of potential binding sites was obtained using a custom perl script based on the TFBS library. C/EBPα peaks were scanned by the Jaspar's C/EBPα (MA0102.2) using a similarity threshold of 80%. If several potential binding sites were found on the same peak with the same matrix, only the ones with the best scores were reported and plotted in the heatmap using the "levelplot" function from the "lattice" R/Bioconductor library (http://bioconductor.org).

Results

C/EBPα is expressed and required for DC differentiation

To assess the potential role of C/EBPα in DC development, we measured C/EBPα transcripts by quantitative RT-PCR in mature cDC ($CD19/CD3/NK^-$, $CD11c^+$, $MHCII^+$) and pDC ($CD19/CD3/NK^-$, $CD11c^{lo/+}$, $PDCA\text{-}1^+$) that were sorted from the spleens of C57BL/6 mice (Figure 1A). Comparisons were made to pre-DC (Lin^-, $B220^{-/lo}$, $CD11c^{lo/+}$, $MHCII^-$, $CD11b/Mac1^-$), B cells ($CD19^+$, $B220^+$), and monocytes ($CD19/CD3/NK^-$, $Gr1^+$, $Mac1^+$) from the same tissue, as well as the stem/progenitor-rich KSL ($cKit^{Hi}$, $Sca1^+$, Lin^-,) fraction and myeloid-related DC progenitors from bone marrow. The latter included CMP (Lin^-, $Sca1^-$, $cKit^{Hi}$, $CD34^+$, $Fc\gamma RII/III^{-/lo}$), GMP (Lin^-, $Sca1^-$, $cKit^{Hi}$, $CD34^+$, $Fc\gamma RII/III^{hi}$), and CDP (Lin^-, $cKit^{lo}$, $Flt3^+$, $MCSFR/CD115^+$, $CD11c^-$) (supplemental Figure 1A). All myeloid-related fractions expressed this gene, whereas C/EBPα transcript levels were undetectable in B cells and low in pDC. Furthermore, C/EBPα protein (p42) could be detected by Western blot and immunostaining in cDC and

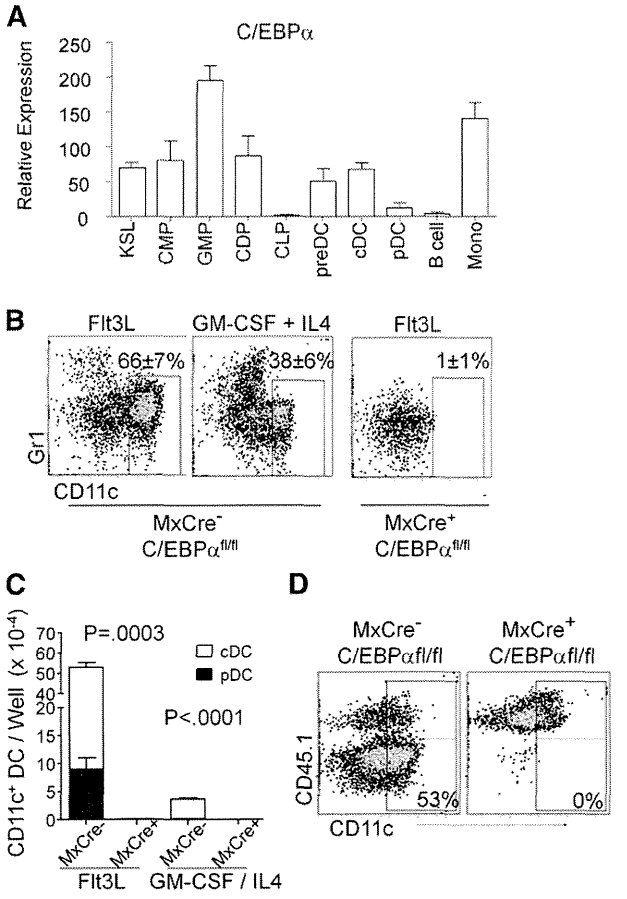


Figure 1. Expression of C/EBPα in DC and DC progenitors. (A) Relative quantitative RT-PCR of RNA from sorted KSL (cKit^{Hi}, Sca1$^+$, Lin$^-$), CMP (Lin$^-$, Sca1$^-$, cKit^{Hi}, CD34$^+$, FcγRII/III$^{-/lo}$), GMP (Lin$^-$, Sca1$^-$, cKit^{Hi}, CD34$^+$, FcγRII/III^{hi}), CDP (Lin$^-$, cKit^{lo}, Flt3$^+$, CD115$^+$, CD11c$^-$), preDC (Lin$^-$, B220$^{-/lo}$, CD11c$^+$, MHCII$^-$, CD11b/Mac1$^-$), cDC (CD19$^-$, CD3$^-$, NK$^-$, B220$^-$, CD11c$^+$, MHCII$^+$), pDC (CD19$^-$, CD3$^-$, NK$^-$, B220$^+$, CD11c$^{lo/+}$, PDCA-1$^+$), B (CD19$^+$, B220$^+$ splenic B cells), and monocytes (CD19$^-$, CD11b/Mac1$^+$, Gr-1$^+$ peritoneal monocytes) to assess expression of C/EBPα. Values are presented relative to that of β2-microglobulin RNA. Data are averages of 4 independent experiments (error bars represent SD). (B) DC loss after deletion of C/EBPα. 10^6 lysed, WBM cells from poly(IC)-injected MxCre$^-$ C/EBPαfl/fl or MxCre+ C/EBPαfl/fl mice were cultured in Flt3L-supplemented media. After 8 days, viable cells were assessed for relative expression of CD11c and Gr1 using flow cytometry. These representative plots show the impact of C/EBPα deletion on the presence of DC (CD11c$^+$). The Gr-1+ myeloid cells can also be seen in these plots. GM-CSF and IL4 cultures with MxCre$^+$ C/EBPαfl/fl WBM cells resulted in no viable cells after 9 days of culture (FACS plot not shown). (C) Shown are the numbers of total live CD11c$^+$ DCs present in the FL or GM-CSF/IL4 cultures for pDC (black, B220$^+$) and cDC (white) from (B). The numbers were generated by hemocytometer counts combined with flow cytometry analysis. These numbers of DCs represent mean ± SD from these 4 experiments where MxCre$^-$ and MxCre$^+$ correspond to MxCre$^-$ C/EBPαfl/fl and MxCre$^+$ C/EBPαfl/fl cells, respectively. (D) Flow cytometric analysis showing CD45.2$^+$ Mx1-Cre$^+$ C/EBPαfl/fl or C/EBPαfl/fl. WBM cells were cultured together with equal numbers of CD45.1$^+$ BM cells and Flt3L (200 ng/mL) for 8 days. Similarly, cultured cells were stained for cell surface markers CD45.1, CD45.2, CD11c, and MHCII followed by flow cytometry and cell counts. DCs derived from WBM were identified as CD45.2$^+$ CD11c$^+$ Gr1$^-$. The data shown are representative dot plots from 4 experiments with similar results.

monocytes, but not in B cells from the spleen (supplemental Figure 1B-C). Thus, this transcription factor is expressed by monocytes, macrophages, myeloid-related progenitors, and DCs.

Granulocyte–macrophage differentiation has been previously found to be dependent on C/EBP, and the patterns of expression described before suggested the same might be true for dendritic cells. To test this possibility, 12-week-old Mx1-Cre$^+$ C/EBPαfl/fl mice and control (Mx1-Cre$^-$ C/EBPαfl/fl) mice were treated with poly(IC). Bone marrow was harvested from these animals 17 to 21 days later, and excision of the C/EBPα gene confirmed by PCR and loss of transcript by quantitative RT-PCR (supplemental Figure 2A). When placed in DC culture conditions containing Flt3L (Flt3 ligand), bone marrow cells from gene-targeted (Mx1-Cre$^+$ C/EBPαfl/fl) bone marrow produced no phenotypic Gr-1^{Hi} myeloid cells or CD11c$^+$ DCs (Figure 1B). An alternate means of DC differentiation is supported by the GM-CSF + IL4 cytokine combination,[36] but C/EBPα-deficient stem/progenitors were also defective under those conditions, with no viable cells recovered from cultures. In addition, cells from these cultures were stained by immunofluorescence for the expression of surface markers CD11c and Mac1/CD11b or CD8 (supplemental Figure 1D-E). Because these cultures were initiated with unfractionated marrow, it was conceivable that C/EBPα is required by accessory cells rather than DC progenitors for cDC and pDC production (Figure 1C). However, accompanying CD45.1$^+$ whole bone marrow (WBM) cells generated DCs in 50:50 mixed cultures set up with either control CD45.1 or CD45.2 C/EBPα-deleted marrow (Figure 1D). Thus, marrow progenitors have a cell autonomous C/EBPα requirement rather than a lack of paracrine signaling for formation of DCs in culture.

DC differentiation in vivo is compromised in the absence of C/EBPα

Because cultures do not always replicate in vivo conditions, and multiple routes of DC differentiation have been described,[1,21,36] we assessed the importance of C/EBPα in a transplantation model in vivo. The stem/progenitor-rich KSL fraction was sorted from poly (IC) treated Mx1-Cre$^+$ C/EBPαfl/fl mice or Mx1-Cre$^-$ C/EBPαfl/fl control mice (CD45.2$^+$) and transferred to sublethally irradiated CD45.1$^+$ recipients. The altered numbers of stem/progenitor cells found in C/EBPα floxed mice compared with controls has been published previously.[28,37] From these mice transplanted with KSL, tissues were harvested 1 month later and the differentiation potential of transplanted cells was assessed. Control Mx1-Cre$^-$ C/EBPαfl/fl KSL produced lymphocytes, monocytes, granulocytes, and DCs (Figure 2, data not shown). Lymphoid lineages were produced normally from Mx1-Cre$^+$ C/EBPαfl/fl–deleted cells, but no phenotypic monocytes, few cDC (0.02%), and only small numbers of pDC (0.09%) were made (Figure 2B). Macrophage/DC progenitors are characterized by the CX3CR1-GFP reporter; we did not cross these mice to the C/EBPαfl/fl mice but found that expression was reduced in sorted CMP by RT-PCR (Figure 2B). In addition, CDP formation was C/EBPα dependent (Figure 2A-B and supplemental Figure 2B-C). These findings extend those made with our cell culture methods and demonstrate a major requirement for C/EBPα in production of DCs and their progenitors in vivo.

