Differential transcriptional control of hematopoiesis in congenital and cyclic neutropenia patients harboring *ELANE* mutations

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Received: August 7, 2023.
Accepted: October 10, 2023.
Early view: October 19, 2023.

https://doi.org/10.3324/haematol.2023.284033

Abstract

Mutations in the *ELANE* gene, encoding the neutrophil elastase (NE) protein, are responsible for most cyclic neutropenia (CyN) cases and approximately 25% of congenital neutropenia (CN) cases. In CN and in CyN, a median of 2.8% of CD34$^+$ cells were early CD49f$^+$ hematopoietic stem cells (eHSC) that did not express *ELANE* and thus escape from the unfolded protein response (UPR) caused by mutated NE. In CyN, the CD49f$^+$ cells respond to granulocyte colony-stimulating factor (G-CSF) with a significant upregulation of the hematopoietic stem cell-specific transcription factors, C/EBPα, *MLL1, HOXA9, MEIS1*, and HLF during the ascending arm of the cycle, resulting in the differentiation of myeloid cells to mature neutrophils at the cycle peak. However, NE protein released by neutrophils at the cycle's peak caused a negative feedback loop on granulopoiesis through the proteolytic digestion of G-CSF. In contrast, in CN patients, CD49f$^+$ cells failed to express mRNA levels of HSC-specific transcription factors mentioned above. Rescue of C/EBPα expression in CN restored granulopoiesis.

Introduction

Cyclic neutropenia (CyN) and severe congenital neutropenia (CN) are autosomal-dominant inherited disorders of hematopoiesis that markedly differ in disease severity.[1] Mutations in the *ELANE* gene are considered largely responsible for most cases of CyN and CN. Because several identical *ELANE* mutations have been identified in both groups of patients,[2] genotyping alone is insufficient to establish a clinical diagnosis. CyN is characterized by regular oscillations of peripheral blood neutrophils from nearly normal to severely low counts, generally within 21-day cycles. Absolute neutrophil count (ANC) at nadir is often less than 200/mm^3 and is associated with fever, mouth ulcers, pharyngitis, sinusitis, and in some cases more severe infections with gram-negative bacteria.[3] By contrast, CN is characterized by recurrent, severe infections that develop in the first months of life, with ANC persistently less than 200/mm^3.[1,4] The availability of recombinant human granulocyte colony-stimulating factor (G-CSF) for clinical use in CN since 1987 and its successful application to CyN patients has changed the outcome for these patients, providing a markedly improved quality of life.[4-7] However, during the course of the disease, 53% of CN patients who harbor *ELANE* mutations and 17% of CyN patients acquire mutations in the gene for the G-CSF receptor, *CSF3R*.[1,8] Together with subsequently acquired mutations in, e.g., *RUNX1, CSF3R* mutations precede the development of myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML).[8-11] The risk of developing leukemia in CN is considered to be 20%.[10] In CyN patients, G-CSF treatment does not abrogate the cycling of neutrophil counts but increases the amplitude of neutrophil counts during the cycle, leading to a shorter period of severe neutropenia and a reduction in the cycle length from 21 days to approximately 14 days.[5] This suggests an influence of G-CSF on the neutrophil cycling pathomechanism. There are reported cases of AML also in CyN patients.[11]

Recently, we and others reported that inhibition of the

proliferation and differentiation of hematopoietic stem and progenitor cells (HSPC) from CN patients harboring *ELANE* mutations is caused by an enhanced unfolded protein response (UPR) in the endoplasmic reticulum (ER) instigated by misfolded mutant NE protein.[1,12] This enhanced UPR is also recapitulated in induced pluripotent stem cells (iPSC) derived from CN patients with *ELANE* mutations.[13,14] However, the UPR does not equally affect all cells, for instance, the least primed early HSC (eHSC) expressing CD49f (integrin-α6)[15] escape from the damage since they do not express *ELANE*.[16] Most CD34$^+$ cells are lineage-restricted progenitors, and true self-renewing CD49f$^+$ eHSC are rare.[17,18] CD49f plays an important, conserved role in stem cell biology that has been reaffirmed by its importance in maintaining the self-renewal of stem cells in more than 30 tissues.[19]

The transcriptional control of differentiation from eHSC to primed myeloid cells is mainly regulated by C/EBPα.[20,21] Knockout of the *cebpa* gene or its 137 kb upstream enhancer in mice showed two major findings: (i) neutropenia in bone marrow (BM) and peripheral blood (PB); (ii) decrease in long-term HSC (LT-HSC) numbers.[22] In co-immunoprecipitation experiments, Collins *et al*. showed that C/EBPα is an essential collaborator of HOXA9 and MEIS1.[23] In addition, C/EBPα regulates the expression of the histone H3 lysine 4 (H3K4) methyltransferase MLL1, which, in turn, coordinates self-renewal, proliferation, and lineage-specific gene expression in HSC.[24] MLL1 dynamically and selectively regulates *Hox* gene expression.[25] Thus, C/EBPα induces the expression of HOXA9/MEIS1 and subsequent proliferation and differentiation of HSC directly or indirectly via MLL1. Combining gene expression analysis with genome-wide assessment of C/EBPα binding and epigenetic configurations, Hasemann *et al*. showed that C/EBPα modulates the epigenetic states of genes important for HSC function.[26] Another study also demonstrated that C/EBPα positively regulates HSC self-renewal, protects adult HSC from apoptosis, and maintains their quiescent state.[27]

In one of our earlier studies on the pathomechanism of CN, we demonstrated that the expression of C/EBPα is greatly reduced in the arrested promyelocytes of CN patients but normal in CyN patients.[28] We found that C/EBPα is directly regulated by the transcription factor lymphoid enhancer-binding factor 1 (LEF-1).[28] The significant reduction of C/EBPα in CN has been confirmed in iPSC derived from CN patients.[13,29]

