Induced cell-autonomous neutropenia systemically perturbs hematopoiesis in Cebpa enhancer-null mice

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Abstract:
The transcription factor C/EBPa initiates the neutrophil gene expression program in the bone marrow. Knockouts of the Cebpa gene or its +37kb enhancer in mice show two major findings: (1) neutropenia in bone marrow and blood; (2) decrease in long-term hematopoietic stem cell (LT-HSC) numbers. Whether the latter finding is cell autonomous (intrinsic) to the LT-HSCs or an extrinsic event exerted on the stem cell compartment remained an open question. Flow cytometric analysis of the Cebpa +37kb enhancer knockout model revealed that the reduction in LT-HSC numbers observed was proportional to the degree of neutropenia. Single cell transcriptomics of wild type mouse bone marrow showed that Cebpa is predominantly expressed in early myeloid-biased progenitors, but not in LT-HSCs. These observations suggest that the negative effect on LT-HSCs is an extrinsic event caused by neutropenia. We transplanted whole bone marrows from +37kb enhancer deleted mice and found that 40% of the recipient mice acquired full blown neutropenia with severe dysplasia and a significant reduction in the total LT-HSC population. The other 60% showed initial signs of myeloid differentiation defects and dysplasia when they were sacrificed, suggesting they were in an early stage of the same pathological process. This phenotype was not seen in mice transplanted with wild type bone marrow cells. Altogether, these results indicate that Cebpa-enhancer deletion causes cell autonomous neutropenia, which reprograms and disturbs the quiescence of HSCs, leading to a systemic impairment of the hematopoietic process.

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RUNNING TITLE: Neutropenia causes LT-HSC loss and dysplasia

KEY POINTS:

- \textit{Cebpa} activates granulocytic differentiation in early myeloid biased progenitors but not in LT-HSCs during steady state hematopoiesis
- Unresolved neutropenia caused by \textit{Cebpa} +37kb enhancer deletion disturbs the LT-HSC pool and leads to severe bone marrow dysplasia

KEYWORDS:
CEBPA, Neutropenia, LT-HSC, dysplasia, hematopoiesis
ABSTRACT
The transcription factor C/EBPa initiates the neutrophil gene expression program in the bone marrow. Knockouts of the *Cebpa* gene or its +37kb enhancer in mice show two major findings: (1) neutropenia in bone marrow and blood; (2) decrease in long-term hematopoietic stem cell (LT-HSC) numbers. Whether the latter finding is cell autonomous (intrinsic) to the LT-HSCs or an extrinsic event exerted on the stem cell compartment remained an open question. Flow cytometric analysis of the *Cebpa* +37kb enhancer knockout model revealed that the reduction in LT-HSC numbers observed was proportional to the degree of neutropenia. Single cell transcriptomics of wild type mouse bone marrow showed that *Cebpa* is predominantly expressed in early myeloid-biased progenitors, but not in LT-HSCs. These observations suggest that the negative effect on LT-HSCs is an extrinsic event caused by neutropenia. We transplanted whole bone marrows from +37kb enhancer-deleted mice and found that 40% of the recipient mice acquired full blown neutropenia with severe dysplasia and a significant reduction in the total LT-HSC population. The other 60% showed initial signs of myeloid differentiation defects and dysplasia when they were sacrificed, suggesting they were in an early stage of the same pathological process. This phenotype was not seen in mice transplanted with wild type bone marrow cells. Altogether, these results indicate that *Cebpa*-enhancer deletion causes cell autonomous neutropenia, which reprograms and disturbs the quiescence of HSCs, leading to a systemic impairment of the hematopoietic process.

INTRODUCTION
Pioneering transplantation studies showed that long-term hematopoietic stem cells (LT-HSCs) possess a multi-lineage differentiation potential towards all hematopoietic lineages after myeloablative conditioning. Bone marrow differentiation occurs along a continuum of cellular states, progressing in a trajectory from LT-HSC towards cell-lineage specific progenitors. Hematopoietic stem and progenitor cells (HSPCs) forming these trajectories are interconnected by transcription factor networks that drive differentiation. Despite their role in differentiation, it remains unclear how transcription factors protect HSCs from exhaustion to preserve bone marrow integrity.
The myeloid lineage transcription factor C/EBPα, encoded by Cebpa, has been studied extensively to understand its role in myeloid differentiation. The expression of Cebpa in myeloid cells is specifically controlled by the +37 kb enhancer (+42 kb in humans)\textsuperscript{9,10}. Genetic knockout of Cebpa\textsuperscript{11} or of its +37kb enhancer\textsuperscript{9,12} \textit{in vivo} (both referred to as Cebpa null mice) causes neutropenia concomitant with reduced LT-HSC numbers. These studies describe C/EBPα as one of the major myeloid regulators that interconnects HSCs with myeloid progenitors. In addition, it has been suggested that C/EBPα has a dual role in LT-HSCs: maintaining LT-HSC quiescence by repressing the self-renewal\textsuperscript{13} and proliferative\textsuperscript{14} gene expression programs, while simultaneously priming early myeloid genes\textsuperscript{11}.

