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ORIGINAL ARTICLE

Overexpression of wild-type or mutants forms of *CEBPA* alter normal human hematopoiesis

O Quintana-Bustamante[1], S Lan-Lan Smith[2], E Griessinger[1], Y Reyal[1], J Vargaftig[1], TA Lister[3], J Fitzgibbon[4] and D Bonnet[1]

CCAAT/enhancer-binding protein-α (C/EBPα/*CEBPA*) is mutated in approximately 8% of acute myeloid leukemia (AML) in both familial and sporadic AML and, with *FLT3* and *NPM1*, has received most attention as a predictive marker of outcome in patients with normal karyotype disease. Mutations clustering to either the N- or C-terminal (N- and C-ter) portions of the protein have different consequences on the protein function. In familial cases, the N-ter form is inherited with patients exhibiting long latency period before the onset of overt disease, typically with the acquisition of a C-ter mutation. Despite the essential insights murine models provide the functional consequences of wild-type C/EBPα in human hematopoiesis and how different mutations are involved in AML development have received less attention. Our data underline the critical role of C/EBPα in human hematopoiesis and demonstrate that C/EBPα mutations (alone or in combination) are insufficient to convert normal human hematopoietic stem/progenitor cells into leukemic-initiating cells, although individually each altered normal hematopoiesis. It provides the first insight into the effects of N- and C-ter mutations acting alone and to the combined effects of N/C double mutants. Our results mimicked closely what happens in *CEBPA* mutated patients.

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INTRODUCTION

Acute myeloid leukemia (AML) is defined by an accumulation of immature myeloblasts in the periphery and bone marrow (BM). Like other cancers, AML is a multistep process characterized by an alteration of different pathways affecting cell proliferation and myeloid differentiation.[1-3] Although the specific relationship between altered proliferation and differentiation in AML remains elusive, a two-hit model has been proposed[4] in which both an uncontrolled proliferation (and/or apoptosis) and a block of differentiation are required to progress to AML with neither of these alone sufficient to cause leukemia. Recent studies suggests that the disruption of CCAAT/enhancer-binding protein-α (C/EBPα) transcription factor may perturb both differentiation and cell cycle[5] because of its role in cell cycle arrest, repression of self-renewal and myeloid differentiation during normal hematopoiesis[6-10] and the fact that the gene encoding this factor, *CEBPA*, is frequently mutated or hypermethylated in sporadic cases of AML.[5,11,12]

CEBPA/C/EBPα is a member of the C/EBP family of bZIP transcription factors encoding two different translational isoforms of 42 and 30 kDa (p42 and p30, respectively) by use of alternative AUG codons within the same open reading frame. The shorter form retains the C-terminal (C-ter) domain but lacks part of the N-terminal (N-ter) trans-activation domain and is unable to block cell cycle and induce granulocytic differentiation.[11,13-15] The effects of p42 on cell cycle control are complex due, at least in part, to the upregulation of $p21^{WAF1}$,[16,17] its interaction with cyclin-dependent kinases 2 and 4 (CDK2 and CDK4),[18] and its repression of E2F.[14,19,20] Myeloid differentiation is equally dependent on collaboration with other specific transcription factors to regulate cell fate.[7-11,21] C/EBPα (as homo-, hetero-dimers) binds directly to lineage-specific promoters[18,22,23] with a requirement for p42 to activate the transcriptional machinery (TBP/TFIIB and CBP/p300).[24,25] It has also been speculated that both cell growth arrest and differentiation may be coupled via its link with the chromatin remodeling protein SWI/SNF.[26,27]

CEBPA is mutated in both familial and in approximately 8% of sporadic AML with a predilection for the normal karyotype subgroup.[28-38] Mutations fall into two groups N-ter truncating mutations that lead to preferential expression of p30 and an alteration in the balance between p42 and p30 isoforms and C-ter mutations, which typical locate at the junction between the basic region and the leucine-zipper and disrupt DNA-binding and dimerization. The pattern of *CEBPA* mutations observed are also variable with single C-ter or N-ter mutations, rarer cases of homozygous mutation as a result of mitotic recombination[38,39] or most frequently as biallelic mutations with the simultaneous occurrence of both N- and C-ter mutations.[28,31,32] This is further complicated by cases where both mutations are present on the same allele. Although it has been assumed that *CEBPA* mutations favor a good outcome, more recent data suggest that this is confined to patients with biallelic mutations.[38,40,41] Along with others, our group have identified families in which affected members have inherited a predisposing N-ter germline mutation, with the acquisition of an additional somatic C-ter mutation[36,42-44] coinciding with the onset of disease[12,36] leading to the hypothesis that N-ter plus C-ter mutations cooperate to induce a full blown leukemia.

[1]Haematopoietic Stem Cell Laboratory, Cancer Research UK, London, UK; [2]Leukaemia and Stem Cell Biology Laboratory, Department of Haematological Medicine, King's College of London, London, UK; [3]Department of Medical Oncology, St Bartholomew's Hospital, Queen Mary University of London, London, UK and [4]Centre of Haemato-Oncology, Barts Cancer Institute, Queen Mary University of London, London, UK. Correspondence: Dr D Bonnet, Haematopoietic Stem Cell Laboratory, CRUK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK.
E-mail: dominique.bonnet@cancer.org.uk
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The mechanism by which *CEBPA* mutants induced leukemogenesis has been addressed in mice using homozygous knock-in studies that express either p30 or p42 C/EBPα alone or both mutated forms[45,46] or more recently by overexpression of the mutated forms in mouse hematopoietic stem/progenitor cells (HSC/HPCs).[47] Despite the importance of these knock-in models, the extent to which these mirror the process of naturally occurring human disease is still uncertain. With the recent success of transforming primary human HSC/HPCs with viral delivery of MLL–ENL fusion oncogenic protein, we decided to investigate the role of the different *CEBPA* mutations in human stem/progenitors using both *in vitro* and *in vivo* assays.[48,49]

MATERIALS AND METHODS

Lentiviral vectors

Genomic DNA from normal BM and an AML patient,[37] who had a 4-bp GGCC insertion at base 363 (N-ter mutation) and an in frame internal tandem duplication of 27 bp at 1096 (C-ter mutation) (Genbank Y11525), were used as template to generate carboxy-terminal FLAG tagged wild-type (WT) *CEBPA*, N-ter, C-ter and NC-ter mutated *CEBPA*, in the pCR2.1-TOPO vector by TOPO TA Cloning (Invitrogen, Paisley, UK). Every FLAG-tagged *CEBPA* form was cloned downstream of an IRES (Internal Ribosome Entry Site)–enhanced green fluorescent protein (EGFP) cassette, previously inserted into the pEntr1A vector (Invitrogen) with the exception of the C-ter mutated form, which was cloned next to an IRES–mCherry cassette. Different *CEBPA*–IRES–EGFP or C-ter *CEBPA*–IRES–mCherry were introduced by recombination into pHR'SIN–SEW lentiviral vector, which had been modified for compatibility with the Gateway System (Invitrogen).