The targets of C/EBPα, HOXA9 and MEIS1, are homeodomain transcription factors that are co-expressed in the most primitive eHSC subpopulation (i.e., CD49f$^+$). Their expression induce robust self-renewal of eHSC and expansion of the HSC pool.[30-32] Diminished HOXA9 function results in a reduction in multilineage long-term repopulating ability and diminished numbers of peripheral blood leukocytes in mice,[33] whereas overexpression of HOXA9 in bone marrow cells induces stem cell expansion.[34] HOXA9 might be the primary physiological determinant of HSC self-renewal.[35]

Using single-cell transcriptomes of HSPC from cord blood, adult bone marrow, and fetal liver to search for human HSC markers with self-renewal capacity, Lehnertz *et al*. identified the master transcription factor HLF as one of the most selectively expressed genes in human HSC and proposed *HLF* as the defining gene of the human HSC state.[36] Giladi *et al*. also identified HLF as the most highly enriched transcription factor in HSC.[37]

Here, we demonstrate that the defective expression of C/EBPα, its targets MLL1, HOXA9, MEIS1, and HLF in CN but not in CyN discriminate between both disorders. During the ascending arm of the CyN cycle, G-CSF induced the proliferation of CD49f$^+$ eHSC and their differentiation into more mature CD34$^+$CD49f$^-$ cells, which respond to the transcription factor C/EBPα to differentiate into neutrophils. Moreover, during the downward arm of the cycle, NE released from neutrophils at the peak of the cycle causes negative feedback through proteolytic digestion of G-CSF, again causing neutropenia.

Methods

Patient samples

Five healthy donors, 13 CyN harboring *ELANE* mutations, and 13 *ELANE*-CN patients were used (*Online Supplementary Table S1*). All patients were treated with subcutaneous injections of Neupogen. BM samples of CN and CyN patients were collected in accordance with an annual follow-up recommendation of the SCNIR and the EHA.[38] The study was conducted under the approval of the Ethical Board of the Medical Faculty, University of Tübingen. Written informed consent was obtained from all participants.

Isolation of human CD34$^+$ cells

Human CD34$^+$ cells were isolated from BM mononuclear cells using Ficoll gradient centrifugation followed by magnetic bead separation using EasySep™ human CD34$^+$ selection kit II (STEMCELL Technologies, #17856).

Multicolor fluorescence-activated cell sorting analysis

Cells were washed with ice-cold phosphate-buffered saline (PBS) and stained with corresponding antibodies in PBS containing 2% fetal bovine serum and 0.02% sodium azide. We used: mouse anti-human CD38 (BD, #563964), mouse anti-human CD34 (BD, #348811), rat anti-human CD49f (BD, #563271), mouse anti-human CD90 (BD, #562685), and mouse anti-human CD45RA (BD, #560673). For intracellular NE protein analysis, cells were subsequently fixed and permeabilized using the Fix&Perm kit (Nordic Mubio, #GAS-002FOC), followed by incubation with a rabbit anti-human NE (abcam, #ab131260) antibody for 15 minutes (min) at room temperature, and subsequent incubation with a goat polyclonal secondary antibody to rabbit IgG-H&L (Alexa Fluor 488) (abcam, #ab150077) for 15 min at room

temperature. Cells were fixed with 0.5% paraformaldehyde and measured using a BD LSRFortessa.
Additional methods are available in the *Online Supplementary Appendix*.

Results

Dynamic cycle-dependent expression of C/EBPα and its target genes in hematopoietic stem cells from cyclic neutropenia patients

We first measured the percentage of CD34⁺CD49f⁺ early HSC in the BM of CN (N=3), CyN (N=8) and healthy donors' (HD) cord blood (CB) cells (N=6) by flow cytometry. Since the percentages of CD49f⁺ cells from CB CD34⁺ cells are comparable to the percentages of CD34⁺CD49f⁺ cells from the BM of children and young adults[39] we used them for comparison. We observed a median of 2.8% (range, 1.2-4.6%) and 2.8% (range, 1.7-7.3%) CD34⁺CD49f⁺ eHSC in CN and CyN patients` BM, respectively. HD CB cells contained 7.3% (range, 3.4-9.3%) CD34⁺CD49f⁺ eHSC. In CyN patients the percentage of CD49f⁺ cells was independent of the granulocyte cycle stage (Figure 1A).
We further evaluated possible cycle-dependent changes