One major technical limitation in these studies is the low resolving power of the technologies used to study rare cell types such as LT-HSCs. Bulk genome-wide transcriptomics measures gene expression signatures at the population level, thereby masking the presence of any rare and transient cell state of physiological importance in the bone marrow. This limitation has been overcome by high-resolution single cell technologies combined with lineage tracing or \textit{in vivo} barcoding. Emerging findings from studies in native hematopoiesis\textsuperscript{4,15,16} place LT-HSCs as a separate and an occasional contributing entity to myelopoiesis in contrast with a continuous HSC to myeloid state, therefore questioning the role of Cebpa as a myeloid priming factor in HSCs.

Here we investigated whether LT-HSC loss in Cebpa null mice is the cause or consequence of neutropenia. We hypothesize that either (1) LT-HSCs harboring an active Cebpa locus are lost upon enhancer deletion, leading to myeloid trajectory shutdown and ultimately neutropenia or, (2) myeloid-biased progenitors expressing Cebpa are lost upon enhancer deletion, causing neutropenia, which systematically disturbs and depletes the LT-HSC pool. To address this question, we combined previously published single-cell datasets from wild type bone marrows with bulk-cell transcriptomics from the +37kb enhancer-deleted mice. Furthermore, we transplanted Cebpa enhancer-deleted cells to study the possible systemic effects on hematopoiesis of the host. Using these approaches we conclude that LT-HSCs do not express detectable levels of Cebpa in unperturbed hematopoiesis and Cebpa null induced neutropenia systemically disturbs LT-HSC quiescence, leading to HSC depletion, bone marrow hypocellularity and severe dysplasia.
MATERIALS AND METHODS

RNA sequencing
Total sample RNA was extracted using Trizol with Genelute LPA (Sigma) as a carrier and SMARTer Ultra Low RNA kit for Illumina Sequencing (Clontech) was used for cDNA synthesis according to the manufacturer’s protocol. cDNA was sheared with the Covaris device and processed according to the TruSeq RNA Sample Preparation v2 Guide (Illumina). Amplified sample libraries were subjected to paired-end sequencing (2 x 75 bp) and aligned against mm10 using TopHat 17. Gene expression levels were quantified by the fragments per kilobase of exon per million fragments mapped (FPKM) statistic as calculated by Cufflinks 18 in the RefSeq Transcriptome database 19. Read counts were determined with HTSeq-count 20 and subsequently used for differential expression analysis in DESeq2 21, with default parameters, in the R environment. Multiple testing correction was performed by the Benjamini-Hochberg procedure on the calculated p-values to control the False Discovery Rate (FDR).

For gene set enrichment analysis (GSEA), a ranking metric was defined for each gene as the log10 of the adjusted p-value calculated by DESeq2 with the sign of the log2 fold change. The ranked gene list was tested against a customized version of the C2 MSigDB collection, incorporating datasets on HSC quiescence from the literature 3,22–24 (Supplementary Table 1).

Single-cell RNA sequencing
A compendium of previously published mouse bone marrow single-cell RNA-Seq datasets (Fluidigm C1 platform) was assembled to evaluate the expression of key progenitor genes of interest described 25–27 (available at: http://www.altanalyze.org/ICGS/Public/Mm-Grimes-Fluidigm-Panorama/User.php). Specifically, wild type in vivo mouse SLAM, LSK, GMP, CMP and lineage-negative Sca1+ CD117+ cells from bone marrow were selected, with labels derived from the noted prior studies (GSE70245, GSE141472). Data were analyzed with RSEM to estimate TPM for all genes as previously described 28. The gene expression data of
selected genes were visualized in the python package *plotly* or GraphPad Prism, represented as log2 TPM values.