An early requirement for C/EBPα in DC differentiation

The results shown here suggest that C/EBPα plays a major role in generating DCs and their progenitors. However, we wanted to pinpoint the stages that require this transcription factor. The KSL fraction, as well as phenotypic CMPs and CLPs could be recovered from poly(IC)-treated Mx1-Cre$^+$C/EBPαfl/fl bone marrow, and these 3 subsets of progenitors were tested for Flt3L-mediated DC differentiation in culture. Cultures were again initiated with a mixture of 500 sorted progenitors and 10^4 WBM cells from normal control CD45.1$^+$ cells (Figure 3). Wells were analyzed for the presence of CD45.2$^+$ CD11c$^+$ DCs, and representative flow cytometry results from day 6 are shown in Figure 3A. Deletion of C/EBPα severely compromised the ability of KSL and CMP subsets of

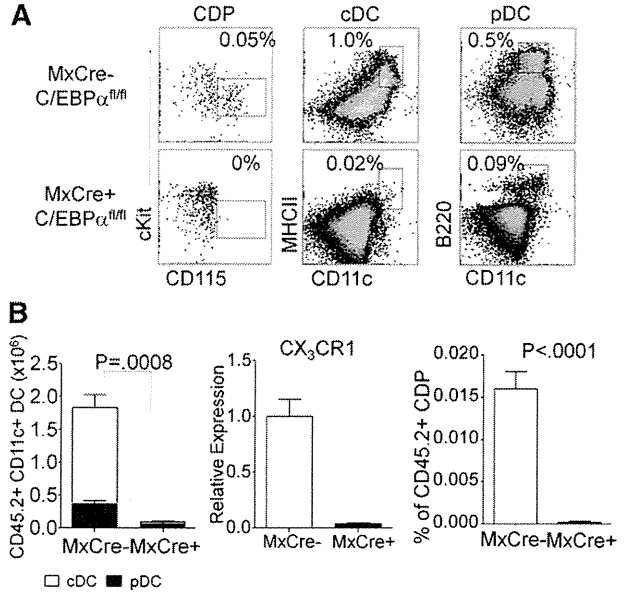


Figure 2. In vivo assay for DC generation from C/EBPα-targeted progenitors. (A) FACS analysis of CD45.2$^+$ donor–derived cells, from 5000 transplanted KSL of C/EBPαfl/fl or Mx1$^-$Cre$^+$ C/EBPαfl/fl–transplanted mice, for CDP (Lin$^-$, Flt3$^+$, FcγRII/III$^-$, cKit$^+$, CD115$^+$, CD11c$^-$) from bone marrow, or cDC (CD19$^-$, CD3$^-$, NK$^-$, CD11c$^+$, MHCII$^+$) and pDC (CD19$^-$, CD3$^-$, NK$^-$, CD11c$^+$, B220$^+$) from the spleen show contribution of donor cells to each population. The data shown are representative dot plots from 3 mice per group for 3 independent experiments with similar results. (B) Absolute numbers of total CD45.2$^+$ CD19$^-$, CD3$^-$, NK$^-$ CD11c$^+$ DCs were calculated from total splenocytes (left). B220$^+$ subset of cells was determined to be pDC (black), and B220$^-$ MHCII+ cells were cDC (white) and plotted accordingly. CMPs were sorted from either MxCre$^-$ C/EBPαfl/fl or MxCre+ C/EBPαfl/fl mice and analyzed for expression of CX_3CR1 by RT-PCR. The percentage of CDP from total CD45.2$^+$ bone marrow are presented from KSL of C/EBPαfl/fl or MxCre$^+$ C/EBPαfl/fl–transplanted mice (right). The numbers represent mean ± SD from 4 similarly transplanted mice. One of 3 experiments giving similar results is presented.

marrow progenitors to generate DCs (Figure 3A-B). Although CLPs are a less efficient source of DCs,[12,38] these progenitors maintained the majority of their ability to produce CD45.2$^+$ CD11c$^+$ DCs in the absence of C/EBPα (Figure 3B). A limiting dilution version of this experimental design revealed that ~1 of 8 Lin$^-$ cKit$^+$ cells from control mice gave rise to CD45.2$^+$ CD11c$^+$ cells compared with ~1 of 32 500 from Mx1-Cre$^+$C/EBPαfl/fl (Figure 3C). To more precisely define C/EBPα dependence of DC formation, Cre-GFP was introduced into sorted KSL, GMP, and CDP from C/EBPαfl/fl mice by retroviral transduction. Double-sorted progenitors were incubated for 24 hours with SCF, Flt3L, and IL6 to allow for Cre-mediated excision before culture with only Flt3L. Although DC production was severely impaired by deletion of C/EBPα in the more primitive progenitors, differentiation from the CDP stage appeared unchanged (Figure 3D). We conclude that C/EBPα is needed for progression to phenotypic CDP and subsequently mature DCs.

C/EBPα is not required for mature DCs in lymphoid tissues

Because some transcription factors have been shown to be required for the maintenance of mature DCs, as with the transcription factor E2-2 in pDC,[23] we wanted to know whether C/EBPα was similarly required for maintenance of DCs. Consequently, stage and cell type specificity were achieved with the CD11c-Cre (also known as Itgax-Cre) deleter mouse strain, in which recombination occurs in >90% of splenic pDC and cDC, but not on earlier precursors that are CD11c-negative. Both major DC populations, pDC and cDC, were present in the spleens of 10-week-old mice carrying both C/EBPαfl/fl and CD11c-Cre genes, and were indistinguishable from those in control C/EBPαfl/fl mice (Figure 4A). Further analyses, using known DC cell surface markers resolved subpopulations with no significant differences between control and DC-specific C/EBPα deletions (Figure 4B). PCR analysis of CD11c$^+$ cells sorted from the spleen confirmed C/EBPα gene excision in these cells (supplemental Figure 3A). Absolute numbers of total CD11c$^+$ DCs were measured in lymphoid-specific tissues, and no differences were found between CD11c-Cre$^+$ × C/EBPαfl/fl or control (C/EBPαfl/fl) mice at ~8 to 10 weeks of age (supplemental Figure 3B). The pattern of cytokine production from C/EBPα-deleted DCs was determined by measuring the mRNA expression in steady-state DCs or after stimulation with LPS. Total RNA was extracted from either CD11c$^+$ CD19$^-$ CD3$^-$-sorted

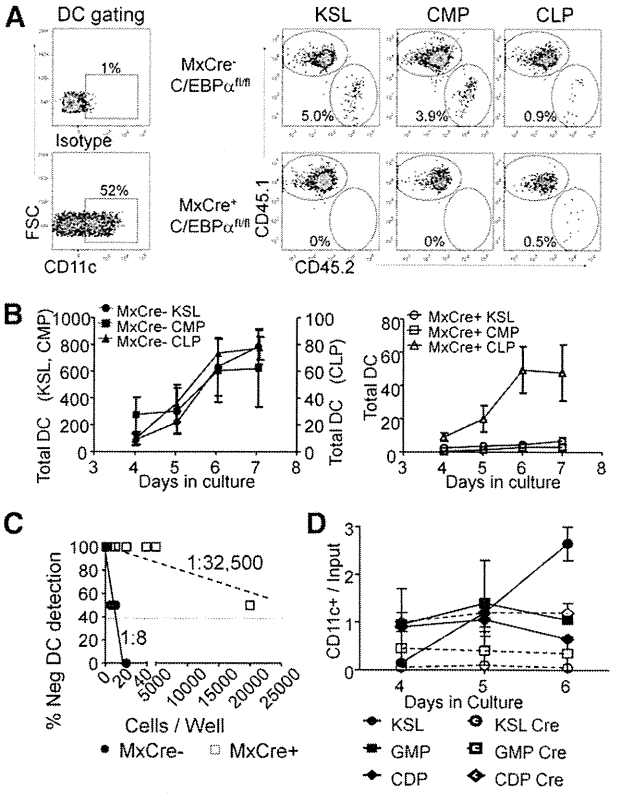


Figure 3. Stage-specific need for C/EBPα in DC progenitors. (A) KSL, CMPs, and CLPs were isolated from the BM of MxCre$^+$ C/EBPαfl/fl or C/EBPαfl/fl mice 2 weeks after 3 poly(IC) injections. 500 progenitor cells were cocultured with 10^4 Ly5.1$^+$ WBM cells in the presence of Flt3L (200 ng/mL). The resulting cultures were analyzed by flow cytometry for Ly5.1, Ly5.2, MHCII, and CD11c after days 3 to 7. In addition, CD45.1$^+$ cells were subjected to the same culture conditions that were used as controls for efficiency of DC differentiation. Day 5 FACS plots are shown as representative data from triplicate wells of 3 independent experiments. (B) The number of DCs generated in each culture was calculated using cell counts and flow cytometry over days and presented as the number of total DCs (average of 3 wells). On the left are sorted populations from C/EBPα fl/fl, with the DC numbers for KSL and CMPs shown on the left axis and CLP production on the right axis. The graphs on the right are from the same progenitors but sorted from MxCre$^+$ C/EBPαfl/fl mice. Data are presented as mean ± SD of one such experiment that was repeated 3 times. (C) Cloning efficiency was assessed by limiting dilution series of sorted CD45.2$^+$ MxCre$^+$ C/EBPαfl/fl or C/EBPαfl/fl Lin$^-$ cKit$^+$ progenitors cocultured with Ly5.1$^+$ Lin$^-$ cKit$^+$ BM cells in the presence of Flt3L(200 ng/mL). Cultures were analyzed on day 8 for the presence of CD45.2+ CD11c+ cells. Data are representative of 2 such experiments. (D) The progenitor populations KSL, GMP, and CDP were isolated from the BM of C/EBPαfl/fl mice. These progenitor cells were infected with retrovirus carrying an empty vector with GFP reporter or Cre with GFP reporter. These cells were then cultured with sorted CD45.1$^+$ Lin$^-$ cKit$^+$ BM cells in the presence of Flt3L. Cultures were assessed for CD45.2$^+$ CD11c$^+$ GFP$^+$ cells at days 4, 5, and 6. The plot shows a yield of CD11c$^+$ cells per input GFP$^+$ progenitor over time. Data are representative of 2 such experiments.