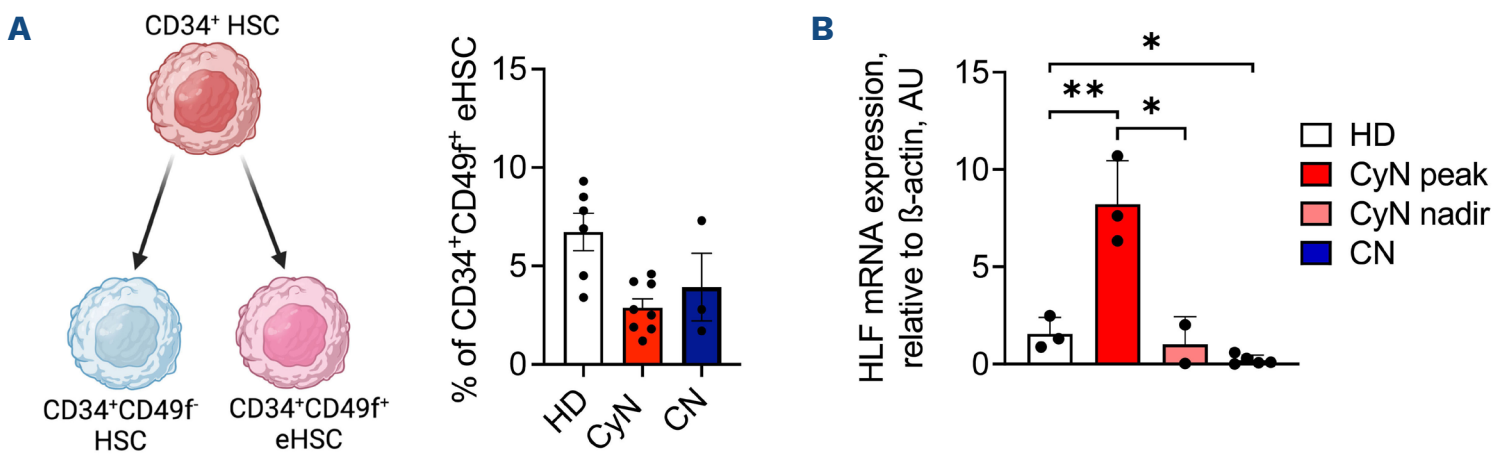

Figure 1. Cycle-dependent dynamics of transcription factors expression in hematopoietic stem and progenitor cells of cyclic neutropenia patients. (A) Left: schema of early CD49f⁺ hematopoietic stem cell (eHSC) specification, where eHSC with self-renewal capacity, but not more committed HSC, express the CD49f marker. Right: percentage of CD34⁺CD49f⁺ cells as assessed by fluorescence-activated cell sorting. Mean ± standard error of the mean are shown. (B, C) mRNA expression of the indicated genes in CD34⁺ bone marrow cells from healthy individuals (HD), cyclic neutropenia (CyN) patients at peak and nadir of neutrophil counts, and congenital neutropenia (CN) patients, measured by quantitative real-time polymerase chain reaction. Data were normalized to β-actin and are represented as mean ± standard deviation in duplicates. Unpaired *t* test, **P*<0.05, ***P*<0.01; ****P*<0.001.

in mRNA expression levels of the stemness marker HLF as well as the transcription factor C/EBPα and its target genes MEIS1, HOXA9, and MLL1, in CD34⁺ HSPC of CyN and CN patients, and healthy individuals. We found that HLF mRNA levels were upregulated at the CyN peak of neutrophil counts, as compared to CyN nadir where HLF levels were comparable to healthy individuals. In CN, HLF expression was significantly downregulated as compared to healthy individuals (Figure 1B; *Online Supplementary Table S2*). C/EBPα mRNA expression was also upregulated at the peak of the cycle and reduced to the levels of healthy individuals at the nadir of the cycle. As already published, we observed almost no C/EBPα expression in CN patients (Figure 1C). Next, we aimed to assess C/EBPα activity during the CyN cycle by measuring the expression of its target genes. Using the UCSC Genome Browser and data from the ReMap Atlas of regulatory regions,[40] we confirmed previous reports that showed C/EBPα binding to the promoters and upstream regulatory regions of HLF, MEIS1, and MLL1 (*Online Supplementary Figure S1A*). In the context of HOXA9 regulation by C/EBPα, it is known that C/EBPα acts as part of a multiprotein complex that contains HOXA9 to promote HOXA9 mRNA expression in an autocrine manner.[41] We found that mRNA levels of all selected factors were strongly upregulated at the peak of the cycle in CyN compared to healthy individuals and were only moderately expressed at the nadir (Figure 1C). In CN, expression of all these factors was diminished when compared to healthy controls (Figure 1C).

Early CD49f⁺ hematopoietic stem cells lack *ELANE* mRNA and neutrophil elastase protein expression

Knowing that CyN and CN patients harbor *ELANE* mutations, and that mutated elastase protein may damage hematopoiesis, we evaluated at which stage of hematopoietic differentiation *ELANE* mRNA and protein are expressed in physiological conditions in healthy individuals. We found the absence of NE protein expression in bone marrow CD49f⁺ eHSC of three HD (Figure 2A). Single-cell RNA-sequencing analysis of CD34⁺ HSC from two HD revealed that only 10% of CD49f⁺ (ITGA6⁺) eHSC co-expressed *ELANE* mRNA (58/560 cells), 90% of the cells express either *ELANE* or *ITGA6*, but not both (Figure 2B). The discrepancy in the *ELANE* mRNA and NE protein expression levels can be explained by different kinetics and efficiencies of *ELANE* mRNA transcription and NE protein translation. The most important finding is that NE protein is absent in CD49f⁺CD34⁺ cells.

Granulocytic differentiation of cyclic neutropenic induced pluripotent stem cells is comparable to healthy donor cells

We further generated induced pluripotent stem cells (iPSC) from peripheral blood mononuclear cells (PBMNC) of three *ELANE*-CyN patients. iPSC showed reliable expression levels of mRNA (*SOX2, NANOG*, and *DNMT3A*) and cell surface proteins (*TRA-1-60* and *SSEA4*) that are markers of pluripotency (*Online Supplementary Figure S2A, B; data not shown*). They also had the potential to spontaneously differentiate into ectoderm and endoderm (*Online Supplementary Figure S2C*). We applied *ELANE*-CN iPSC, already available in the laboratory,[14,29] to compare their hematopoietic and granulocytic differentiation potential between CN and CyN using the embryoid body (EB) based hematopoietic differentiation.[42] Flow cytometry analysis of iPSC-derived hematopoietic cells obtained at day 14 of differentiation revealed that the majority of HSC were early HSC (CD34⁺CD43⁺) in CN iPSC (Figure 3A), which corresponded to up to 50% CD49f⁺ eHSC in CyN and HD iPSC (Figure 3B). On day 28 of differentiation, the percentage of mature neutrophils was severely reduced in iPSC derived from CN patients while, interestingly, the proportion of neutrophils produced by CyN iPSC was comparable to HDr iPSC (Figure 3C). The mRNA expression levels of C/EBPα and MEIS1 in iPSC-derived HSC collected on day 14 of differentiation recapitulated the results obtained from primary HSC. C/EBPα levels were severely downregulated in HSC derived from CN iPSC as compared to CyN HSC (Figure 3D). MEIS1 levels were markedly higher in CyN HSC as compared to CN and HD HSC (Figure 3E, left). Intriguingly, HOXA9 expression in iPSC-derived HSC was very low in all samples (Figure 3E)