Lentiviral supernatant was produced after co-transfecting 293T cells with pMD.2 VSV-G envelope plasmid, pCMV-dR8.74 helper plasmid and *CEBPA* lentiviral vectors by calcium-phosphate transfection protocol.[50] Virus suspension was collected, filtered and concentrated by ultracentrifugation. Lentiviral titers were determined by GFP or mCherry analysis of transduced Hela cells on a LSR II flow cytometer (BD Pharmingen, Oxford Science Park, UK). Protein lysates of transduced human Lineage-negative (Lin^-) cord blood (CB) cells were used to identify exogenous expression of flagged-C/EBPα by standard western blot using an anti-FLAG antibodies (M2, Sigma, Gillingham, UK).

Purification and transduction of mouse and human hematopoietic progenitors

Mouse hematopoietic progenitors ($mLin^-$) were purified from BM mononuclear cells of C57BL6/6J mice by lineage cell depletion (MACS, MiltenyiBiotec, Bergisch Gladbach, Germany). The mouse cells were then pre-stimulated in StemSpam serum-free expansion medium (StemCells Technologies, Vancouver, Canada) with 50 ng/ml mouse stem cell factor (R&D Systems, Minneapolis, MN, USA), 100 ng/ml human interleukin (IL)-11, 100 ng/ml human Flt-3 ligand and 10 ng/ml human IL-3 (PeproTech, Rocky Hill, NJ, USA) for 4–6 h. After which, $mLin^-$ cells were transduced with the different lentivirus. On the other hand, CB was collected from mothers attending the Royal London Hospital (London, UK), after informed consent and via a protocol approved by the Local Research Ethics Committees. Mononuclear cells were obtained by Ficoll density centrifugation and ammonium chloride red cell lysis. Density-separated CB mononuclear cells were depleted for lineage marker positive cells via the StemSep system (StemCells Technologies) according to the manufacturer's instructions to generate Lin^- cells. Lin^- cells were pre-stimulated in StemSpam serum-free expansion medium and supplemented with 50 ng/ml human stem cell factor, 50 ng/ml human Flt-3 ligand, 20 ng/ml human thrombopoietin and 10 ng/ml human IL-6 (PeproTech) for 4–6 h. Lentiviral supernatants were added at a multiplicity of infection of 30 (for single transduction) or 20 (for each lentivirus during the double transduction). All the transductions were carried out over-night in the presence of 4 mg/ml of polybrene (R&D Systems, Abingdon, UK). The efficiency of transduction was analyzed at 4 days by eGFP or/and mCherry expression.

Colony-forming cell and LTC-IC assays

Infected Lin^- cells were plated in triplicate in methylcellulose media (Methocult H4434 and Methocult M3434 for human and mouse, respectively, StemCells Technologies) to assess colony-forming units (CFUs). Cells from primary plates were transferred to secondary and tertiary plates in new methylcellulose media. Transduced and total colonies were scored at 7 and 14 days for mouse and human, respectively, following plating and classified according to their morphology.

Long-term culture-initiating cell assays (LTC-IC) were performed according to the manufacturer's instructions. Briefly, Lin^- cells were plated after transduction on irradiated M2-10B4 stromal cells for 5 weeks. The cells were then plated in methylcellulose media (Methocult H4435, StemCells Technologies) with colonies scored 14 days later.

Liquid cultures assays

Two types of liquid culture were used one for maintaining stem/progenitors and the second for inducing myeloid differentiation. For the maintenance of HSC/progenitors, transduced Lin^- cells were cultured in Iscove's modification of Dulbecco's medium (Gibco, Invitrogen, Paisley, UK)/10% fetal calf serum supplemented with 20 ng/ml stem cell factor, 50 ng/ml IL-3, 20 ng/ml IL-6 and 10 ng/ml granulocyte colony-stimulating factor (PeproTech). Fresh media was added every week. For myeloid-promoting differentiation, transduced cells were cultured as previously described for 2 weeks[48] in Iscove's modification of Dulbecco's medium/15% fetal calf serum supplemented with 2 ng/ml IL-3 and 20 ng/ml stem cell factor.

Immunodeficient mouse transplantations

All animal experiments were performed in compliance with Home Office and institutional guidelines. NOD/SCID/β2 microglobulin null mice (NOD/SCID/β2) were bred at Charles Rivers Laboratories (Moorgate, UK), housed in micro-isolators and fed sterile food and acidified water. Mice aged 8–12 weeks were sub-lethally irradiated at 375 rads (Cesium 137 source) up to 24 h before intravenous (i.v.) injection of cells. NOD/SCID/β2 mice were transplanted intravenous with $0.5–1 \times 10^5$ human Lin^- cells after transduction. The animals were killed after 4, 7 or 8 weeks, then femurs, pelvis and tibias were flushed and all cells were pooled for analysis. In most animals, BM aspirations were performed 4 weeks after transplantation. When secondary transplants were performed, pooled BM cells from primary recipients, killed at 7 to 8 weeks, were depleted of mouse cells by Mouse/Human Chimera Enrichment kit (StemCells Technologies) to a $>90\%$ purity of human cells. In all, 1×10^6 human-enriched cells from 7 weeks primary mice were transplanted intrabone and analyzed after 5 weeks, or $0.9–5 \times 10^6$ human cells from 8 weeks primary mice were transplanted intravenous and analyzed 6 weeks later.

Flow cytometry

Cell suspensions were analyzed in phosphate-buffered saline/2% fetal calf serum on a FACS LSR II (BD Pharmingen), where transduced cells were identified based on their expression of eGFP and/or mCherry. Surface markers were detected with fluorescent human-specific antibodies from BD Pharmingen (anti-CD14 APC-H7, anti-CD14 PE-Cy7, anti-CD15 APC, anti-CD19 APC, anti-CD19 PE, anti-CD33 PE, anti-CD34 PE-Cy7, anti-CD34 APC and anti-CD45 PE-Cy7). Apoptosis was analyzed in cell suspensions in binding buffer (BD Pharmingen) after annexin V-AlexaFluor647 (Molecular Probes, Invitrogen, Paisley, UK) and 4,6-diamidino-2-phenylindole incubation. Intracellular staining was performed to study cell cycle. Cells were fixed by phosphate-buffered saline/2% paraformaldehyde and permeabilized with phosphate-buffered saline/0.2% Triton X-100 before adding anti-Ki67 AlexaFluor647 (Molecular Probes, Invitrogen). Fixed cells were resuspended in phosphate-buffered saline/2% fetal calf serum solution with 4,6-diamidino-2-phenylindole with the data analyzed using FlowJo software (Tree Star, Oten, Switzerland).

Quantitative real-time PCR analysis

RNA was isolated using RNAeasy kit (Qiagen, Crawley, UK) from transduced cells after sorting on MoFlo cell sorter (Beckman Coulter, High Wycombe, UK).

RNA was reversed transcribed with SuperScript III (Invitrogen) according to the manufacturer's instructions. The complementary DNA was subjected to quantitative real-time PCR using Fast SYBR Green Master Mix (Applied Biosystems, Cheshire, UK) and gene-specific primers (Supplementary Table S1). Quantitative real-time PCRs were run on 7500 Fast System and analyzed on SDS software (Applied Biosystems). Results were normalized to glyceraldehyde 3-phosphate dehydrogenase expression and expression of control samples according to the $2^{\Delta\Delta Ct}$ method.

Statistical analysis

Data are presented as the mean ± s.d. Statistical analysis was performed using one-way analysis of variance test with Statgraphics Plus software (Statpoint Technologies, Warrenton, VA, USA). *In vivo* experiments were compared according a Negative Binomial Distribution with R software (R Foundation for Statistical Computing, http://www.R-project.org/).