For the analysis in Figure S1, published single-cell RNA-seq data of HSPCs from the bone marrow of 10 female 12-week-old C57BL/6 mice were retrieved. Raw counts were downloaded from GEO database (GSE81682), with labels derived from the broad gating strategy used in the original study (available here: http://blood.stemcells.cam.ac.uk/data/all_cell_types.txt). Data were imported and processed using the Seurat R package. Cells with fewer than 200000 total counts, fewer than 4000 detected genes or more than 10% mitochondrial reads were excluded. Expression data were log-normalized and the 5000 most variable features were selected for dimensionality reduction with UMAP. *Cebpa* expression was projected on the UMAP and compared to the expression of other selected genes (*Mecom, Hlf*) to investigate its association with different cell populations.

**Mice and transplantation procedures**

The strains of +37kb enhancer-1.2kb and +37kb enhancer-1.15kb deleted mice were generated using zygotes derived from C57/BL6 mice by CRISPR/Cas9 editing and maintained as previously described. The CRISPR/Cas9 single guide RNAs were directed against the +37kb enhancer of *Cebpa*, using the following sequences:

5' : TGAAGCCTACACTACCTTTGT and AGAGGTAGGAACTCCATTCC
3' : AGAGCCTCGCTCAAGCCCAT and TTGAGACATCTGGTAACCTT

Recipient mice were exposed to a 5.5 Gy of gamma radiation. Given that the native +37kb*HOM* mouse exhibits 3-5 fold increase in the cKIT+ Lineage negative progenitor fraction of the bone marrow, we transplanted one million of WT CD34.2 total bone marrow cells and 250,000 of HOM CD34.2 cells to compensate for the fold difference. Non-transplanted mice were sacrificed for FACS analysis between 4-8 weeks of age. For transplantation experiments, bone marrow from 4 weeks old wild type or +37kb enhancer-deleted CD45.2 mice were harvested in PBS/5%FCS and injected intravenously in tails of (CD45.1) female mice. All mice were sacrificed in a CO2 chamber. Animal studies were approved by the Animal Welfare/Ethics Committee of the EDC in accordance with legislation in the Netherlands (approval No. EMC 2067, 2714, 2892, 3062).
Flow-cytometry and sorting

Flow cytometry was carried out on the LSRII and the FACSCanto II ((BD Biosciences). FACS Arialll (BD Biosciences) was used for cell sorting, using the following fluorescent antibodies: Markers for mature hematopoietic cells [CD11B APC/GR1-FITC/B220-PE/CD3 PB]; Pan-hematopoietic marker [CD45.1 PE; CD45.2 APC-CY7]; LIN biotinylated cocktail [CD11B, GR-1, B220 and CD3] streptavidin-pacific orange; LSK [cKIT-APC/SCA1-PB]; LT-HSCs [CD48-FITC/CD150-PE-CY7]. Lineage negative selection was carried out using a cocktail of antibodies targeting antigens expressed on mature hematopoietic cells including CD11B, GR-1, B220 and CD3. All antibodies were purchased from BD Biosciences or Biolegend (Supplementary Table 2). The LSK population was gated from Live (DAPI-), CD45+, Lineage- cKIT+ SCA-1+ bone marrow cells and the sorted LSK fraction was collected in 500μl PBS with 5% FCS, spun down and re-suspended in 800μl of Trizol and used for RNA-seq.

RESULTS

Neutropenia results in LT-HSC number reduction in +37kb Cebpa-enhancer-deleted mice