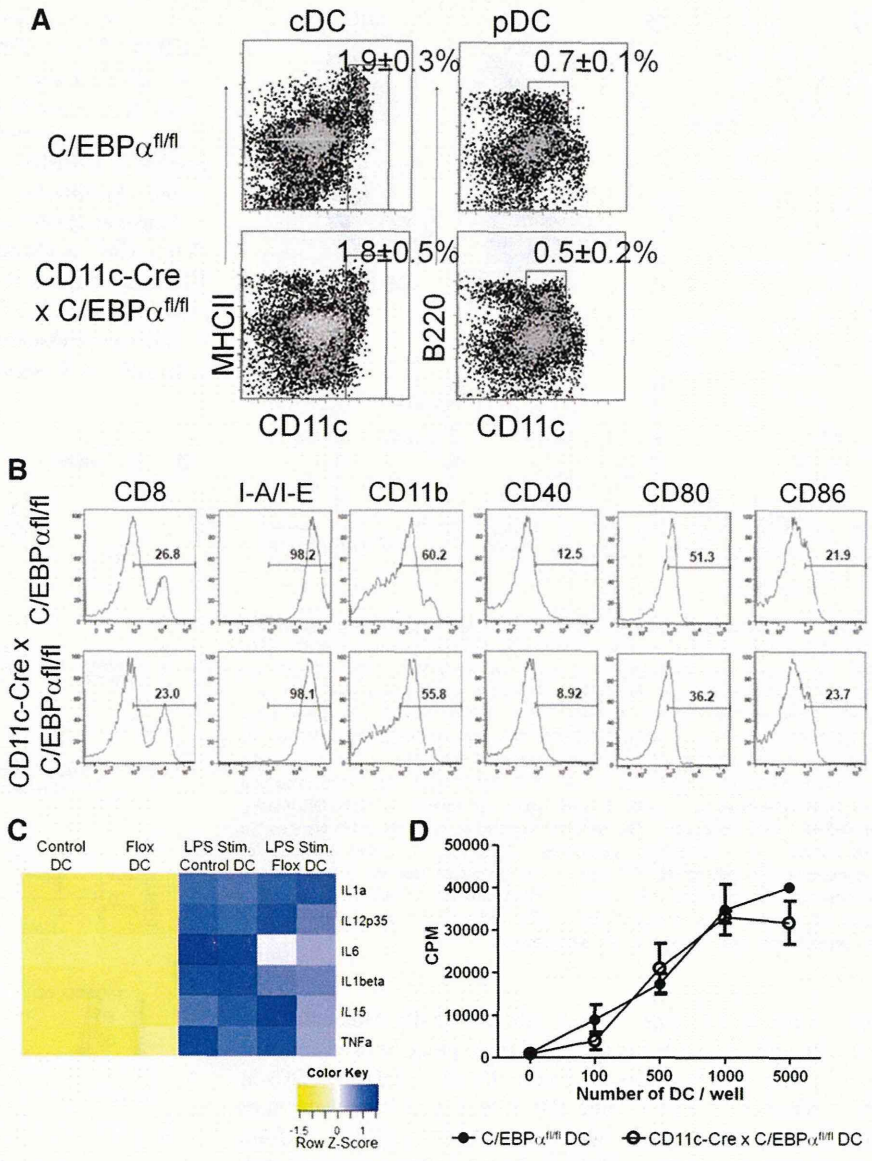


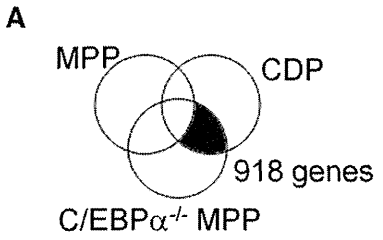
Figure 4. Targeted C/EBPα deletion has no effect on late-stage DC development. (A) CD11c-Cre$^+$ × C/EBPαfl/fl and C/EBPαfl/fl control mice were analyzed by flow cytometry between 8 and 12 weeks of age. Flow cytometry analysis of DC populations from the spleens of mice of the indicated genotypes are shown as dot plots. cDC are defined as CD19$^-$, CD3$^-$, NK$^-$, CD11c$^+$, and MHCII$^+$, whereas pDC were gated as CD19$^-$, CD3$^-$, NK$^-$, CD11c^{lo}, and B220$^+$. Boxes are used to indicate the position of the DC populations in each group with the average percentage per spleen. FACS plots were taken from 3 similar experiments. (B) Histograms of flow cytometric analysis of CD11c$^+$ splenic DC from CD11c-Cre$^+$ C/EBPαfl/fl or C/EBPαfl/fl mice for subpopulations of DCs for CD4, CD8, CD11b, CD40, CD80, CD86, IL3Rα, and M-CSFR. Percentages are representative of similar experiments. (C) CD11c-Cre$^+$ x C/EBPαfl/fl or C/EBPαfl/fl splenic DCs were sorted from 12-week-old mice. These cells were stimulated with LPS for 10 hours and then RT-PCR was performed for expression of cytokine transcripts. RT-PCR analysis displayed in a heatmap for transcripts from steady-state and LPS stimulated DC from both control and flox-deleted DCs. Data are shown from 2 similar experiments. (D) Control or C/EBPα-depleted DCs were sorted as CD11c$^+$ CD19$^-$, and CD3$^-$ into 72-hour cultures along with magnetic bead column–enriched CD3$^+$ splenic cells to induce the proliferation of autologous T-lineage cells. The proliferative response of T-lineage cells was measured by tritiated thymidine incorporation after 16 hours. Results are representative of 2 experiments, and each value represents the mean from triplicate wells.

splenic DCs or similar cells incubated with LPS (0.5 μg/mL) for 10 hours before RNA isolation. LPS significantly upregulated the expression of IL1α, IL1β, and TNFα transcripts, and, to a lesser extent, the amount of IFNγ and TGFβ1 mRNA (not shown) compared with their steady-state counterpart DCs (Figure 4C). No significant differences in cytokine profiles were observed between control and C/EBPα-deleted DCs with the exception of IL6. In addition, no differences were observed in C/EBPα-deleted splenic DCs in phagocytosis assays (supplemental Figure 3C) or in the ability to activate T-lineage cells to proliferate (Figure 4D). As an assessment of potential DC defects, we double-sorted CD19$^-$ CD3$^-$ NK$^-$ CD11c$^+$ cells from the spleens of C/EBPαfl/fl control or C/EBPα DC–deleted mice and evaluated them for the expression of DC-related transcripts using a commercially available RT-PCR kit (SABiosciences, RT2 Profiler PCR array, PAMM-406A). This confirmed that C/EBPα was deleted and significant expression differences were found in only 6 of 84 DC-related transcripts (supplemental Figure 3D and supplemental Table 1). These data suggest that under these conditions, C/EBPα is not needed for maintenance of mature DCs.

C/EBPα regulates gene expression needed for DC formation

To explore potential mechanisms for C/EBPα regulation of DC formation, we analyzed array data from C/EBPα$^{-/-}$ MPPs and wild-type common CDPs. Genes whose expression differed between these 2 populations were removed if they were also expressed in control MPPs because these were likely not C/EBPα-dependent (Figure 5A). This approach was used to discover C/EBPα-dependent genes that associate with the earliest steps in DC differentiation. These genes are promising candidates for regulation by C/EBPα during the transition from C/EBPα$^{-/-}$ MPPs to CDPs (supplemental Table 2), and a subset of those genes most differentially regulated are shown with the lower bound of fold change values given (Figure 5A).

The differentiation of DCs is controlled by the expression of growth factor receptors that together with transcription factors direct cell fates.[39,40] We wanted to test whether C/EBPα was responsible for the induction of well-characterized DC-related transcription factors, so we analyzed the expression patterns of these factors and the interaction of C/EBPα with their promoters, or up- and downstream regulatory elements. To do this, sorted CMPs were used to



Up-regulated		Down-regulated	
Gene	LB of FC	Gene	LB of FC
Elane	17.51	C1qb	19.27
Eif2s3y	14.23	Gata3	16.37
Dlg2	12.58	Igh-VJ558	13.76
Nrp1	11.59	Als2	13.64
Cybb	8.72	Slc16a12	13
Ddx3y	8.35	Epb4.1l5	12.73
Fam105a	7.9	Pcdh7	11.56
Gpc4	7.72	Tmem215	11.3
Tmem151a	7.17	Pot1a	11.1
Igh-6	6.23	Nynrin	9.3