RESULTS

In order to investigate the effect of the different *CEBPA* mutations, genomic DNA from normal BM and an AML patient,[37] with a 4-bp GGCC insertion at base 363 (N-ter mutation) and an in frame internal tandem duplication of 27 bp at 1096 (C-ter mutation) (Genbank Y11525) were used as template to generate lentiviral vectors with WT *CEBPA*, N-ter, C-ter and N- and C-ter mutated *CEBPA* (see Materials and methods section) (Supplementary Figure S1A). The expression of the corresponding C/EBPα forms was confirmed by western blot in transduced Lin^- cells (Supplementary Figure S1B).

Effect of the transduction of different forms of C/EBPα on human hematopoietic progenitor compartment (CFU-C/LTC-IC)

In the first instance, we examined the effect of overexpressing individual mutations (N-ter or C-ter), WT and control (empty vector) to determine the potential consequence of each mutation alone on human hematopoietic progenitor development. CB-derived Lin^- cells were transduced (Supplementary Figures S1B and S1C) and the hematopoietic progenitor compartment tested by CFU assay.

Surprisingly, using human cells, overexpression of WT C/EBPα as well as C-ter mutation (Figure 1a) led to a significant reduction in the total number of CFUs compared with control whereas the total number of CFUs was not modify by the N-ter mutation.

When looking at the percentage of erythroid and myeloid progenitors present in each group, we show that overexpression of the N-ter favor myeloid differentiation (Figure 1a) whereas WT and C-ter mutation block colony formation with a predominant effect on erythroid colony formation. This blockage of erythroid colony formation has been documented for WT on human CD34+ previously.[51] When trying to dissect which myeloid colonies was mostly affected by the N-ter mutation, we did not see any difference in the ratio of CFU-G, CFU-M or CFU-GM in the N-ter compare with control (Supplementary Figure S4). Serial replating experiments demonstrate that cells overexpressing C-ter have a lower self-renewal potential than control, N-ter or WT (Figure 1b). The LTC-IC capacity of the transduced cells also showed distinctive patterns. The different *CEBPA* mutations had opposing effects with N-ter slightly increasing the number of LTC-IC whereas the C-ter as with the WT overexpression did not give rise to any LTC-IC (Figure 1c).

Our data does not follow the results of Kato *et al*.,[47] obtained in mice where the C-ter was able to increase self-renewal. To determine whether it was due to differences in the constructs used or due to the intrinsic differences between human and mouse cells, we decided to overexpress our constructs in mouse stem/progenitors hematopoietic cells. As shown in Figure 2, we show an increase in the number of colonies with both N-ter and C-ter with the C-ter cells and to a lesser degree the N-ter being able to give rise to serial colonies indicative of the increase in self-renewal of these cells. Thus, it appears that intrinsic properties between mouse and human are responsible for the differences observed and not due to the difference in mutant constructs used.

We thus went back using human cells for the rest of the analysis.

These first results apportioned distinct functions for N- and C-ter *CEBPA* mutations and that overexpression of the N-ter mutation alone is sufficient to induce myeloid differentiation while

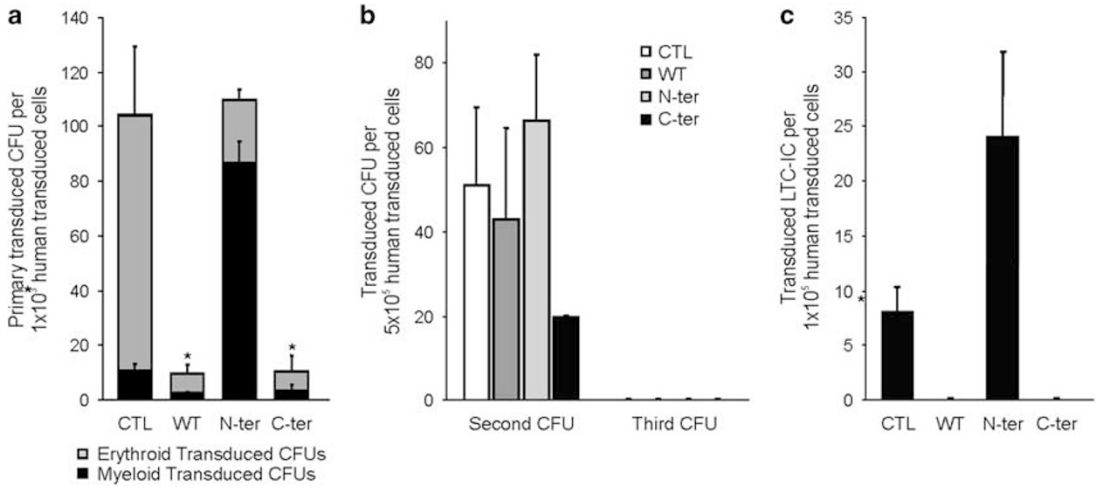

Figure 1. Effect of different mutated forms of C/EBPα on human progenitors. (**a**) Analysis of effect of expression of WT C/EBPα, mutated C/EBPα on N-ter and on C-ter on human hematopoietic progenitors by CFUs assay. In all, 2×10^3 human hematopoietic progenitors (Lin^- cells) after transduction were plated in H4434 medium; 2 weeks later number, type of CFUs and expression or not of GFP were determinate using an inverted fluorescent microscope. The number of erythroid (grey) and myeloid (black) colonies per transduced cells (GFP+) are reported. (**b**) Maintenance of human hematopoietic progenitors by expression of C/EBPα mutant. Serial replating from primary or secondary CFU assays were done to evaluate secondary or tertiary CFUs, respectively. Each CFU assays was plated in H4434 medium and quantify after 2 weeks. Numbers of transduced CFU for Control, WT C/EBPα, N-ter and C-ter are represented. (**c**) Differences in HSCs after transduction were determinate by LTC-IC assay. In all, 1×10^4 human Lin^- cells, after transduction, were plated on irradiated stromal cell layer (M2-10B4 cell line) for 5 weeks, then preserved progenitors was evaluated by CFU assay in H4435 medium. Results are shown as mean ± s.d., with significant differences of $^*P \leqslant 0.05$. All the data are from a minimum of triplicates of two independent experiments.