To study the causal relationship of neutropenia and loss of LT-HSCs, we investigated the allelic dosage effect of Cebpa enhancer deletion on the numbers of neutrophils and LT-HSCs using Cebpa +37kb enhancer heterozygous (+37kbHET) deleted and +37kb homozygous (+37kbHOM) deleted mouse strains 9. The neutrophil (Mac1+Gr1+) frequency and absolute numbers in the peripheral blood and bone marrow of the +37kbHET and the +37kbHOM mice correlated with their mono-allelic and bi-allelic enhancer deletion, respectively (Figure 1a-1d). Therefore, 50% enhancer activity reduced the neutrophil output approximately by half (median frequency: 44.6%) (Figure 1c), while a full enhancer deletion completely abrogated neutrophil production (median frequency 0%). The reduction of neutrophils occurring in the +37kbHOM mice was confirmed by hematoxylin and eosin (H&E) staining of bone marrow sections (Figure 1e) and by the decreased frequency of S100A8 cells (Figure 1f). To investigate whether enhancer deletion affects the LT-HSC pool, we studied the LT-HSC population using the SLAM CD150 and CD48 markers (LT-HSCs: LSK/CD150+CD48-) in the +37kbHET and +37kbHOM mice. The LT-HSC pool was not affected in the +37kbHET mice despite the 50% enhancer dosage (Figure 1g and 1h). Although there was a near 2-fold reduction in LT-HSC numbers, this difference was not statistically significant. In contrast, a 10-fold
reduction of LT-HSC numbers at 100% enhancer dosage reduction was observed in the +37kb$^{\text{HOM}}$ mice (Figure 1g and 1h). The fact that mono-allelic enhancer deletion does not affect LT-HSC numbers, but reduces the neutrophil compartment by half, suggests that $\text{Cebpa}$ only becomes expressed in neutrophil-primed progenitors and is inactive in LT-HSCs. Thus, the quantitative changes in the LT-HSC pool of the +37kb$^{\text{HOM}}$ mice could be explained by an indirect effect of complete ablation of neutrophils, whereas partial neutropenia in the +37kb$^{\text{HET}}$ is insufficient to inflict these changes. Alternatively, myeloid progenitors may be more sensitive to reduced C/EBPa levels than LT-HSCs, which would only become depleted upon biallelic deletion of the +37 kb enhancer.

**Cebpa is predominantly expressed in early myeloid-biased progenitors**

To study $\text{Cebpa}$ expression along differentiation from LT-HSCs towards multipotent and myeloid progenitors, we utilized single-cell RNA sequencing datasets generated by a Fluidigm-based platform. In total, 1,110 bone marrow cells were analyzed (27 libraries, ~50 cells per library and >2 million reads/cell on average) and assigned to a specific bone marrow cell type. The identified cellular states were classified into LT-HSC (SLAM) population, HSPC-1 and HSPC-2 (both LSK), and multi-lineage progenitors, which constitute a mixture of bi-potent and uni-potent restricted myeloid progenitors, as previously described $^{25-27}$. To investigate at what differentiation stage $\text{Cebpa}$ becomes expressed, we first used known gene markers associated with HSC quiescence, namely $\text{Hlf}$ $^{3}$ and $\text{Mecom}$ $^{31}$, and early myeloid differentiation ($\text{Elane}$, $\text{Mpo}$) (Figure 2a). We found a positive correlation between $\text{Hlf}$ and $\text{Mecom}$, while $\text{Cebpa}$ negatively correlated with $\text{Hlf/Mecom}$ (Figure 2b). Accordingly, $\text{Cebpa}$ expression was almost negligible in LT-HSCs (detected in 0.8 % of LT-HSCs), while it appeared in a subset of HSPCs expressing early myeloid lineage genes, indicating priming of the myeloid lineage at very early stages of differentiation (Figure 2c). The detection limit of this technique is 0.25 transcripts per million (TPM), which could possibly exclude cells with very low but present levels of $\text{Cebpa}$. However, these results were confirmed in another dataset $^{29}$ generated by a different single cell sequencing strategy (Figure S1). Altogether, our single cell analysis does not support a cell-autonomous role for $\text{Cebpa}$ in LT-HSCs. Thus, the reduction in LT-HSC numbers observed in the $\text{Cebpa}$ null mice seems to occur systemically as a consequence of neutropenia.
Transcriptional programs of LT-HSC quiescence and neutrophil lineage-priming are deregulated in +37kb\(^{\text{HOM}}\) HSPCs

The results so far indicate that the enhancer deletion decreased the pool of LT-HSCs through a systemic effect in the presence of neutropenia. To study mechanisms linking the two observed events, we applied RNA sequencing on sorted HSPCs using LSK markers on +37kb\(^{\text{WT}}\) (n=3) and +37kb\(^{\text{HOM}}\) (n=3) bone marrow cells. We confirmed that +37kb enhancer deletion in HSPCs reduces Cebpa expression relative to wild type controls (Figure 3a). Differential expression analysis identified dysregulated genes related to neutrophil differentiation (S100a9, Camp) and HSC quiescence (Hlf) (Figure 3b, Supplementary Table 3). Differentially expressed genes in +37kb\(^{\text{HOM}}\) LSKs were further investigated by gene-set enrichment analysis (GSEA) (Supplementary Tables 4-5). Genes associated with neutrophil ontogeny were downregulated in +37kb\(^{\text{HOM}}\) LSK cells (Figure 3c), indicating an early block in myeloid differentiation. Interestingly, we found an early myeloid gene set (Cpa3, Mpo, Cd48) (Figure 3d) to be upregulated in +37kb\(^{\text{HOM}}\) HSPCs, suggesting that an early myeloid-biased population upstream of Cebpa-HSPCs is primed for myelopoiesis. Based on these findings we conclude that Cebpa-HSPCs are intermediate progenitors that link early myeloid biased HSPCs to myeloid committed progenitors and they represent the cell of origin for the induced neutropenia in Cebpa null mice.