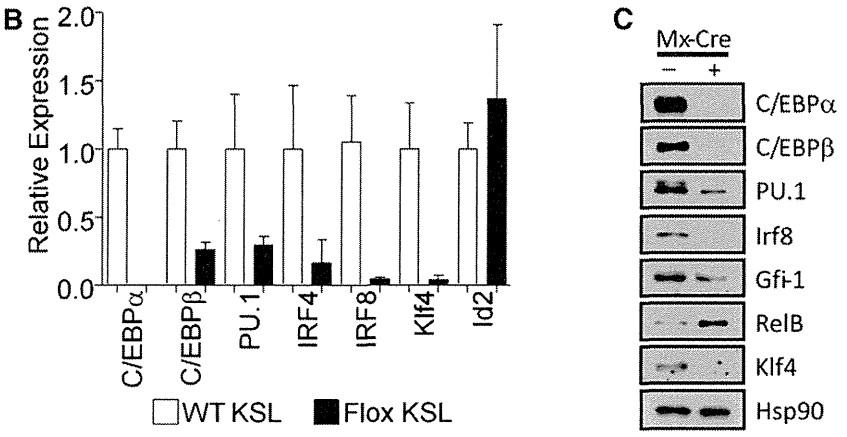


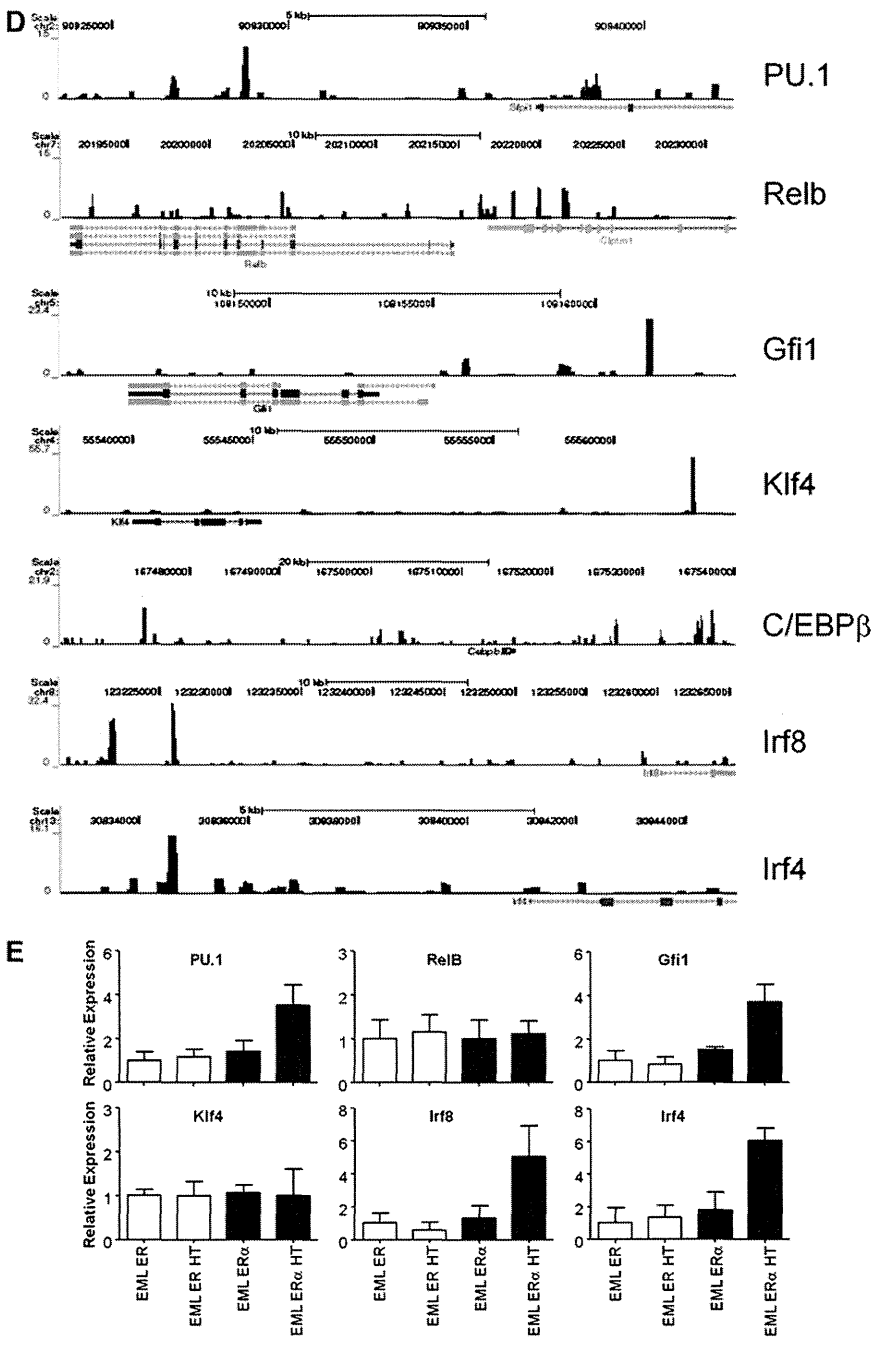
Figure 5. C/EBPα binds the promoter or transcriptional start site of many genes needed for transition from MPP to CDP. (A) Array data from C/EBPα$^{-/-}$ MPPs were compared with that of CDPs, whereas differences between normal MPPs and CDPs were subtracted from this analysis, uncovering genes that are located in the shaded section of the Venn diagram. The most differentially up- and downregulated genes while transitioning to CDP are presented in the right two panels. (B) Expression of C/EBPα, C/EBPβ, PU.1, IRF4, IRF8, Klf4, Id2, and RelB by relative quantitative RT-PCR of RNA from sorted CMPs (cKit^{Hi}, Sca1^{+}, Lin^{-} CD34^{+} FcγRII/III$^{lo/-}$); white bars represent controls and black bars represent floxed progenitors. Values normalized to the control, nonexcised flox CMPs, except with RelB where samples were normalized against the floxed deleted sample. Data are averages of 3 independent experiments (error bars represent SEM). (C) Lin^{-} cKit^{+} progenitors were sorted from the bone marrow of control or C/EBPα floxed mice. Cells were double-sorted from each group and used for Western blot analysis to determine transcription factor expression compared with HSP-90 (bottom panel). (D) C/EBPα ChIP-seq of CMPs was analyzed for specific genes important during DC differentiation. Plots show ChIP-fragment density at each position in the regions of the genes PU.1, Relb, Gfi1, Klf4, C/EBPβ, Irf8, and Irf4. ChIP-seq profiles are given for genes that were considered altered from (B) and (C). (E) Relative quantitative RT-PCR of transcripts, isolated from EML-ER and EML-C/EBPα-ER, were measured from cell lines cultured for 24 hours with 4-HT to assess expression of PU.1, RelB, Gfi1, Klf4, Irf8, and Irf4. The y-axis indicates the relative expression of the transcription factor relative to that of control treated EML. Data are averages of 2 independent experiments (error bars represent SD).

generate cDNA to determine the expression of DC transcription factors with the loss of C/EBPα (Figure 5B). DC-related transcription factors C/EBPβ, PU.1, IRF4, IRF8, and Klf4 were all decreased with the loss of C/EBPα in CMPs. However, Relb was increased and Id2 was unchanged in C/EBPα-deleted CMPs relative to control cells (not shown); similar analysis for KSL was also conducted (supplemental Figure 4A). In addition, the expression of many of these factors were confirmed through Western blot (Figure 5C), suggesting that C/EBPα regulation of the RNA of many of these transcription factors is reflected in their protein levels. Because PU.1 has been previously shown to be required for DC differentiation,[13] our data suggest that C/EBPα could play an important collaborative role with PU.1 during the earliest stages. As evidence for this codependence, overexpression of only PU.1 was not sufficient to rescue DC differentiation from KSL in the absence of C/EBPα, but it did rescue DC differentiation from PU.1 conditionally-deleted KSL (supplemental Figure 4B). Furthermore, to determine whether these changes might be directly regulated by C/EBPα, the interaction of C/EBPα with the regulatory elements of these factors was confirmed by ChIP-seq from sorted progenitors. This analysis was performed on sorted CMP and ChIP-seq profiles for C/EBPα after IgG subtraction was done in the regions of interest. Significant signals were detected in regulatory regions close to these essential transcriptional regulators (Figure 5D). Interestingly, the newly identified Zbtb46, zinc transcription factor specific for cDC, also showed a significant signal upstream of this factor's start site.[41,42] Finally, to determine whether induction of C/EBPα can mediate expression of these DC-related factors, we induced C/EBPα in an EML-inducible system. Estrogen receptor (ER) control clones that contain the ER peptide without C/EBPα, and C/EBPα-ER–expressing clones were cultured in the presence of SCF with or without 4-hydroxytamoxifen (HT). Cells were treated for 24 hours with 4- HT and transcripts were isolated for expression of direct C/EBPα targets. Quantitative RT-PCR showed enrichment of the PU.1, Gfi1, Irf4, and Irf8 upon 4-HT treatment in the C/EBPα-ER–expressing cells, but not in the absence of 4-HT or the ER control cells (Figure 5E). With that data taken together, we have identified a significant number of genes that are associated with DC differentiation and are dependent on C/EBPα. ChIP-seq data suggest that a number of these are likely to be directly regulated by C/EBPα, including those previously defined transcription factors essential for DC differentiation.

Discussion

Limited information was available about expression and function of C/EBPα expressed in several DC subsets, although both are known

Figure 5. (Continued).



to be particularly important for early events in hematopoiesis and myeloid differentiation.[19] Furthermore, C/EBPα is frequently compromised in hematopoietic diseases and its loss could have consequences for immune system development or function.[26] Indeed, we now show that C/EBPα is required for discrete steps in DC production, specifically formation from myeloid progenitors through common DC progenitors. Other genes that could contribute to this process were identified by microarray analyses relative to C/EBPα, and ChIP-seq data showed how multiple factors may cooperate to replenish the innate immune system.

Until now, it was thought that C/EBPα functions as a negative regulator of DC formation.[43] This conclusion resulted from artificial overexpression of C/EBPα in primary human cells or cell lines. In addition to the fact that unphysiological levels of C/EBPα were achieved, those approaches would not have been informative about particular steps in the process for which this transcription factor is required. A large body of evidence suggests that C/EBPs regulate lineage choice decisions in a highly stage- and dosage-dependent manner.[3,28] Thus, it was essential to explore these questions with lineage- and stage-specific deletion models.

Although macrophage colony-stimulating factor (M-CSF) is not required for DC formation, expression of the M-CSF receptor (M-CSFR) is used to distinguish both MDPs and CDPs.[5-7] There is evidence that C/EBPα induces expression of CD115, so it might be expected to find no phenotypic CDPs in C/EBPα knockouts.[44] Although that was indeed the case, it is important to stress that downstream stages of DC differentiation were also compromised. Interestingly, recent studies of progenitors and terminally differentiated

cells have shown that DCs are more transcriptionally linked to CMPs than GMPs.[45] Because CMPs are more likely to be precursors for DC progenitors,[46] we found that cells with CMP characteristics were present with C/EBPα deletion. However, they too were unable to generate mature DCs in the absence of C/EBPα.

DC formation is complex, and DCs with similar properties can be made from both myeloid and lymphoid progenitors. The consensus from studying murine models is that the major pathway is through DC progenitors, which arise from the myeloid progenitors and are independent from monocytes.[1,38] This process is likely to be altered under normal, disease, and experimental circumstances, making it difficult to determine the relative importance of optional differentiation pathways. We now show that this major route through myeloid progenitors is C/EBPα-dependent. Consistent with the decreased expression of C/EBPα in CLPs, these lymphoid progenitors retained DC potential even in the absence of this transcription factor.

Our unique strategy for identification of genes required for DC formation was highly effective, and many of those upregulated from C/EBPα-deficient MPP to CDP stages were directly bound by C/EBPα in ChIP-seq analysis. Several other transcription factors, including Irf4, Irf8, RelB, Gfi1, Id2, and E2-2, are required for specification and/or differentiation of selected DC subsets, whereas C/EBPα and PU.1 appear to have unique importance.[21] Our data suggest that C/EBPα regulates the expression of many of these DC-specific transcription factors through direct binding to the regulatory elements of these genes in progenitors. That some of these factors cooperate rather than complement each other was shown by our finding that PU.1 overexpression did not restore DC potential in C/EBPα-deficient progenitors. As another distinction, PU.1, but not C/EBPα, is required for later stages of DC maturation.

Our new findings show that C/EBPα supports DC formation under normal steady-state conditions. However, previous studies demonstrated that related family members have compensatory functions that are revealed during disease and inflammatory circumstances.[27,47] Therefore, alternative mechanisms of DC differentiation may be used in inflammatory environments, such as monocyte activation to DC,[11] non–lymphoid tissue DC commitment,[48,49] or when C/EBPα is mutated, silenced, or methylated, as in leukemia.[26]

Overall, our study emphasizes the necessity of C/EBPα to mediate the early events of steady-state DC differentiation. C/EBPα can bind and regulate the expression of many transcription factors previously shown to be necessary for DC differentiation. As one example, PU.1 likely cooperates with C/EBPα during this progression of commitment. These data demonstrate that C/EBPα is needed within a transcriptional network for the earliest differentiation events in normal steady-state DC development.