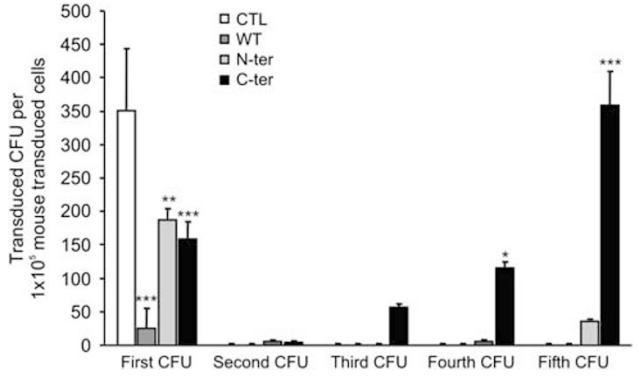

Figure 2. Effect of different mutated forms of C/EBPα on mouse progenitors. Effect of expression of WT C/EBPα (dark grey bars), mutated C/EBPα on N-ter (light grey) and on C-ter (black) was evaluated on mouse hematopoietic progenitors by CFUs assay. In all, 2×10^3 mouse hematopoietic progenitors (mLin$^-$ cells) after transduction were plated in M3434 medium; 1 week later number and type of CFUs were determinate. Serial replating was performed to evaluate secondary, tertiary, fourth or fifth CFUs, respectively. Numbers of transduced CFU by control (white bar), WT C/EBPα (dark grey), N-ter (light grey) and C-ter (black) are represented. The increase in colonies after serial replating observed could be explained by the presence of live immature cells in the methylcellulose, which were able after replating to mature and give rise to CFUs. Results are shown as mean ± s.d., with significant differences of $*P \leqslant 0.05$, $**P \leqslant 0.01$ or $***P \leqslant 0.001$.

maintaining immature progenitors. This is in line with its potential role as pre-leukemic event favoring proliferation of myeloid precursor cells and thus enhancing their chance to acquire further mutations, consistent with its predisposing function in familial AML.

Effect of the different forms of C/EBPα on cell cycle and apoptosis

In order to test the effect of the different forms of C/EBPα on the maintenance of HSC/progenitors, we cultured the cells *in vitro* in a supportive stem cell medium for up to 14 days and cell cycle was analyzed by DNA content and Ki67 expression (Figure 3a and Supplementary Figure S2A). WT and C-ter C/EBPα led to a block in cell cycle, maintaining transduced cells in G0 (Figure 3a), whereas the N-ter promoted the transition from G0 to G1 with a potential block in G1 phase. These results were in concordance with the expression analysis of cell cycle regulator genes ($p21^{WAF}$ (p21), INK4C (p18)) (Supplementary Figure S2B). Indeed, p18 and p21, which are known inhibitors of cell cycle progression show elevated expression in WT, as p18 in C-ter whereas the potential block in G1 phase seen in N-ter is less clear. We also could not exclude that the block in G0 observed here in WT and C-ter was also due, at least in part, to their effect in inducing differentiation.

The disappearance of WT and C-ter transduced cells appears to be *Bcl-2* independent and not due to apoptosis, as the percentage of apoptotic cells in the transduced population of human hematopoietic progenitors determined by annexin-V staining and 4,6-diamidino-2-phenylindole permeability was decreased in WT, C-ter and N-ter cells (Figure 3b), consistent with the anti-apoptotic role of C/EBPα.[52] These results were confirmed by quantitative real-time PCR (Figure 3c), where an increase in BCL2 expression in WT, N-ter and C-ter was observed, indicating that neither N-ter nor C-ter mutations affected the regulation of BCL2.

Effect of double mutations in human progenitors

The patterns of *CEBPA* mutations in AMLs are varied with patients having N-ter or C-ter alone or having both N- and C-ter mutations either on the same allele or more frequently as biallelic mutations. In order to examine the role of the biallelic versus monoallelic mutation, CB Lin$^-$ cells were transduced with either N-ter/C-ter together or a corresponding NC-ter construct. First, we assessed the effect of N-ter/C-ter and NC-ter expression on colony-forming cells. As previously shown for WT and C-ter alone (Figure 1a), the N-ter/C-ter combination reduced the number of total CFUs (Figure 4a). The total number of colony-forming cells in NC-ter was similar to control. The combination of N-ter/C-ter induced a reduction in erythroid colonies as for WT and C-ter (Figure 4a) whereas NC-ter had no effect. To examine the effect of these mutations on self-renewal, we performed secondary and tertiary replating from primary CFUs (Figure 4b). No hematopoietic progenitors were detected on secondary replating for the N-ter/C-ter C/EBPα while the NC-ter slightly increase the self-renewal potential of the cells as evidenced by an increase in secondary colonies and the presence of tertiary colonies. Accordingly, in LTC-IC assays (Figure 4c) the N-ter/C-ter transduced cells had no primitive progenitor potential while the NC-ter cells, demonstrating a sixfold increase in LTC-ICs number compared with control. Despite the presence of the N-ter mutation, an arrest in cell cycle as previously demonstrated for both WT and C-ter C/EBPα was detected in the N-ter/C-ter cells (Figure 4d). Both double mutation combinations showed a similar cell survival to the other C/EBPα constructs tested (data not shown).

These results document differences between biallelic and monoallelic mutations. For the NC-ter, the inclusion of the mutation on C-ter seems to damper the effects observed with N-ter alone as despite having an increase in self-renewal and LTC-IC, no difference was observed at the induction of the myeloid differentiation contrary to what was observed for the N-ter alone. The N-ter/C-ter double mutant behaves similarly to the C-ter alone with blockage of cell cycle and a decrease in self-renewal despite the presence of the N-ter mutation. These results provide the first experimental data to explain the favorable clinical outcome observed exclusively in bi-allelic mutated patients and suggest a potential role for NC-ter favoring an increase in self-renewal. Nevertheless, we could not also exclude that differences in the transcription factor level, (N-ter/C-ter mutants having almost as twice the level as the NC-ter) might have also on impact on the different phenotype observed.

Effect of different C/EBPα forms in myeloid differentiation

To determine the effects of the different forms of C/EBPα on myeloid differentiation, the production of monocytes, granulocytes and granulocytes precursors were assessed following the induction of myeloid differentiation (Figure 5a). A large proportion of granulocytic precursors could be detected in controls with some mature monocytes and granulocytes. In comparison, WT C/EBPα promoted granulocytic differentiation whereas the C-ter mutation blocked the terminal differentiation of monocytes and to a lesser degree of granulocytes. The N-ter has no effect on granulocytic precursors but seems to induce terminally differentiated granulocytes at the expense of monocytes. Interestingly, NC-ter was similar to control with only a slight decrease in monocytes. In comparison N-ter/C-ter double transduced cells were reminiscent of C-ter mutants. We further examined the level of endogenous C/EBPα and PU.1 transcript key regulator of myeloid differentiation. It appears that the overexpression of N-ter/C-ter induced a 32-fold increase in the endogenous level of C/EBPα whereas other constructs have little effect. Both N-ter and C-ter alone and to a lesser extend NC-ter induce a significant increase in PU.1 whereas with the combination of N-ter/C-ter, this effect disappears (Supplementary Figure S3).