A

	HSPC CD34⁺CD38⁻	HSC CD34⁺CD38⁻ CD45RA⁻ CD90⁺	CD49f⁺ HSC CD34⁺CD38⁻ CD45RA⁻ CD90⁺CD49f⁺	MPP CD34⁺CD38⁻ CD45RA⁻ CD90⁻	CMP/MEP CD34⁺CD38⁺ CD45RA⁻
HD 1	7,29	1,34	0,0	62,1	99,6
HD 2	13	0,8	0,0	66,8	97,8
HD 3	15,8	1,0	0,0	43,3	97,6

B

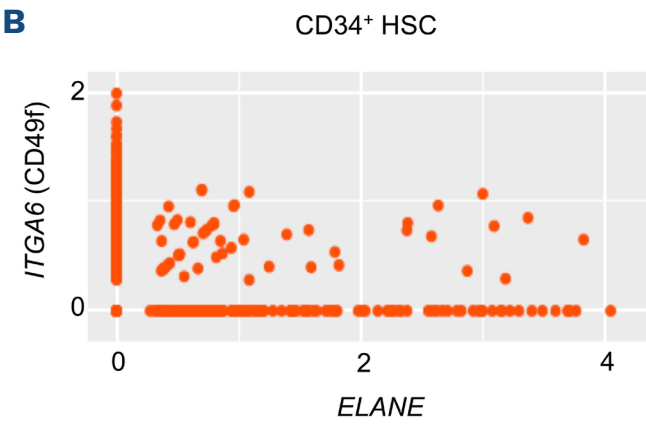

Figure 2. *ELANE* mRNA and protein expression in hematopoietic stem cell subsets. (A) Percentage of neutrophil elastase (NE) expressed in different human stem and progenitor cell subsets in the bone marrow of 3 healthy donors (HD) measured by flow cytometry. (B) Co-expression plot of ITGA6 (y-axis) and ELANE (x-axis) expression in single CD34⁺ hematopoietic stem cells (HSC) from 2 HD. Values plotted were normalized to read counts. MPP: multipotent progenitors; CMP: common myeloid progenitors; MEP: megakaryocytic-erythroid progenitors.

and more than ten times lower than in primary cells from e.g., CyN patients (Figure 1E). This is in agreement with reports from other laboratories, that HOXA9 expression in iPSC or in ESC is very low.[43] Therefore, it is hard to make any solid conclusions based on these data. However, there is a clear difference in the expression levels of C/EBPα, and MEIS1 and thus myeloid differentiation between CyN and CN iPSC.