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Authorship

Contribution: All authors designed research; R.S.W., D.B., G.A., A.C., C.B., K.D.S.A.W., M.Y., H.Z., T.I., and C.J.H. performed research; R.S.W., D.B., G.A., A.C., T.B., C.B., T.I., K.A., and D.G.T. analyzed data; and R.S.W., D.B., G.A., K.A., and D.G.T. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Inflamatory Bowel Diseases

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The Cytotoxic Effects of Certolizumab Pegol and Golimumab Mediated by Transmembrane Tumor Necrosis Factor

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The Cytotoxic Effects of Certolizumab Pegol and Golimumab Mediated by Transmembrane Tumor Necrosis Factor α

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Background: Anti–tumor necrosis factor α (anti-TNF-α) agents have been successfully applied for the treatment of rheumatoid arthritis, Crohn's disease, and other chronic inflammatory diseases. Not only the neutralization of soluble TNF-α but also the effect on transmembrane TNF-α is important mechanisms of action of anti-TNF-α agents. This study investigated the cytotoxic effects of new anti-TNF-α agents, certolizumab pegol and golimumab, which are mediated by transmembrane TNF-α.

Methods: Transmembrane TNF-α–expressing Jurkat T cells that did not express TNF receptors were used. The binding ability of each anti-TNF-α agent to transmembrane TNF-α, antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity, and the apoptotic effect were examined.

Results: Certolizumab pegol and golimumab bound to transmembrane TNF-α. Golimumab induced antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity, which was comparable to infliximab and adalimumab. However, certolizumab pegol did not induce antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity. Certolizumab pegol directly induced nonapoptotic cell death in transmembrane TNF-α–expressing cells. Golimumab induced a weaker apoptotic effect than infliximab and adalimumab.

Conclusions: The cytotoxic effects of anti-TNF-α agents on TNF-α–expressing cells are considered to be associated with the clinical effect of these agents on granulomatous diseases. The direct cytotoxic effect of certolizumab pegol on TNF-α–producing cells may contribute to its clinical efficacy in Crohn's disease. Golimumab may be less effective for granulomatous diseases.

(Inflamm Bowel Dis 2013;19:1224–1231)

Key Words: apoptosis, cytokines, biologic therapies

Anti–tumor necrosis factor α (anti-TNF-α) agents have been successfully applied for the treatment of rheumatoid arthritis, Crohn's disease (CD), and other chronic inflammatory diseases.[1,2] Infliximab, etanercept, and adalimumab are clinically approved worldwide for the treatment of these diseases, and ample data on their clinical profiles are available. These 3 agents are equally effective for rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis, but etanercept is not effective for CD, granulomatosis with polyangitis, and sarcoidosis.[3] These differences in clinical efficacy cannot be explained solely by the neutralization of soluble TNF-α, which is the commonly shared action of these 3 agents. TNF-α is generated in a precursor form, transmembrane TNF-α.[4–6] TNF-α–converting enzyme (TACE) processes and releases soluble TNF-α.[7–10] Accumulating evidence suggests that not only soluble TNF-α but also transmembrane TNF-α is involved in inflammatory responses. The differential effects of anti-TNF-α agents on transmembrane TNF-α may explain the difference in clinical efficacies.[11]

We developed a unique and simple system of transmembrane TNF-α–expressing Jurkat T cells that stably expressed an uncleavable form of transmembrane TNF-α on their cell surfaces and were negative for TNF receptors to investigate the specific effects of anti-TNF-α agents on transmembrane TNF-α. We previously reported that infliximab, etanercept, and adalimumab exerted different effects on transmembrane TNF-α–expressing cells using this system. All 3 agents bound to transmembrane TNF-α and mediated antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Infliximab and adalimumab, but not etanercept, induce apoptosis and cell cycle arrest.[12] Infliximab is a chimeric protein that is composed of a murine variable region and a human IgG1 constant region. Adalimumab is a fully human monoclonal antibody. Etanercept is composed of the extracellular portion of the 2 human type 2 TNF receptors linked to the Fc portion of human IgG1.[13] As full-length bivalent antibodies, infliximab and adalimumab, can bind 2 TNF-α simultaneously. The multimer

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formation of transmembrane TNF-α may be essential for apoptosis induction. The cross-linking of etanercept that is bound to cell surface transmembrane TNF-α by anti-human IgG antibodies induces apoptosis.[14]

New anti-TNF-α agents, certolizumab pegol and golimumab, have been recently approved for clinical use. Certolizumab pegol is a monovalent Fab′ antibody fragment that is covalently linked to polyethylene glycol (PEG). Certolizumab pegol is presumed to be unable to induce ADCC, CDC, or apoptosis because it lacks an Fc region, and it does not cross-link transmembrane TNF-α as a monovalent Fab′ fragment. So it is intriguing that certolizumab pegol is effective for CD.[15] Golimumab is a fully human IgG1 monoclonal antibody that is specific for TNF-α.

This study examined the effects of certolizumab pegol and golimumab on transmembrane TNF-α–expressing cells. We demonstrated that certolizumab pegol directly induced cell death in transmembrane TNF-α–expressing cells through a different mechanism than apoptosis. In addition, golimumab exerted ADCC and CDC activities that were equivalent to infliximab and adalimumab. However, golimumab demonstrated a less potent apoptotic effect.

MATERIALS AND METHODS

Cell Line and Reagents

Jurkat cells, which are a human lymphoblastoid T-cell line, were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 50 IU/mL penicillin, and 20 mg/mL streptomycin (culture medium) at 37°C in a 5% CO_2-humidified atmosphere. Rituximab (Rituxan), infliximab (Remicade), etanercept (Enbrel), and adalimumab (Humira) were purchased from Chugai Pharmaceutical (Tokyo, Japan), Mitsubishi Tanabe Pharma (Osaka, Japan), and Pfizer Japan (Tokyo, Japan), respectively. Certolizumab pegol and golimumab were a gift from UCB Pharma SA (Brussels, Belgium) and Janssen Biotech (Horsham, PA), respectively. Fluorescein-conjugated goat affinity-purified $F(ab')_2$ fragment to human IgG Fc was purchased from Cappel Laboratories (Durham, NC). Biotinylated anti-PEG antibody was obtained from Epitomics (Burlingame, CA). Avidin–fluorescein was purchased from R&D Systems (Minneapolis, MN).

Generation of Mutant Transmembrane TNF-α

Mutant transmembrane TNF-α, which is resistant to TACE-mediated cleavage, was generated using site-directed mutagenesis as described previously.[16] A mutant transmembrane TNF-α in which 3 cytoplasmic serine residues were replaced by alanine was also generated using site-directed mutagenesis.[14]

Assay for the Binding of Anti-TNF-α Agents to Transmembrane TNF-α

Mock-transfected and transmembrane TNF-α–transfected Jurkat cells were incubated with infliximab, etanercept, adalimumab, certolizumab pegol, golimumab, or the control antibody rituximab (all at 1 μg/mL) for 30 minutes at 4°C in phosphate-buffered saline containing 2% fetal bovine serum (fluorescence-activated cell sorting [FACS] buffer). The cells were washed 3 times with an FACS buffer. The cells that were incubated with infliximab, etanercept, adalimumab, and golimumab were stained with a fluorescein-conjugated goat $F(ab')_2$ fragment to human IgG Fc as a secondary antibody for 30 minutes at 4°C. Cells that were incubated with certolizumab pegol were incubated with an anti-PEG-biotin antibody for 30 minutes at 4°C and stained with avidin–fluorescein for 45 minutes at 4°C. Fluorescence intensities were measured using an FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Antibody-dependent Cell-mediated Cytotoxicity

The capacity of anti-TNF-α agents to induce effector cell–dependent lysis of transmembrane TNF-α–expressing cells was evaluated using flow cytometry as described previously.[17] Briefly, transmembrane TNF-α–expressing Jurkat T cells were labeled with membrane dye, PKH26 (Sigma-Aldrich, St. Louis, MO), to permit discrimination during incubation with effector cells. Labeled target cells were preincubated with anti-TNF-α agents or rituximab. Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood from healthy individuals using centrifugation on Lymphocyte Separation Medium gradients (ICN Biomedicals, Aurora, OH). Thereafter, peripheral blood mononuclear cells (effector cells) with effector cell:target cell (E:T) ratios from 8:1 to 1:8 were added to target cells and incubated at 37°C for 3 hours. The final concentration of anti-TNF-α agents and rituximab was 1 μg/mL. TO-PRO 3 iodide (Invitrogen, Carlsbad, CA) was added just before analysis on an FACSCalibur flow cytometer to detect cellular death.

Complement-dependent Cytotoxicity

Uncleavable transmembrane TNF-α–expressing Jurkat cells were used as targets. Serial 10-fold dilutions (final concentrations, 0.01–10 μg/mL) of anti-TNF-α agents and rituximab were prepared and added to each well of Jurkat cells. Target cells were incubated in RPMI 1640 with 10% fresh or heat-inactivated human serum in 24-well culture plates for 4 hours at 37°C in a 5% CO_2 incubator. The treated cells were stained with 10 μg/mL propidium iodide (PI) (Wako, Osaka, Japan). Cell lysis was analyzed using flow cytometry.

Cell Viability Assay

Mock-transfected and uncleavable transmembrane TNF-α–transfected Jurkat cells (5×10^4 cells in 100 μL per well) were placed in 96-well plates and stimulated for 24, 48, or 72 hours with 0.1, 1, or 10 μg/mL of anti-TNF-α agents or rituximab in culture medium containing 10% heat-inactivated serum. The WST-8 reagent in the Cell Counting Kit-8 (Dojin Laboratories, Kumamoto, Japan) was added to the cells (10 μL per well) followed by a 3- to 4-hour incubation. The number of viable cells was determined using differences in absorbance measurements (450 and 620 nm) on an enzyme-linked immunosorbent assay autoreader. The fraction of viable cells represented the ratio of WST-8 values from drug-treated cells to untreated cells.

Apoptosis Assay

Uncleavable transmembrane TNF-α–transfected Jurkat cells were stimulated for 24 hours with 0.01, 0.1, 1, or 10 μg/mL of anti-TNF-α agents or rituximab in culture medium containing 10% heat-inactivated serum. Apoptosis and cell death were analyzed using a MEBCYTO Apoptosis Kit (MBL, Nagoya, Japan) as described previously.[14]

Measurement of Caspase-3 Activity

Uncleavable transmembrane TNF-α–transfected Jurkat cells were treated with 10 μg/mL of anti-TNF-α agents or rituximab for 48 hours in culture medium containing 10% heat-inactivated serum. The levels of active caspase-3 were determined by enzyme-linked immunosorbent assay (Quantikine Human Active Caspase-3; R&D Systems).