Effect of different C/EBPα forms in HSC/HPC

To determine the effect of the different forms of C/EBPα on HSC/HPCs maintenance, the percentage of CD34$^+$CD38$^-$ and

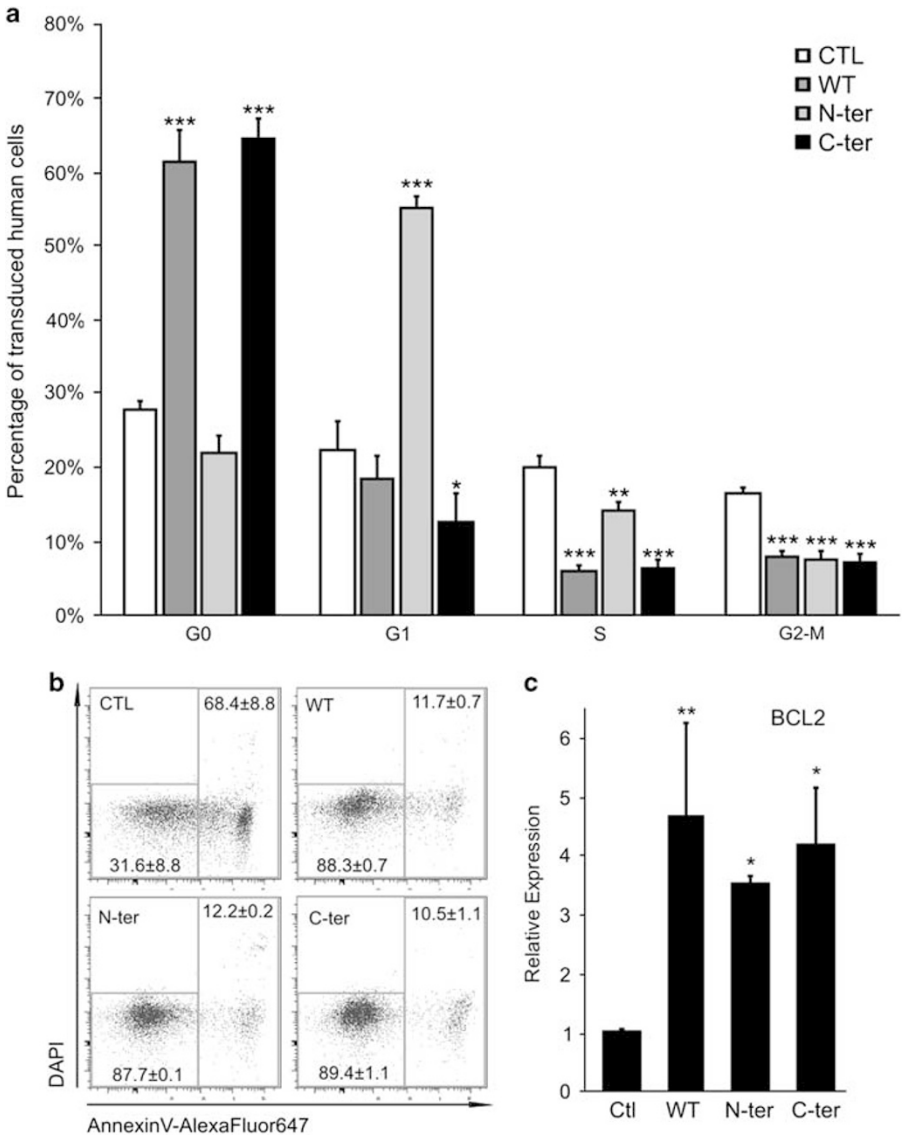

Figure 3. Expression of WT and mutants C/EBPα modifies proliferation and apoptosis of human hematopoietic progenitors. (**a**) Analysis of cell cycle of transduced cells at 2 weeks in culture. Different cell cycle stages were identified by fluorescence-activated cell sorting according expression of Ki67 and DNA amount by 4,6-diamidino-2-phenylindole (DAPI). Cell cycle profile of human Lin^- transduced by control (white bars), WT C/EBPα (dark grey), N-ter (light grey) and C-ter (black) is shown. Data are from three independent experiments done for triplicate. (**b**) Transduction by different C/EBPα forms increases human Lin^- cell survival. Representative dot-plot representations of DAPI and Annexin V distinguish alive (left square) and dead cells (right square). Means ± s.d. are indicated. (**c**) Quantitative real-time PCR analysis of Bcl-2 in transduced human cells. Sorted transduced cells were analyzed in two independent experiments. Results are expressed as mean ± s.d., with significant differences of $*P \leqslant 0.05$, $**P \leqslant 0.01$ or $***P \leqslant 0.001$.

$CD34^+CD38^+$ were estimated (Figure 5b). We observed a significant reduction of the both $CD34^+CD38^-$ and $CD34^+CD38^+$ compartment in WT, N-ter, C-ter and N-ter/C-ter, whereas in NC-ter group we observed a maintenance of the $CD34^+CD38^-$ compare with control with a concomitant twofold increase in $CD34^+CD38^+$ cells.

Expression of C/EBPα mutants alters human repopulating cells

Finally, we assessed the effect of different *CEBPA* mutants on transplantation potential of human Lin^- after transduction into NOD/SCID/β2. The level of human engraftment in all groups were quite similar ranging from 21.3 ± 4.8 in control, 28.9 ± 10.4 in WT, 26.6 ± 5.8 in N-ter, 28.8 ± 5.8 in NC-ter, 28.3 ± 13.4 in C-ter and 22.2 ± 9.2 in N-ter/C-ter after 8 weeks. The engraftment of the transduced human cells was analyzed at different times points (Figure 6a). In the control group, the percentage of transduced cells fell from 38 to 15% between 4 and 8 weeks. WT *CEBPA* expressing cells were low at 4 and 8 weeks (<1%) reflecting its tumor-suppressor activity. The percentage of N-ter or C-ter transduced cells were comparable (15%) at 4 weeks but in both cases had decreased by 8 weeks (2.5%). The N-ter/C-ter cells were not detected (<0.01%) at either time point demonstrating that all mutants still have some tumor-suppressor activities except potentially for the NC-ter. Indeed, only NC-ter transduced cells were detected at similar levels as the control group (42–22% at 4 and 8 weeks, respectively).

The phenotype of transduced cells was analyzed by fluorescence-activated cell sorting (Figure 6b and Table 1). In control group, there was an equal proportion of myeloid and B cells at