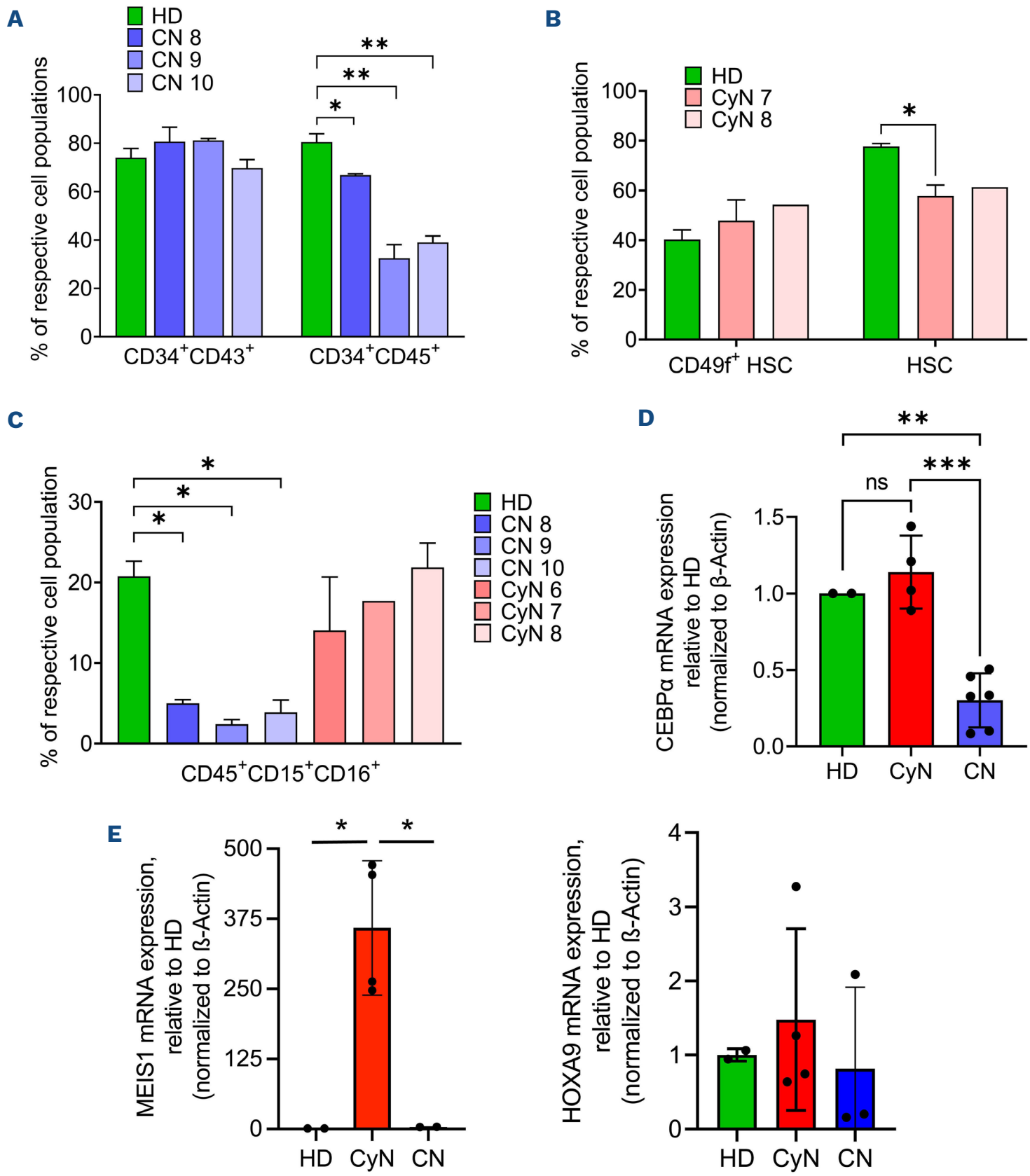

Figure 3. Evaluation of granulocytic differentiation of induced pluripotent stem cells derived from congenital neutropenia and cyclic neutropenia patients harboring *ELANE* mutations. (A) Evaluation of early hematopoietic differentiation of induced pluripotent stem cells derived (iPSC) derived from 1 healthy donor (HD) and 3 congenital neutropenia (CN) patients using flow cytometry. Data are represented as mean ± standard deviation (SD) from 2 independent experiments, in duplicates; unpaired *t* test, **P*<0.05; ***P*<0.01. (B) Evaluation of early hematopoietic differentiation of iPSC derived from 1 healthy donor (HD) and 2 cyclic neutropenia (CyN) patients using flow cytometry. Data are represented as mean ± SD from 2 independent experiments, in duplicates, Unpaired *t* test, **P*<0.05. (C) Granulocytic differentiation of iPSC generated from 1 HD, 3 CN and 3 CyN patients. The percentage of $CD45^+CD15^+CD16^+$ neutrophils was assessed by flow cytometry of suspension cells on day 28 of embryoid body-based differentiation. Data are represented as mean ± SD from 2 independent experiments, each in duplicates. Unpaired *t* test, **P*<0.05. (D, E) Relative mRNA expression of C/EBPα (D), MEIS1 and HOXA9 (E) in $CD34^+$ cells derived from iPSC of CyN patients and CN patients to HD cells measured by quantitative real-time polymerase chain reaction. Data were normalized to β-actin and are represented as mean ± SD in duplicates. Unpaired *t* test, **P*<0.05.

Rescue with C/EBPα restored defective granulopoiesis in primary hematopoietic stem cells of two *ELANE* congenital neutropenia patients

We next tested whether restoration of the diminished C/EBPα expression in primary CD34$^+$ HSPC of CN patients harboring *ELANE* mutations would affect their granulocytic differentiation *in vitro*. We used an RFP$^+$-lentiviral vector to overexpress C/EBPα in CD34$^+$ HSPC obtained from three *ELANE*-CN patients and then differentiated transduced cells to neutrophils in liquid culture (Figure 4A). After 14 days of differentiation, we found a reduction in granulocyte-monocyte progenitor cells (GMP/MB: RFP$^+$CD45$^+$CD33^{high}), while promyelocytes/myelocytes (PM/MY: RFP$^+$CD45$^+$CD33^{DIM}CD16$^-$) and band cells/polymorphonuclear neutrophils (BC/PMN: RFP$^+$CD45$^+$CD33^{DIM}CD16$^+$) were markedly higher in C/EBPα transduced cells as compared to RFP control (Figure 4B; *Online Supplementary Figure S3A*). Cytospin morphology of one *ELANE*-CN patient confirmed the improved granulocytic differentiation in C/EBPα transduced cells, as compared to RFP control (Figure 4C).

Strong correlation of cyclic neutrophil elastase plasma levels with absolute neutrophil count in a cyclic neutropenia patient

Finally, we investigated whether the cyclic behavior of NE protein expression is also observed in CyN plasma. We collected blood samples from one CyN patient over three cycles and assessed NE values using a NE-specific enzyme-linked immunosorbent assay (ELISA). This assay revealed a dramatic difference in NE levels according to the phase of the cycle, with high expression during the cycle peak and very low levels at the cycle nadir (Figure 5A). NE protein levels strongly correlated with ANC during the cycles (Figure 5A, B). The NE levels in the plasma of three healthy individuals not treated with G-CSF were 66.98+1.94 ng/mL and of three individuals treated with 5 mg/kg/day of G-CSF for 3 days were 374.3+123.0 ng/mL (Figure 5A). In order to prove the cycle-dependent correlation between NE levels and ANC, more patients should be analyzed in the future.

Discussion

By searching for the different pathomechanisms of CN and CyN, we found that C/EBPα expression discriminates between CN and CyN. C/EBPα, one of the key factors in granulopoiesis,[20] was greatly reduced in CN, but not in CyN. In addition to C/EBPα, mRNA expression levels of its target genes *MLL1, MEIS1, HOXA9*, and *HLF* were substantially decreased, or even absent, in CD34$^+$ cells from CN patients