Apoptosis DNA Ladder Assay

Uncleavable transmembrane TNF-α–transfected Jurkat cells were treated with anti-TNF-α agents or rituximab for 48 hours in culture medium containing 10% heat-inactivated serum. DNA was extracted from treated cells using the Enhanced Apoptotic DNA Ladder Detection Kit (Bio Vision, Irvine, CA) according to the manufacturer's protocol. Extracted DNA (2 μg) was separated using 1.2% agarose gel electrophoresis, and the DNA fragments were stained with ethidium bromide.

Statistical Analysis

The results are expressed as the means ± SD. Statistical evaluation was performed using Tukey's multiple comparison test.

RESULTS

Certolizumab Pegol and Golimumab Bound Transmembrane TNF-α

The binding of certolizumab pegol, golimumab, and other anti-TNF-α agents to transmembrane TNF-α was assessed. Golimumab bound transmembrane TNF-α similar to infliximab, etanercept, and adalimumab (Fig. 1A). Certolizumab pegol also bound transmembrane TNF-α (Fig. 1B).

Golimumab, But Not Certolizumab Pegol, Mediated ADCC and CDC

Peripheral blood mononuclear cells (effector cells) were incubated with transmembrane TNF-α–expressing Jurkat cells at an increasing E:T ratio in the presence of 1 μg/mL each of anti-TNF-α agents to evaluate ADCC (Fig. 2A). Golimumab-induced significant and E:T ratio-dependent target cell lysis at E:T ratios from 2:1 to 8:1 to a similar degree as infliximab and adalimumab. In contrast, certolizumab pegol did not induce ADCC. Etanercept-mediated ADCC was less than infliximab, adalimumab, and golimumab, but no significant differences between these agents were observed. The anti-TNF-α agents were incubated with transmembrane TNF-α–expressing Jurkat cells in the presence of fresh normal human serum or heat-inactivated

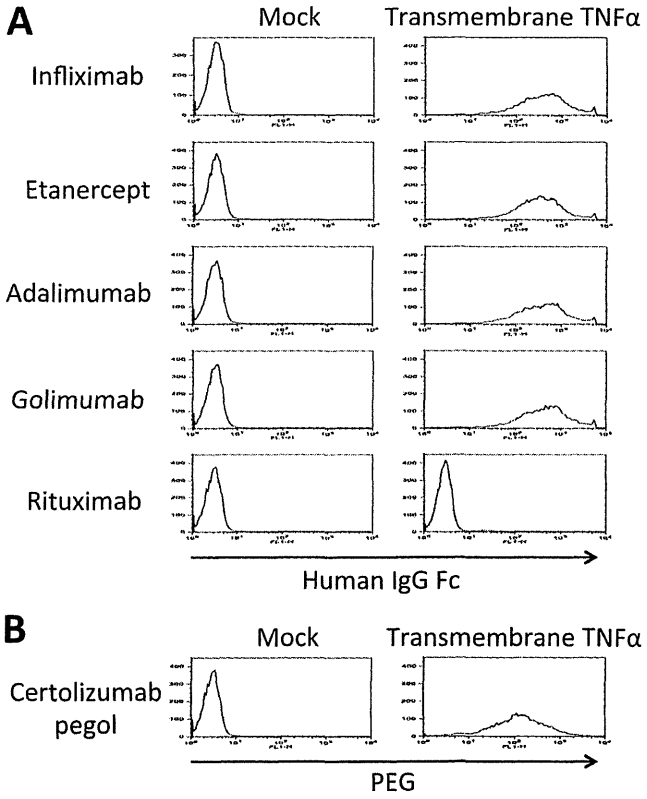

FIGURE 1. Binding activity of certolizumab pegol and golimumab to transmembrane TNF-α on the cell surface. Mock-transfected and transmembrane TNF-α–transfected Jurkat cells were incubated with the anti-TNF-α agents. (A) Cells incubated with infliximab, etanercept, adalimumab, golimumab, or rituximab were then stained with a fluorescein-conjugated $F(ab')_2$ fragment to human IgG Fc. (B) Cells incubated with certolizumab pegol were incubated with a biotinylated anti-PEG antibody and stained with avidin–fluorescein. The histograms show the binding of anti-TNF-α agents and rituximab to mock-transfected and transmembrane TNF-α–transfected Jurkat cells. FL-1, fluorescence channel 1.

human serum for 4 hours to evaluate CDC (Fig. 2B). Golimumab exerted significant cytotoxic activity at concentrations of 0.1 μg/mL or more, which was similar to infliximab and adalimumab. However, certolizumab pegol did not induce CDC. Etanercept tended to induce CDC at 1 and 10 μg/mL, but this induction was not statistically significant. Our previous study demonstrated that etanercept produced significant CDC at 10 and 100 μg/ml.[12]

Certolizumab Pegol and Golimumab Directly Induced Reductions in the Viability of Transmembrane TNF-α–Expressing Cells, Which Was Less Potent Than Infliximab and Adalimumab

The direct effects of certolizumab pegol and golimumab, which were independent of effector cells and complements, were examined. Certolizumab pegol was not expected to induce apoptosis and cell cycle arrest because of its inability to cross-link

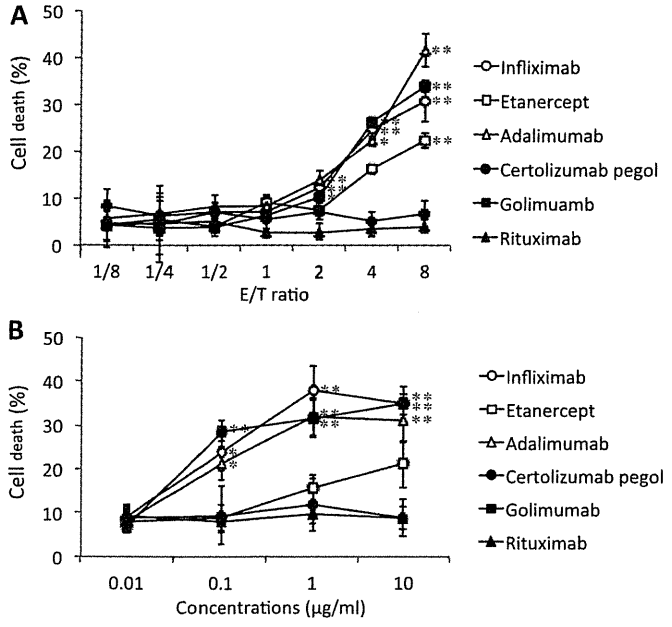

FIGURE 2. ADCC and CDC of certolizumab pegol and golimumab. (A) ADCC of anti-TNF-α agents. The proportions of dead cells in target cells are indicated. Values are the means ± SD of triplicate cultures. (B) CDC of anti-TNF-α agents. Values are the percentages of dead cells incubated in 10% fresh serum minus the percentages of dead cells incubated with 10% heat-inactivated serum. Values are the means ± SD of triplicate cultures. * = $P < 0.05$; ** = $P < 0.005$ versus rituximab using Tukey's multiple comparison test.

transmembrane TNF-α as a monovalent Fab′ fragment. Golimumab was expected to induce apoptosis and cell cycle arrest similarly to infliximab and adalimumab because these agents exhibit similar structures. Cell viability assays, which reflect both cellular death and cell growth arrest, were performed. Transmembrane TNF-α–expressing Jurkat cells were stimulated with 0.1, 1, or 10 μg/mL anti-TNF-α agents for 24, 48, and 72 hours and the resulting viable cell numbers were determined (Fig. 3B). Contrary to our anticipation, 1 and 10 μg/mL certolizumab pegol significantly reduced viable cell numbers, but this reduction was less pronounced compared with the full-length monoclonal antibodies. Golimumab induced a smaller reduction in viable cell numbers than infliximab and adalimumab. Significant differences between 1 and 10 μg/mL golimumab versus infliximab and adalimumab were observed after 24, 48, and 72 hours in culture. None of the 5 anti-TNF-α agents, including certolizumab pegol and golimumab, affected viable cell numbers in mock-transfected Jurkat cells that did not express transmembrane TNF-α (Fig. 3A).

Certolizumab Pegol Directly Induced Cellular Death in a Novel Manner, and Golimumab Induced Less Apoptosis Than Infliximab and Adalimumab

Apoptosis assays examined the direct effects of certolizumab pegol and golimumab in more detail. Transmembrane TNF-α–expressing Jurkat T cells were stimulated for 24 hours with 0.01, 0.1, 1, or 10 μg/mL anti-TNF-α agents. The stimulated cells were stained with annexin V and PI and analyzed using flow cytometry (Fig. 4A–C). Certolizumab pegol increased the proportion of annexin V–positive/PI-positive cells (Fig. 4B), but it did not increase annexin V–positive/PI-negative cells (Fig. 4C). This result indicated that certolizumab pegol induced cellular death without phosphatidylserine (PS) flipping, which is a morphological feature of apoptosis. Golimumab-treated cells exhibited typical apoptotic plots that were similar to infliximab and adalimumab (Fig. 4A). However, the proportion of annexin V–positive/PI-negative cells was significantly lower than infliximab and adalimumab (Fig. 4C). The cytotoxic effects of the 5 anti-TNF-α agents were exerted through transmembrane TNF-α because these agents did not produce cytotoxic effects on mock-transfected Jurkat cells (Fig. 5B, C). The direct effects of anti-TNF-α agents were examined on Jurkat cells that were transfected with a mutant transmembrane TNF-α in which 3 cytoplasmic serine residues were replaced by alanine. We previously demonstrated that these 3 serine residues in the cytoplasmic domain of transmembrane TNF-α were essential for the apoptotic effect of infliximab.[14] The apoptotic effects of infliximab, adalimumab, and golimumab were abolished by the substitution of the cytoplasmic serine residues. However, certolizumab pegol–induced cell death was not affected (Fig. 5A–C).

Certolizumab Pegol Had No Effects on Caspase-3 Activation and Apoptotic DNA Fragmentation, and Golimumab Had Weaker Effects Than Infliximab and Adalimumab

We next examined caspase activation and DNA fragmentation by direct effects of anti-TNF-α agents. Transmembrane TNF-α–expressing Jurkat cells were incubated for 48 hours with 10 μg/mL anti-TNF-α agents or rituximab. Then the cells were lysed and the levels of active caspase-3 were determined by enzyme-linked immunosorbent assay (Fig. 6A). The DNA was extracted and separated using electrophoresis (Fig. 6B). Treatment with infliximab, adalimumab, and golimumab increased the levels of active caspase-3 and induced apoptotic DNA fragmentation. Golimumab had weaker effects than infliximab and adalimumab. Certolizmab pegol, etanercept, or rituximab had no effects.