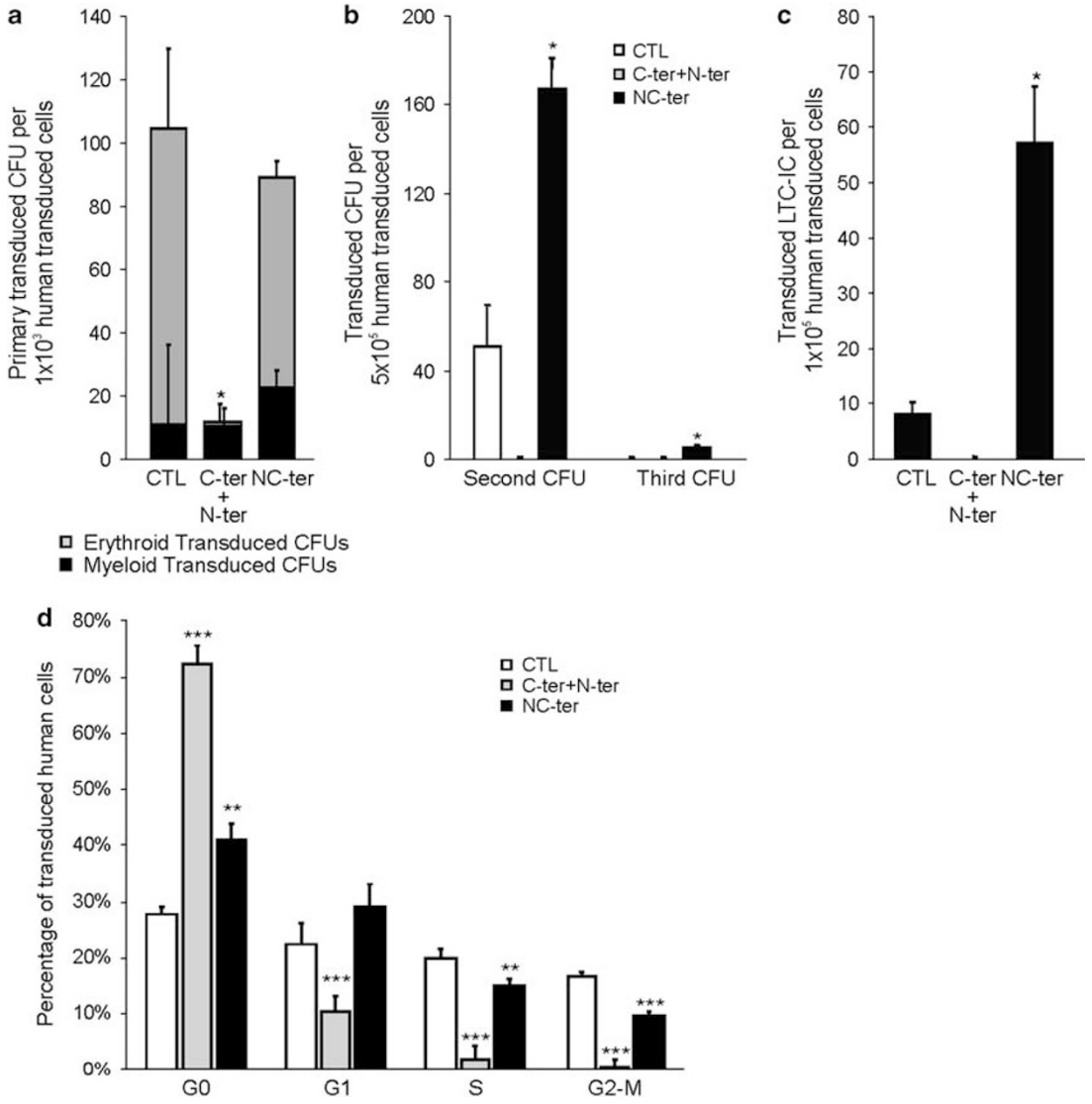

Figure 4. Biallelic and double monoallelic mutations on C/EBPα modifies differently human progenitor compartment and cell cycle. (**a**) Effect of C-ter with N-ter mutated C/EBPα (biallelic mutations) or NC-ter mutated C/EBPα (double monoallelic mutation) in human hematopoietic progenitors were determines by CFU assays. The number of erythroid (grey) and myeloid (black) CFUs in the tranduced cells are presented. (**b**) Serial replating of control, N-ter + C-ter or NC-ter mutated C/EBP transduced progenitors are presented. (**c**) LTC-IC assay to evaluate effect in HSC after expressing C-ter + N-ter or NC-ter C/EBPα. (**d**) Cell cycle profile of control, N-ter + C-ter or NC-ter expressing after 14 days of culture. Results are shown as mean ± s.d., with significant differences of $*P \leqslant 0.05$, $**P \leqslant 0.01$ or $***P \leqslant 0.001$. All the data are from a minimum of triplicates of two independent experiments.

4 weeks, which as expected shifted to lymphoid cells ($hCD19^+$) at 8 weeks. WT C/EBPα expressing cells were mainly myeloid cells at both time points, consistent with the requirement of C/EBPα in inducing human myeloid differentiation *in vivo*. The N-ter C/EBPα demonstrated similar increase in myeloid production at 4 weeks, which disappeared at 8 weeks, contrary to the C-ter, which show no increase in myeloid at 4 weeks but an increase at 8 weeks. NC-ter behaves similarly to control in contrast to N-ter/C-ter construct where cells were not detectable.

At 8 weeks, we further analysis of the type of myeloid cell present and identified granulocytes ($hCD33^{low}/hCD15^+$), granulocytic precursors ($hCD33^+/hCD15^+$) and monocytes ($hCD33^+/hCD14^+$) (Figure 6c). All the constructs except C-ter behave similarly to control (N-ter/C-ter was not studied as no transduced cells were present at 8 weeks). For the C-ter overexpression, we observed contrary to the *in vitro* differentiation assay, an increase in granulocytes at the expense of monocytes and granulocytic precursors.

To test whether we could induce leukemia, and to insure that we were able to transduce long-term repopulating cells with our lentivector constructs, we performed secondary transplants from some of these first recipient mice. Purified human cells from transplanted NOD/SCID/β2 mice were injected (intravenously or intrabone) in new irradiated NOD/SCID/β2 mice and human engraftment analyzed 5–6 weeks later. Human transduced cells were only detected in the control and NC-ter group (Table 1) with a predominance of lymphoid cells ($hCD19^+$) (data not shown). Thus, comparable to control, the NC-ter transduced cells can retain long-term repopulating activity *in vivo*, the rest of C/EBPα mutated forms lose their repopulating capacities, probably via myeloid differentiation and/or cell cycle arrest and thus on their own are unable to transform normal HSC/HPC.

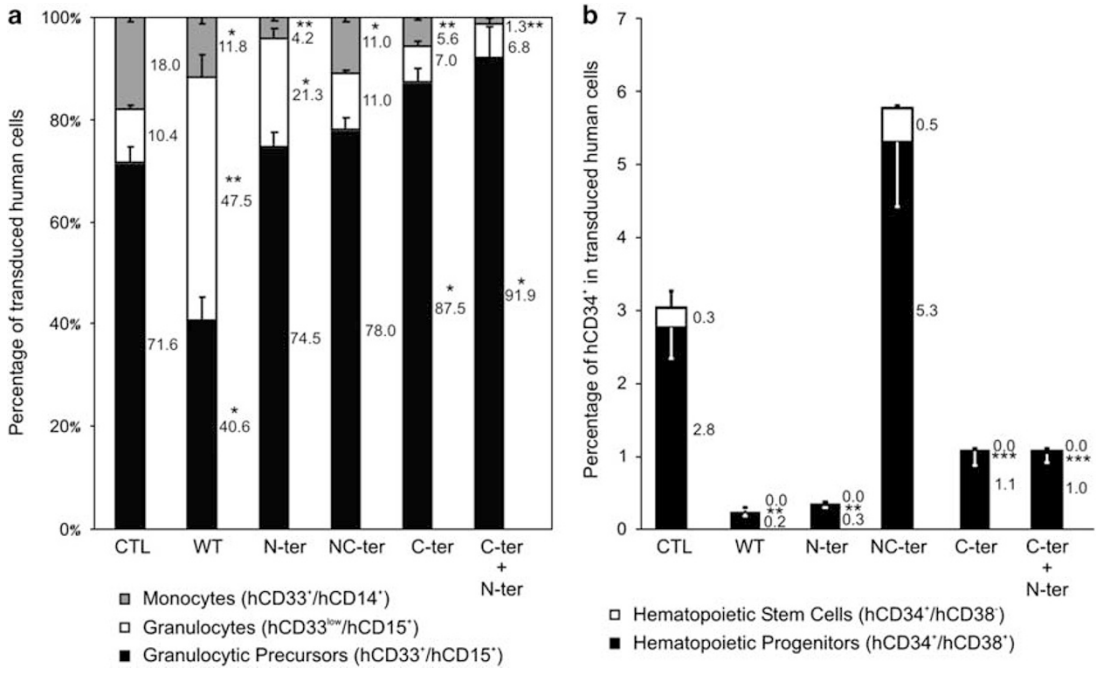

Figure 5. Modification of myeloid differentiation by of WT and mutants C/EBPα (**a**) Human Lin$^-$ cells were cultured in myeloid conditions for 2 weeks. Fluorescence-activated cell sorting analysis evaluated percentage of different populations of myeloid lineage in transduced cells: monocytes (hCD33$^+$/hCD14$^+$, grey), granulocytes (hCD33^{low}/hCD15$^+$, white) and granulocytic precursors (hCD33$^+$/hCD15$^+$, black). (**b**) Percentage of CD34 + CD38 - (white) and CD34 + CD38 + (black) in transduced cells after 2 weeks of culture in myeloid conditions. Percentage of each subset is indicated. Data show triplicates of three independent experiments. Significant differences are indicated by $*P \leqslant 0.05$, $**P \leqslant 0.01$ or $***P \leqslant 0.001$.