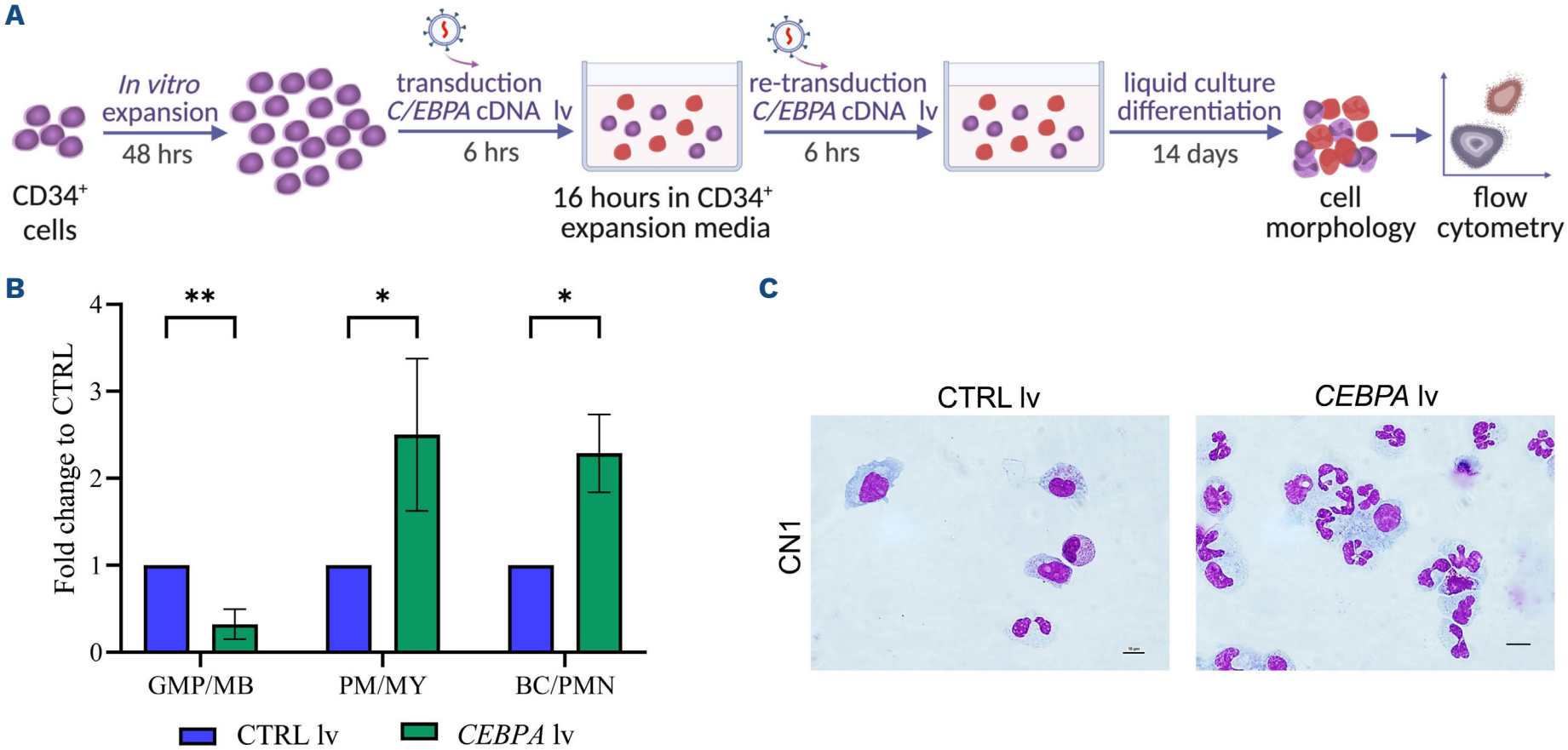

Figure 4. Ectopic expression of C/EBPα rescues neutrophilic differentiation of CD34$^+$ hematopoietic stem and progenitor cells of congenital neutropenia patients. (A) Schematic of the experimental procedure: CD34$^+$ hematopoietic stem and progenitor cells (HSPC) of 3 *ELANE* congenital neutropenia (CN) patients were expanded *in vitro*, transduced with lentivirus particles containing C/EBPα cDNA or a control (CTRL) virus with the red fluorescent marker RFP, and differentiated in liquid culture for 14 days. (B) Flow cytometry analysis of granulocyte-monocyte progenitors/myeloblasts (GMP/MB; RFP$^+$CD45$^+$CD33^{high}), promyelocytes/myelocytes (PM/MY; RFP$^+$CD45$^+$CD33^{DIM}CD16$^-$) and band cells/polymorphonuclear cells (BC/PMN; RFP$^+$CD45$^+$CD33^{DIM}CD16$^+$) at day 14 of differentiation. Data are represented as fold-change increase to control RFP transduced cells and as mean ± standard deviation, in duplicates. Unpaired *t* test, *P<0.05, **P<0.01. (C) Representative images of May-Grunwald-Giemsa-stained preparations of differentiated cells on day 14 of culture (60X magnification, scale bars =10 μm).