DISCUSSION

This study demonstrated that certolizumab pegol directly induced the death of transmembrane TNF-α–expressing cells. This cellular death was mediated by transmembrane TNF-α because certolizumab pegol bound transmembrane TNF-α (Fig. 1B) and no cellular death was induced in Jurkat cells that lacked transmembrane TNF-α (Fig. 5A–C). The most distinctive feature of certolizumab pegol is the PEG conjugation, but PEG is unlikely to elicit cytotoxic effects. PEG is nontoxic, and it is used for the modification of many biopharmaceuticals.[18] Some PEGylated drugs bind to cell surface molecules, such as PEG-conjugated recombinant granulocyte colony-stimulating factor, but cytotoxicity in target cells has not been reported.[19] Cellular signaling through transmembrane

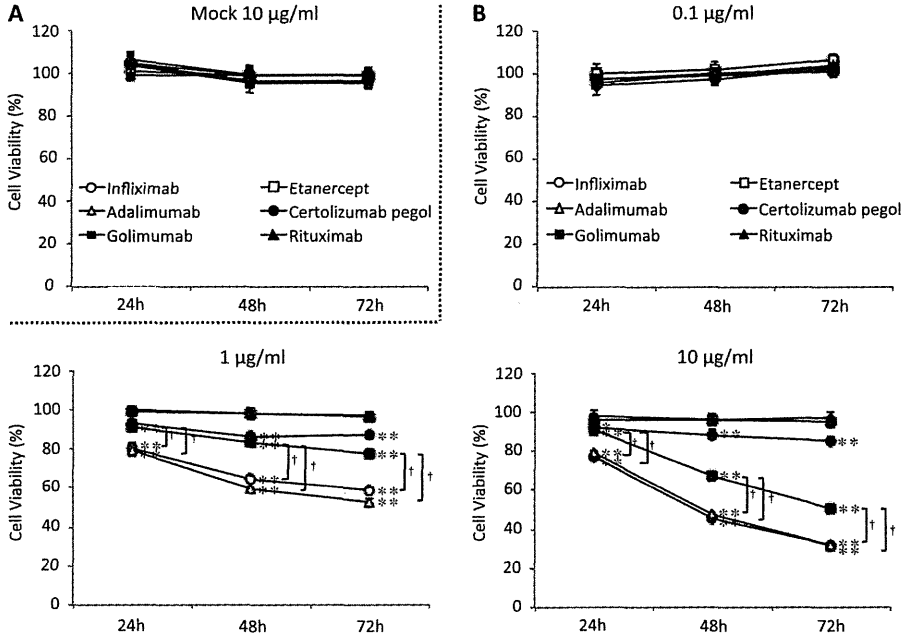

FIGURE 3. Cell viability assays. Mock-transfected (A) or transmembrane TNF-α–transfected (B) Jurkat cells were cultured with anti-TNF-α agents. Relative viable cell numbers were calculated by taking the number of viable cells in the absence of drugs as 100%. Data represent the means ± SD of 6 cultures. * = $P < 0.001$; ** = $P < 0.0001$ versus rituximab using Tukey's multiple comparison test. † = $P < 0.0001$ using Tukey's multiple comparison test.

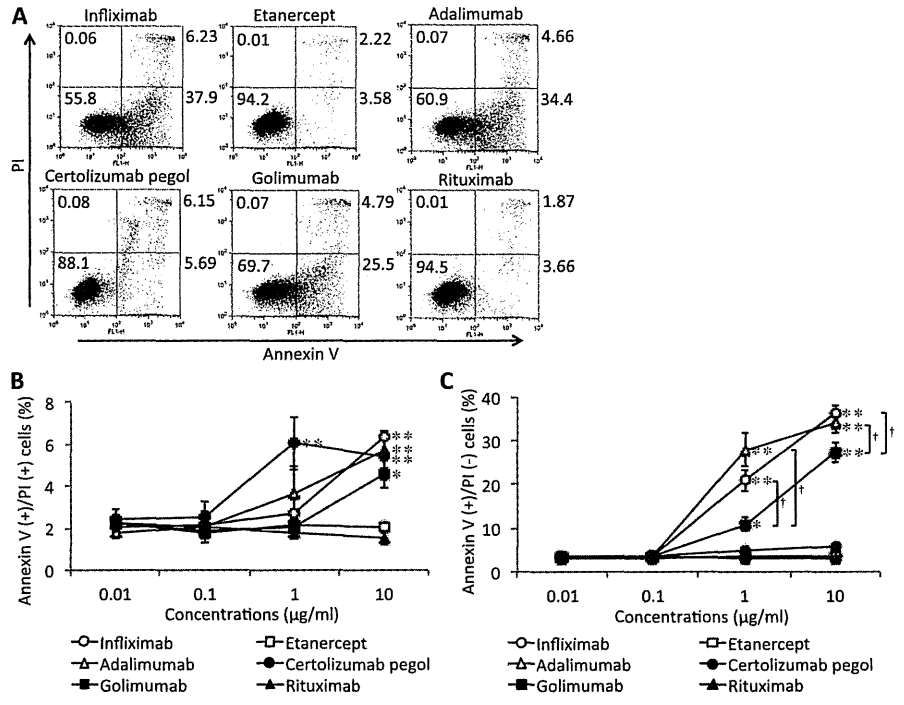

FIGURE 4. Apoptosis assays. Transmembrane TNF-α–transfected Jurkat cells were stimulated with anti-TNF-α agents and then stained with FITC-conjugated annexin V and PI. (A) Representative dot plots of cells stimulated with 10 μg/mL anti-TNF-α agents. The proportion of cells residing in each quadrant is expressed as a percentage. The proportions of annexin V–positive/PI-positive (B) and annexin V–positive/PI-negative (C) cells are indicated. Data represent the means ± SD of triplicate cultures. * = $P < 0.05$; ** = $P < 0.005$ versus rituximab using Tukey's multiple comparison test. † = $P < 0.01$ using Tukey's multiple comparison test.

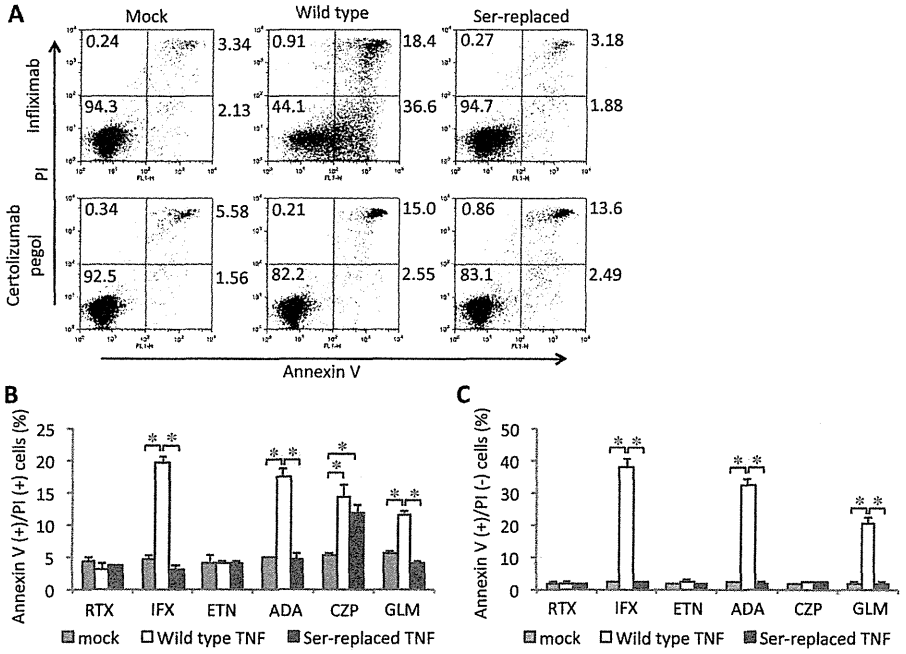

FIGURE 5. Direct cytotoxic effects of anti-TNF-α agents on mock-transfected and Ser-replaced transmembrane TNF-α–transfected cells. Mock-transfected, wild-type uncleavable transmembrane TNF-α–transfected, or Ser-replaced transmembrane TNF-α–transfected Jurkat cells were stimulated with anti-TNF-α agents and then stained with FITC-conjugated annexin V and PI. (A) Representative dot plots of cells stimulated with infliximab or certolizumab pegol. The proportions of annexin V–positive/PI-positive (B) and annexin V–positive/PI-negative (C) cells are indicated. Data represent the means ± SD of triplicate cultures. * = $P < 0.0001$ using Tukey's multiple comparison test. RTX, rituximab; IFX, infliximab; ETN, etanercept; ADA, adalimumab; CZP, certolizumab pegol; GLM, golimumab.

TNF-α is likely essential for the induction of cellular death after the binding of certolizumab pegol to transmembrane TNF-α.