DISCUSSION

In our model, overexpression of WT C/EBPα reduced HSC and progenitors *in vitro* and *in vivo*, by inducing terminal myeloid differentiation, preferentially of granulocytic lineage, and arresting the cells in G0, consistent with the block in both granulocytic and monocytic differentiation imposed by C/EBPα silencing.[53] These data further confirmed the tumor-suppressor function of WT CEBPα. These data also indicated that the level of WT C/EBPα is important for the normal hematopoiesis and is similar to other CEBP transcription factor family members whose upregulation accompanying immunoglobulin heavy-chain locus translocations, give rise to B-cell precursor acute lymphoid leukemia.[54,55] The balance between normal and mutated WT C/EBPα may well be a feature of AML development. Indeed, in addition to the mutations in *CEBPA*, C/EBPα expression can be controlled in a number of ways, through the effects of specific fusion proteins,[35] hypermethylation[56] or post-translation modifications.[57]

Although it was initially assumed that all *CEBPA* mutations are associated with a good prognosis favorable outcome now appears to be restricted to patients with biallelic mutation. We therefore chose to evaluate not only N and C-ter alone but also the different combination of double mutants. Mutations had different effects on normal hematopoiesis, which was not wholly surprising given the complex role of C/EBPα in both cell cycle regulation via p21^{WAF}, CDK2–CDK4 interaction and E2F repression,[14,16–18] and on myeloid differentiation.[7–9,11,21] The N-ter *CEBPA* mutations (N-ter) failed to induce an arrest in G0 but seems to block the cell in G1 most likely reflecting the critical role for the N-ter domain in E2F repression.[11] The N-ter alone showed a slight increase in immature myeloid progenitors (as shown by an increase in LTC-IC), a favorable granulocytic differentiation versus monocytic, which is in agreement with recent studies by the Stocking group.[51] However, despite this slight increase in immature myeloid progenitors, only a low long-term engraftment was observed *in vivo*, indicating that the proposed dominant-negative effect of the N-ter mutant is weak, and may merely enrich the pool of myeloid progenitors in which secondary mutations arise and from which the leukemic-initiating cell can emerge. Consistent with this interpretation it is noteworthy that carriers of the germline N-ter mutation develop leukemia after a relatively long latency period.[42] In our case, we were unable to look at long-term effect as after 8 weeks, we could not detected any engraftment of the N-ter overexpressing cells.

It has previously been shown that mice with a disruption of the C-ter domain fail to promote myeloid differentiation.[11] In accordance with these studies, we show that the C-ter mutation arrests cells in G0 and increase the number of myeloid precursors mostly by blocking monocytic differentiation. *In vivo* the C-ter mutation showed a decrease in repopulating capacity compared with control implying that additional lesions are necessary to offer the proliferative advantage needed for these myeloid progenitors in order to acquire leukemic-initiating cell potential.

It appears therefore that N-ter and C-ter mutations alone might not be capable of inducing a full leukemia, which is consistent with the long latency period necessary to obtain overt leukemia described in both the knock-in mouse models[45,46] or in the overexpression experiment.[47] These data are also consistent with clinical findings whereby mutations in *FLT3*/ITD, *NRAS* and *WT1* are coincident with *CEBPA* mutations in AML patients[34,58,59] suggesting that these or other oncogenic events (like *TET2*),[60] are required for the development of the leukemic-initiating cell in a *CEBPA* mutant background.

Despite the capacity of N-ter to maintain more immature progenitors (that is, LTC-ICs) *in vitro* and C-ter to induce a block in terminal differentiation, the combination of the two has no additive effect. The monoallelic NC-ter mutation induce a mild *in vitro* phenotype. Indeed, there was an increase in immature progenitors because of the N-ter mutation, a cell cycle arrest and a modest decrease in monocytic differentiation recurrent of the C-ter mutation. Nevertheless, *in vivo*, the cells behave similarly to