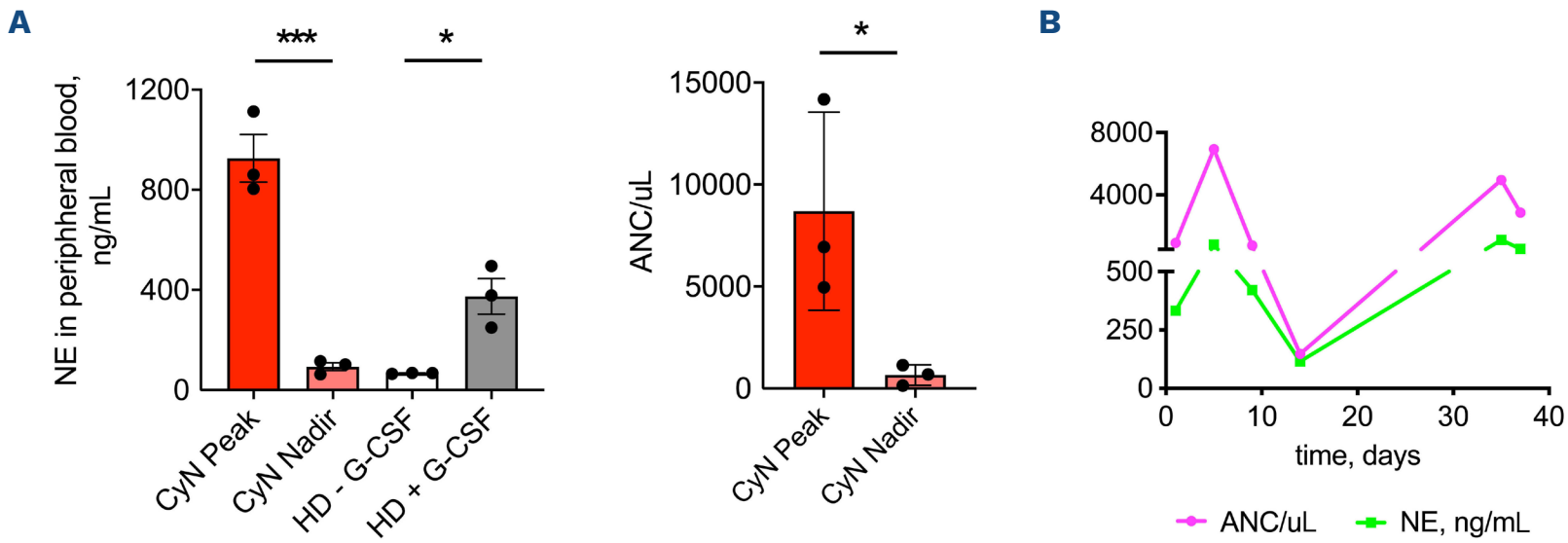

Figure 5. Correlation of neutrophil elastase plasma levels and absolute neutrophil count in cyclic neutropenia cycle. (A) Neutrophil elastase (NE) plasma levels (left panel) assessed by enzyme-linked immunosorbant assay and absolute neutrophil counts (ANC) (right panel) in peripheral blood at peaks and nadirs of neutrophil cycles of 1 cyclic neutropenia (CyN) patient over 3 cycles. NE plasma levels were also evaluated in healthy donors (HD) treated or not with recombinant human granulocyte colony-stimulating factor (rhG-CSF). Data are represented as mean ± standard deviation. Unpaired *t* test, **P*<0.05, ****P*<0.001. (B) Time course of NE levels and ANC in 1 CyN patient.

compared with CD34$^+$ cells from healthy controls (Figure 1C). Indeed, self-renewal, proliferation, and differentiation of HSC are dependent on the epigenetic activation, e.g,. by histone-acetylation of transcription factors such as C/EBPα, the C/EBPα-target genes MLL1, MEIS1, and HOXA9[23-25,30,31,33,35] and HLF, all of them abrogated in CN. We need to emphasize, that we were able to acquire a relatively small number of patient samples for this investigation and further studies should be performed on large patient cohorts.

The severely diminished expression of HSC-specific transcription factors CEBPA and MEIS1 in CN, as compared to CyN, could be recapitulated and confirmed in iPSC established from these group of patients. Intriguingly, HOXA9 was expressed at least ten times lower in iPSC-derived HSC than in primary CD34$^+$ cells. The low expression of HOXA9 in iPSC or in ESC was already reported by others[43] supporting our data in Figure 3E. Ramos-Meja *et al*. identified HOXA9 as the most downregulated gene in hESC-derived HSPC as compared with CB-derived CD34$^+$ cells, HOXA9 expression was 60-fold lower in differentiating day 15 EB than in CB-CD34$^+$ cells. The principal problem of low HOXA9 expression in iPSC cells was not the subject of our study. The low expression of C/EBPα in iPSC derived from CN patients was already reported.[13]

In contrast to CN patients, at the peak of the cycle, CD34$^+$ cells from CyN patients, which included CD49f$^+$ eHSC, expressed C/EBPα, MLL1, MEIS1, HOXA9, and HLF at levels significantly higher than those in healthy controls (Figure 1B, C). Increased expression of these stem-cell-specific transcription factors during the ascending arm of the neutrophil cycle in CyN induces self-renewal, proliferation, and differentiation of CD49f$^+$ eHSC, which give rise to more mature HSC, myeloid progenitor cells, and mature neutrophils. The high expression of HLF at the peak of the cycle (Figure 1B) ensures the high self-renewal capacity of the CD49f$^+$ eHSC in CyN and can be used as a biomarker for the presence of self-renewing, naïve HSC. Indeed, HLF is one of the most selectively expressed genes in human HSC with self-renewal capacity and the defining factor of the human HSC state.[36,37] Intriguingly, the same *ELANE* mutation can cause CN and CyN. We demonstrated that the UPR-induced inhibition of proliferation and differentiation of HSC reported in CN is incomplete in CyN[16] reflecting the fact that some eHSC escape from UPR-related damage. In this study, we showed that these escaper cells are CD34$^+$CD49f$^+$ eHSC. The asymmetrical self-renewal of CD34$^+$CD49f$^+$ eHSC gives rise to equal numbers of CD49f$^+$ eHSC and committed CD34$^+$CD49f$^-$ HSC that differentiate to progenitor cells and ultimately to mature neutrophils.[17] CD49f$^+$ cells are eHSC with the capacity for self-renewal that only exist at low frequencies within the CD34$^+$ cell population.[15,16] The CD49f$^+$ escaper cells in CN and in CyN are not affected by UPR, since they do not express *ELANE* but respond to G-CSF.[15,46] Indeed, single-cell RNA-sequencing analyses of individual CD34$^+$ cells revealed that *ELANE* is not co-expressed with integrin α6 (CD49f antigen), indicating that CD49f$^+$ eHSC do not express *ELANE* (Figure 2B). Additionally, we found no NE protein in CD49f$^+$ eHSC by FACS analysis (Figure 2A). Avellino *et al*. also reported that *ELANE* is not expressed in LT-HSC,[22] further supporting our hypothesis that escaper eHSC are not affected by the UPR. In CN, CD49f$^+$ eHSC are not capable of asymmetric division, proliferation, and differentiation due to the lack of hematopoiesis-specific transcription factors, C/EBPα, MLL1, MEIS1, HOXA9, and HLF. However, rescue of C/EBPα expression by ectopic expression of C/EBPα, cDNA in CD34$^+$ cells of three *ELANE* CN patients led to strongly improved differentiation to neutrophils *in vitro*, confirming the crucial role of C/EBPα in the pathomechanism of CN and in discriminating between CN and CyN.