The direct cytotoxicity of certolizumab pegol was different from the other anti-TNF-α agents. It was not affected by the substitution of 3 serine residues in the cytoplasmic domain of transmembrane TNF-α (Fig. 5A–C), which are essential for the apoptosis-inducing outside-to-inside (reverse) signals of infliximab, adalimumab, and golimumab.[14] Furthermore, neither PS flipping, caspase-3 activation, nor DNA fragmentation, which were the features of apoptosis, accompanied the cellular death that was induced by certolizumab pegol (Figs. 4A, C and 6A, B). These results indicate that certolizumab pegol–induced cellular death is not apoptotic. Apoptosis is not the sole form of regulated cell death. Death receptors, including CD95 (FAS), TNF receptor, and TNF-related

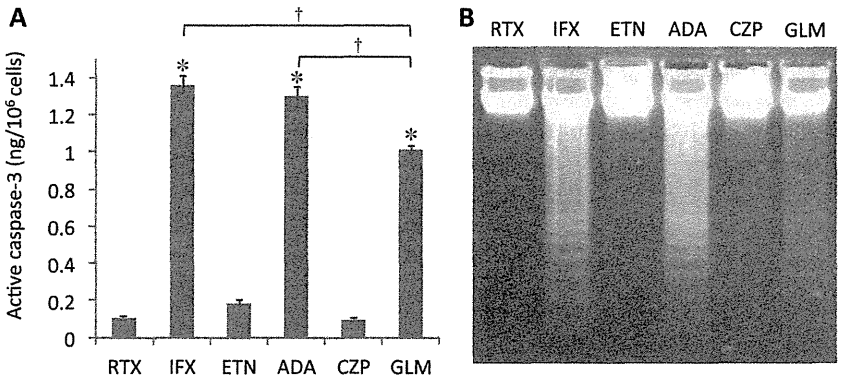

FIGURE 6. Caspase-3 activation and apoptotic DNA ladder. Transmembrane TNF-α–transfected Jurkat cells were treated with 10 μg/mL anti-TNF-α agents or rituximab for 48 hours in culture medium containing 10% heat-inactivated serum. (A) The level of active caspase-3 was determined by enzyme-linked immunosorbent assay. Data represent the means ± SD of triplicate cultures. * = $P < 0.0001$ versus rituximab using Tukey's multiple comparison test. † = $P < 0.0001$ using Tukey's multiple comparison test. (B) The DNA was extracted from treated cells and separated using 1.2% agarose gel electrophoresis. The DNA fragments were stained with ethidium bromide. RTX, rituximab; IFX, infliximab; ETN, etanercept; ADA, adalimumab; CZP, certolizumab pegol; GLM, golimumab.

apoptosis-inducing ligand receptor, and pathogen recognition receptors, such as Toll-like receptors, induce apoptosis and necrosis.[20] Type II CD20–specific monoclonal antibodies, which are used in the treatment of lymphoid disorders, evoke a lysosome-mediated nonapoptotic cell death in human lymphoma and leukemia cells.[21]

The direct cytotoxic effect may be 1 mechanism of the clinical efficacy of certolizumab pegol in CD. It is widely accepted that apoptosis induction in effector cells is an important contributing factor for the successful treatment of CD.[22,23] However, certolizumab pegol did not induce ADCC and CDC (Fig. 2A, B), and the direct cytotoxic effect was less compared with the full-length antibodies (Fig. 3B). Therefore, the overall cytotoxicity of certolizumab pegol on transmembrane TNF-α–expressing cell was lower compared with the other anti-TNF-α agents. Etanercept, which is not effective in CD,[24] did not exert a direct cytotoxic effect, but it did induce ADCC and CDC. One possibility is that the direct cytotoxic effect is essential for clinical efficacy in CD, but the induction of ADCC and CDC is not. Alternatively, other properties might contribute to the efficacy of certolizumab pegol for CD. Infliximab, adalimumab, and certolizumab pegol, but not etanercept, inhibit the cytokine production by monocytes.[25] Certolizumab pegol distributes into inflamed tissue more effectively than infliximab and adalimumab, which might be because of PEGylation and the relatively smaller molecular weight of certolizumab pegol.[26] The mechanism of certolizumab pegol efficacy in CD is not clear, but the direct cytotoxic effect of certolizumab pegol may contribute.

This study demonstrated that golimumab induced apoptosis through transmembrane TNF-α. We expected golimumab to exert a similar apoptotic effect as infliximab and adalimumab because golimumab also exhibits a full-length bivalent IgG1 structure. However, the apoptotic effect of golimumab through transmembrane TNF-α was lower than infliximab and adalimumab. This was apparent in PS flipping, caspase-3 activation, and apoptotic DNA ladder formation (Figs. 4A, C and 6A, B). The affinity of golimumab to soluble TNF-α and the capacity to neutralize soluble TNF-α were higher than infliximab and adalimumab.[27] It is considered that less frequent (every 4 weeks) subcutaneous dosing is possible because a lower serum concentration of golimumab is enough to neutralize soluble TNF-α to a similar level as infliximab and adalimumab. Our experimental results suggest that the cytotoxic effect of golimumab on TNF-α–producing cells on a less frequent dosing schedule may be much lower than infliximab and adalimumab. Golimumab may be less effective than infliximab and adalimumab for granulomatous diseases, such as CD, in which the effects that are mediated by transmembrane TNF-α may be required.[12] Recently, 2 patients with CD-associated spondylarthritis and 1 patient with AS and comorbid colitis were treated with golimumab. These patients were switched from adalimumab to golimumab, and they experienced an improvement in rheumatic symptoms but marked flares in their inflammatory bowel diseases.[28]

The direct cytotoxic effects of anti-TNF-α agents through transmembrane TNF-α have been examined previously in vitro. Nesbitt et al[25] reported that infliximab, adalimumab, and etanercept, but not certolizumab pegol, increased the proportion of annexin V–positive cells in activated lymphocytes and monocytes. Why etanercept could induce apoptosis in their experimental system was not clear, but the differences of cell types, cell preparation, and culture protocols might explain the discrepancy. In addition, TNF receptor signaling inhibition by anti-TNF-α agents might influence their experimental system. Our experimental system used the cells without TNF receptors on their surface. Stimulation of the TNF receptors by transmembrane TNF-α of neighboring cells and soluble TNF-α should also be taken into account in addition to the transmembrane TNF-α–mediated effects in Nesbitt's experimental conditions, which may be more complicated compared with our system.

Certolizumab pegol did not induce ADCC and CDC in our experimental system. In contrast, golimumab induced ADCC and CDC similarly to infliximab and adalimumab (Fig. 2A, B). The activities of golimumab and certolizumab pegol on ADCC and CDC could be expected because golimumab is a full-length antibody with the Fc portion of IgG1, and certolizumab pegol is a monovalent Fab′ antibody that lacks the Fc portion.

Etanercept was significantly worse than full-length antibodies at inducing CDC (Fig. 2B). Full-length antibodies and etanercept commonly possess the Fc portion of IgG1. The Fc portion contains CH2 domain, which activates C1, the first component of complement activation. However, etanercept does not carry the CH1 domain, which is a platform for C3 activation, the most important step in complement activation. Moreover, lack of a hinge region of etanercept results in structural rigidity compared with natural antibody, which may be conformational hindrance to the proper access of complement.[12] In a system using mouse NS0 myeloma cells and rabbit complement, etanercept induced CDC comparable to infliximab and adalimumab.[25] The reason for the discrepancy is not clear, but possible explanation is that CDC occurs more easily in their system using mouse cells and rabbit complement than in our system using human cells and complement because membrane complement regulatory proteins do not work for the complement of different species.

Considering that our assay system uses a cell line with accumulated transmembrane TNF-α expression, our study is not without limitations. It would be difficult to infer that our data may have an in vivo relevance. Further studies are needed to investigate the relevance of transmembrane TNF-α–mediated effects by anti-TNF-α agents in patients.

In conclusion, the cytotoxic effects of new anti-TNF-α agents, certolizumab pegol and golimumab, that were mediated by transmembrane TNF-α were examined using transmembrane TNF-α–expressing Jurkat T cells that did not express the TNF receptor. The results demonstrated that certolizumab pegol directly induced the death of transmembrane TNF-α–expressing cells. This cellular death was mediated by a mechanism different than the apoptosis that was induced by full-length antibodies. This cytotoxic effect may be 1 mechanism of the efficacy of certolizumab pegol in CD. Golimumab induced ADCC and CDC in transmembrane

TNF-α–expressing cells similarly to infliximab and adalimumab, but golimumab-induced apoptosis was less potent. The delineation of the biological features of anti-TNF-α agents against transmembrane TNF-α would contribute to the understanding of the differential clinical effects of anti-TNF-α agents and promote the discovery of promising antiinflammatory therapeutic modalities.

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Antibiotic Rotation for Febrile Neutropenic Patients with Hematological Malignancies: Clinical Significance of Antibiotic Heterogeneity

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Abstract

Background: Our unit adopted the single administration of cefepime as the initial treatment for febrile episodes in neutropenic patients with hematological malignancies. However, recently, cefepime-resistant gram-negative bacteremia, including those with extended-spectrum β-lactamase (ESBL)-producers, was frequently observed in these patients. Therefore, we instituted a rotation of primary antibiotics for febrile neutropenic patients in an attempt to control antibiotic resistance.

Methods: This prospective trial was performed from August 2008 through March 2011 at our unit. After a pre-intervention period, in which cefepime was used as the initial agent for febrile neutropenia, 4 primary antibiotics, namely, piperacillin-tazobactam, ciprofloxacin, meropenem, and cefepime, were rotated at 1-month intervals over 20 months. Blood and surveillance cultures were conducted for febrile episodes, in order to assess the etiology, the resistance pattern (particularly to cefepime), and the prognosis.

Results: In this trial, 219 patients were registered. A 65.9% reduction in the use of cefepime occurred after the antibiotic rotation. In the surveillance stool cultures, the detection rate of cefepime-resistant gram-negative isolates, of which ESBL-producers were predominant, declined significantly after the intervention (8.5 vs 0.9 episodes per 1000 patient days before and after intervention respectively, $P<0.01$). Interestingly, ESBL-related bacteremia was not detected after the initiation of the trial (1.7 vs 0.0 episodes per 1000 patient days before and after intervention respectively, $P<0.01$). Infection-related mortality was comparable between the 2 periods.

Conclusions: We implemented a monthly rotation of primary antibiotics for febrile neutropenic patients. An antibiotic heterogeneity strategy, mainly performed as a cycling regimen, would be useful for controlling antimicrobial resistance among patients treated for febrile neutropenia.

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Introduction

Febrile neutropenia is one of the most serious adverse events in patients with hematological malignancies and chemotherapy. A prompt initiation of empirical antibiotic therapy is favorable for patients with febrile neutropenia, regardless of the detection of bacteremia. Based on guidelines [1,2], our unit had adopted single administration of cefepime, a fourth-generation cephalosporin, as the initial treatment for febrile episodes in neutropenic patients. However, recently, cefepime-resistant gram-negative bacteremia, including those with extended-spectrum β-lactamase (ESBL)-producers, was frequently seen in these patients [3]. ESBL-producing bacteria have been frequently detected not only in our hematology unit, but in all the units at our hospital. Therefore, we considered rotating primary antibiotics for the treatment of febrile neutropenic episodes as a measure to suppress the antibiotic resistance seen in our unit.

Most antibiotic rotation trials, known as cycling therapies, have been conducted in adult intensive-care units (ICUs), where infectious agents are exposed to heavy antimicrobial pressure. Many cycling therapies implemented in clinical settings have been reported to date. Most of these trials were conducted in order to reduce the selective pressure of antibiotic-resistant gram-negative bacteria, which was a major concern for patient prognosis in an ICU setting. The efficacy of antibiotic cycling on the recovery of antimicrobial susceptibility and patient mortality is controversial. Several trials have successfully reported a recovery of susceptibility to specific antibiotics or a reduction in detection rates of