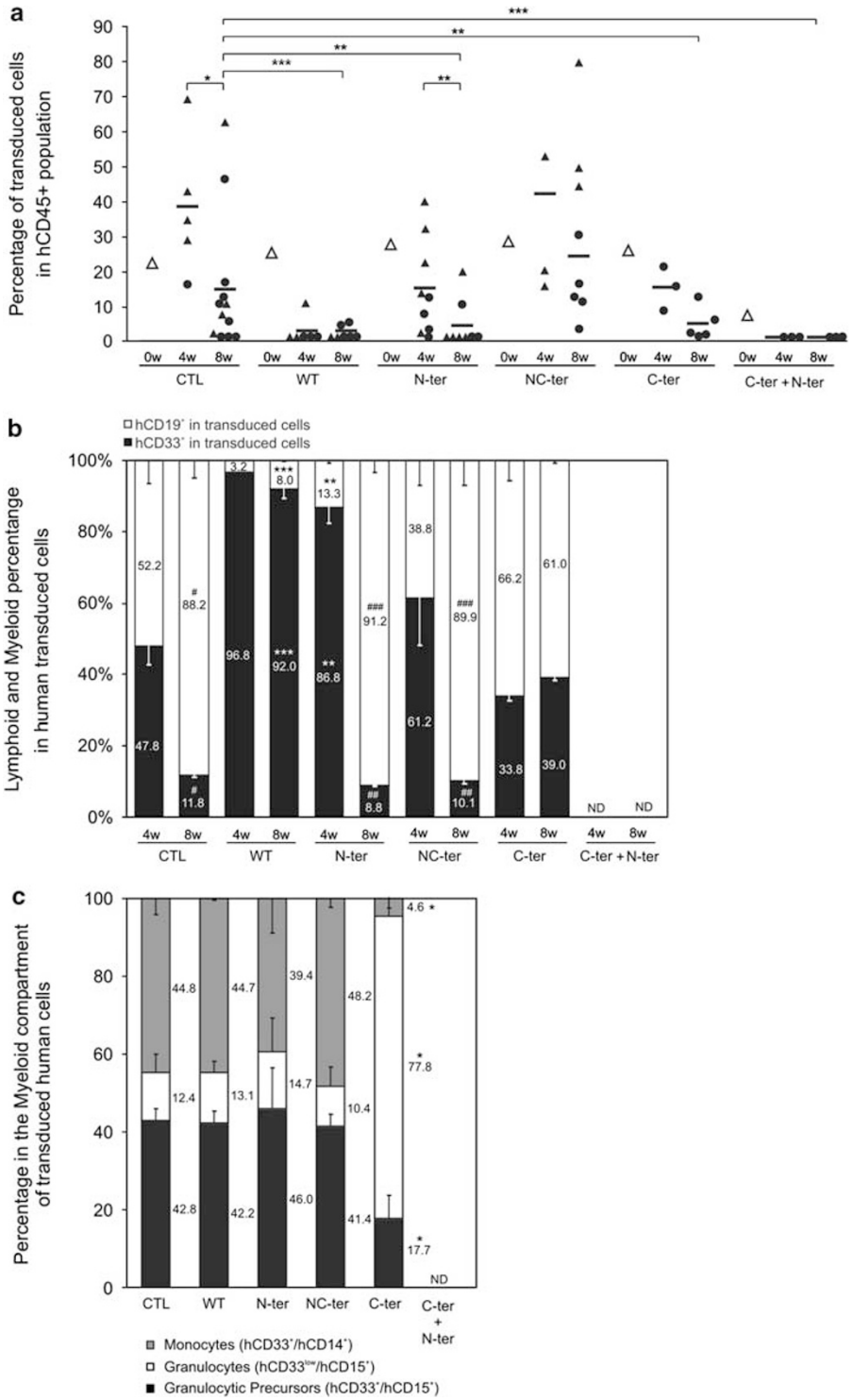

Figure 6. WT and mutants C/EBPα engraftment in immunodeficient mice. (**a**) *In vivo* kinetic of transduced cells in human hematopoietic population. Sub-lethally irradiated NOD/SCID/β2 microglobulin null mice (NOD/SCID/β2) were transplanted with human Lin$^-$ cells after transduction. Percentage of transduced cells in human engraftment was analyzed at different time points by fluorescence-activated cell sorting (FACS). Transduction efficiency of transplanted populations (white triangle), mean of transduced cells percentage at different time points (black dash) and transduced cells percentage of individual mice (black triangle or circle) are shown. Animal analyzed at 4 or 8 weeks by BM aspirations are identified by black triangles; circles indicated that the mice have been culled first and BM cells flushed for the analysis. Data from two independent experiments are shown. (**b**) Hematopoietic lineage analysis of transduced cells *in vivo* at different time points. Bar graph showing percentage of lymphoid (hCD19$^+$, white bars) and myeloid (hCD33$^+$, black bars) transduced cells. Numbers represent myeloid–lymphoid ratio in transduced human cells. (**c**) Percentage of transduced monocytes, granulocytes and granulocyte precursors present in the myeloid compartment of engrafted mice 8 weeks post-transplant. Numbers represent the ratio of each fraction in transduced human cells. Results are shown as mean ± s.d. Data from four independent experiments are shown. Significant differences with the control (CTL) group are indicated by $^*P \leqslant 0.05$, $^{**}P \leqslant 0.01$ or $^{***}P \leqslant 0.001$. Significant differences with 4w analysis are indicated by $^{\#}P \leqslant 0.05$, $^{\#\#}P \leqslant 0.01$ or $^{\#\#\#}P \leqslant 0.001$. ND, not detected; w, week.

Table 1. Summary of secondary transplants with transduced human cells

Mouse	Group	Transplanted hCD45+ (x106) cells from primary recipients		Secondary recipients	
		Doses of $hCD45^+$ (x106) cells	GFP^+ (%)	$hCD45^+$ (%)	$GFP+$ in $hCD45^+$ (%)
1	CTL	1.30	42.3	0.00	
2	CTL	4.00	46.1	0.09	76.70
3	CTL	1.00	7.57	0.31	4.26
4	CTL	1.00	7.57	0.49	4.26
5	CTL	1.00	7.57	0.44	5.08
6	WT	0.90	0.42	0.02	0
7	WT	4.50	0.39	0.20	0
8	WT	4.50	0.39	0.06	0
9	WT	4.50	0.39	0.03	0
10	N-ter	3.35	22.8	0.02	0
11	N-ter	3.35	22.8	0	–
12	NC-ter	5.00	23.6	0	–
13	NC-ter	5.00	23.6	11.70	55.20
14	NC-ter	5.00	23.6	0.12	17.10
15	NC-ter	1.00	4.36	0.63	2.06

Abbreviations: CTL, control; GFP, green fluorescent protein; N-ter, N-terminal; NC-ter, NC-terminal.

control. For the biallelic N-ter/C-ter mutation, myeloid differentiation was promoted without reaching terminal differentiation with the C-ter mutation imposing an arrest in cell cycle. This might provide an explanation for the favorable outcome associated with biallelic mutation pattern. Nevertheless, *in vivo*, these cells fail to give rise to any short- or long-term repopulation. These data suggest thus additional mutations might be present in patients with biallelic N-ter/C-ter mutation observed in familial cases of mutated C/EBPα contrary to the notion that in these patients, the C-ter mutation acts as a second hit model sufficient to promote the full blow leukemia.[12,36]

Although forced overexpression of mutated forms demonstrated distinct phenotype, we could not exclude that residual WT copy present in the umbilical CB had modulated the effects of our overexpression mutant forms. The downregulation of the WT C/EBPα in the original CB by itself is difficult to achieve without interfering with the N-ter and C-ter mutations. However, in our experiments forced expression originated from a viral promoter and expression was considerably higher than endogenous C/EBPα. Nevertheless, it appears that with the biallelic N-ter/C-ter form, the endogenous expression of C/EBPα was increased substantially after induction of myleloid differentiation *in vitro* suggesting a potential compensatory feedback loop. This might explain at least in part the data obtained here. Nevertheless, it is clear that our results showed some clear differences between mouse and human hematopoietic cells related to the effect of mutated C/EBPα. Indeed, we show that by using our constructs, we were able to confirm the results obtained by Kato *et al*.,[47] using the same overexpression strategy demonstrating that the nature of the cells and not the difference in constructs used was responsible for the difference of action.

In conclusion, our data underline the critical role of C/EBPα in human hematopoiesis and demonstrate that C/EBPα mutations (alone or in combination) are insufficient to convert normal human HSC/HPCs into leukemic-initiating cells, although individually each altered normal hematopoiesis in a manner characteristic of leukemic transformation. It provides the first insights into the effects of N- and C-ter mutations acting alone but also of the combined effects of N-ter/C-ter double mutants. Contrary to the results obtained recently where the MLL–ENL fusion protein was able to transform primary human hematopoietic cells[48,49] after a 15 weeks latency, we were not able to propagate the transduced cells *in vivo* long enough for a potential secondary mutation to occur. Indeed after 6 to 8 weeks, no human repopulating ability could be detected indicating the potential limitations of the xenotransplantation model to recapitulate the multistep transformation process of a leukemia to arise from human normal primary HSC/HPCs.[61–63] One question, which is still open is how many mutations do exist in these C/EBPα mutated AML patients and how many are needed to trigger leukemia. Based on how the N- and C-ter mutations behave together, the greater challenge in the future is not merely to address the nature of the individual contribution but how mutations compensate and complement each other's function when partnered together.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

OQ-B designed and performed research, analyzed and interpreted data, performed statistical analysis and wrote paper. SL-LS, EG, YR and JV performed part of the research experiments, TAL provided vital materials and JF interpreted data and wrote paper, DB designed research and analyzed and interpreted data and wrote paper.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)