During the downward arm of the cycle, there is a decrease

in HSC-specific transcription factor expression caused by negative feedback mediated by an inhibitor/s released by mature neutrophils at the peak of the cycle. Early reports suggested that, in CyN, disruption of such an autoregulatory feedback loop would explain cycling.[45,46] Horwitz *et al*. favored a feedback model in which mature neutrophils elaborate an inhibitor of myelopoiesis whose concentration depends upon the number of neutrophils present.[47] Indeed, we were able to demonstrate that the concentration of NE in CyN correlates with the number of neutrophils in one CyN patient (Figure 5A, B), suggesting that the NE released from neutrophils at the peak of the cycle is a strong candidate mediator of the negative feedback loop. This correlation needs to be confirmed by the evaluation of more patients in the future. NE is already known to provide feedback regulation of granulopoiesis through direct proteolytic action on G-CSF and the G-CSF receptor.[48,49] We further demonstrated that treatment with NE digests human recombinant G-CSF.[50] Li and Horwitz hypothesized that the CN phenotype is unlikely to result from haploinsufficiency in the proteolytic activity of mutated NE toward native substrates.[51] Their study also proposed the "chalone" hypothesis, in which neutrophils homeostatically regulate their production by inhibiting granulopoiesis. Efforts to track down the chalone led to the purification of NE protein.[52] The relatively high numbers of neutrophils at the peak of the cycle release NE causing proteolytic digestion of G CSF. Intriguingly, in CN, levels of NE in mature neutrophils and in plasma were decreased, whereas in CyN they were comparable to healthy individuals,[53] which is dependent on the cycle stage (Figure 5A, B). Therefore, there is a sufficient amount of proteolytically active NE in the plasma of CyN patients, especially at the peak of the cycle, to promote the digestion of G-CSF. Indeed, Watari *et al*. showed G-CSF levels below the detection limit at the peak of the cycle and 165 pg/mL at the nadir of the cycle in a patient with CyN.[54] The lack of biologically active G-CSF at the peak leads to a decrease in the expression of transcription factors in CD49f$^+$ eHSC (escaper cells), which is normally triggered by G-CSF. The amount of NE released by neutrophils at the peak is sufficient to abrogate the proliferation and differentiation of CD49f$^+$ eHSC. Moreover, the majority of NE-expressing, more mature CD34$^+$CD49f$^-$ cells are affected by the UPR.

In healthy individuals, the NE protein released by neutrophils and the degree of proteolytic digestion of G-CSF is not sufficient to completely block the proliferation of the relatively high number of CD34$^+$ cells but instead functions only as an autoregulatory feedback loop for granulopoiesis. At the nadir of the cycle, high UPR activity in CD34$^+$ cells containing CD49f$^+$ eHSC, as measured by elevated ATF6, BiP, and PERK expression,[16] again leads to the damage of the newly generated NE-expressing CD34$^+$ HSPC. Because of its low release at the nadir attributable to the low neutrophil counts, NE no longer affects G-CSF levels, and thus G-CSF is available for the induction of proliferation and differentiation of the remaining CD49f$^+$ eHSC during the ascending arm of the cycle. The reduction in the cycle length from 21 to 14 days upon G-CSF therapy might be attributable to G-CSF-induced increases in the proliferation of CD49f$^+$ eHSC during the ascending arm of the cycle. Indeed, CD49f$^+$ eHSC can respond to treatment with G-CSF.[46] Therefore, the cycling of neutrophils is caused by cycling G-CSF-triggered transcriptional activities of CD49f$^+$ eHSC, which are not affected by the UPR. Therefore, high levels of the stem cell factors, HLF, C/EBPα, MLL1, HOXA9, and MEIS1, at the peak of the cycle induce proliferation and self-renewal of HSC which differentiate to progenitor cells and neutrophils.

In CN, G-CSF is unable to induce the proliferation of CD34$^+$ cells because they are impacted by the UPR and the CD49f$^+$ cells fail to express stem cell-specific transcription factors. This disables the generation of sufficient numbers of HSC and neutrophils. CN patients require treatment with high dosages of G-CSF to overcome this UPR-mediated block of granulocytic differentiation. How much G-CSF is required to produce more than 1,000 neutrophils/μL is, therefore, most likely dependent on the degree to which G-CSF is degraded by NE.

In summary, by searching for different molecular pathomechanisms of CyN and CN, we identified that C/EBPα expression and its targets HLF, MLL1, MEIS1, and HOXA9, in CD34$^+$ HSC discriminate between CN and CyN. Whereas in CN patients, expression of these factors was defective or absent, in CyN we observed a cycle-dependent increase in the expression of these five factors, leading to the self-renewal, proliferation, and differentiation of CD49f$^+$ eHSC. Ectopic expression of C/EBPα in CN rescued the differentiation to neutrophils. In CyN, CD49f$^+$ eHSC within the CD34$^+$ cell population escape UPR damage because they do not express *ELANE*. This reveals the high transcriptional activity of CD49f$^+$ eHSC (escaper cells) in response to G-CSF at the peak of the cycle, which ensures their differentiation to sufficient neutrophil numbers. However, the neutrophils at the peak of the cycle release NE causing proteolytic digestion of G CSF leading to a lack of biologically active G-CSF and a subsequent decrease in the expression of transcription factors in CD49f$^+$ eHSC (escaper cells). On the basis of these findings, we propose that the therapeutic options for patients suffering from CN or CyN would be either to inhibit the mutated NE activity with, for example, an elastase inhibitor,[55] or to delete or correct the *ELANE* gene using gene therapy.[56] Another approach could be to treat CN patients with a newly designed G-CSF that is resistant to proteolytic digestion by NE.[50]

Disclosure

No conflicts of interest to disclose.

Contributions

KW designed the study. KW and JS supervised the experimentation and wrote the manuscript. AZ and NB-B performed main experiments and analyzed the data. MR performed FACS analysis. BD, AZa and MN assisted with iPSC experiments. MK and SK performed scRNA-seq data analysis. MK performed

prediction of C/EBPα binding to target genes. KH assisted with qRT-PCR and FACS. CZ and JK provided patients material.

Acknowledgments

We thank K. Hähnel and R. Bernhard for their excellent technical support.

Funding

This work was supported by the Intramural Fortüne program of the Medical Faculty of the UKT (to MK, AZ), Madeleine Schickedanz Kinderkrebsstiftung (to JS, KW, MK, MN), DFG (to JS, AZ), BMBF (to JS, KW, CZ, SK), and the COST Action European Network for Innovative Diagnosis and Treatment of Chronic Neutropenias (EuNet-INNOCHRON) (to KW, CZ, MK, JS). We acknowledge the support by the Open Access Publishing Fund of the University of Tübingen.

Data-sharing statement

All data are available on request from the corresponding author.

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