To investigate pathways related to LT-HSC depletion, we retrieved datasets from published hematopoietic studies and pooled them with the MSigDB datasets \(^{3,23,32-34}\). Of the most significantly (FDR <0.025) enriched pathways, 6 gene sets were enriched for loss of HSC quiescence and exhaustion (Figure 3e). The identified gene sets (Figure 3f; Figure S2a-f) included transcription factors related to HSC dormancy (Hlf, Mecom, Tcf15) \(^{3,31,35}\), HSC retention factors (Ptpn21, Cxcr4) \(^{36,37}\), cluster of differentiation (Cd) markers for HSC regeneration and engraftment (Cd81, Cd274) \(^{38,39}\), and the Polycomb chromobox proteins for HSC self-renewal regulation (Pbx6, Pbx7) (gene lists in Supplementary Table 1). Deregulation of ribosomal genes (Figure 3g) in our dataset is in accordance with loss of HSC quiescence. Thus, LT-HSC loss in the presence of neutropenia may result from quiescence exit and subsequent exhaustion. Altogether, the transcriptomic analysis of sorted LSK cells from +37kb\(^{\text{HOM}}\) mice suggests that myeloid priming in HSPCs occurs before Cebpa activation.
and that a neutrophil differentiation block in these progenitors potentially leads to HSC quiescence exit.

**Transplantation of +37kb\textsuperscript{HOM} bone marrow into wild type recipients confirms that neutropenia is induced by the +37kb enhancer deletion**

Next, we studied the systemic effects of the +37kb\textsuperscript{HOM} bone marrow on normal hematopoiesis in recipient mice. A sub-lethal irradiation approach was used to overcome the low survival rate caused by the weak chimism known to occur when transplanting \textit{Cebpa} null bone marrow cells into lethally irradiated recipients \textsuperscript{11}. We used donor bone marrow cells from two different +37kb\textsuperscript{HOM} mouse strains, to ensure that the observed phenotype was not caused by an off-target effect. The strains differed in the genomic size of enhancer deletion generated by CRISPR, i.e. +37kb\textsuperscript{HOM} deletion of 1.15kb (CD45.2 +37kb\textsuperscript{HOM-1.15kb} deletion) or +37kb\textsuperscript{HOM} deletion of 1.2kb (CD45.2 +37kb\textsuperscript{HOM-1.2kb}) (Figure S3a-S3b). Sub-lethally irradiated recipient (CD45.1) mice were transplanted with total bone marrow of CD45.2 +37kb\textsuperscript{WT} (N=8), CD45.2 +37kb\textsuperscript{HOM-1.15kb} (N=7) or CD45.2 +37kb\textsuperscript{HOM-1.2kb} (N=8) mice (Figure 4a). As expected, peripheral blood samples withdrawn twelve weeks after transplantation showed a weaker chimism in mice transplanted with +37kb\textsuperscript{HOM} bone marrow compared to those transplanted with wild type bone marrow (Figure 4b). For cellular reconstitution, we determined the frequency of myeloid cells (Mac1+Gr1+), B-cells (B220+) and T-cells (CD3+) by flow cytometry for mice transplanted with +37kb\textsuperscript{WT} or +37kb\textsuperscript{HOM} BM cells (Figure 4c-f, Figure S4a-c).

Populations from the T-cell, myeloid and B-cell lineages were present in the recipients from the three cohorts, indicating that hematopoiesis is functional in the recipient (Figure 4d upper panel and 4f upper panel). However, +37kb\textsuperscript{HOM} donor cells (Figure 4e lower panel and Figure 4f lower panel) lacked myeloid reconstitution as compared to +37kb\textsuperscript{WT} (Figure 4c lower panel and Figure 4d lower panel). Therefore, transplanted +37kb\textsuperscript{HOM} bone marrow cells in a wild type niche did not recover neutrophil differentiation, confirming that neutropenia in +37kb\textsuperscript{HOM} enhancer-deleted mice is cell autonomous. In the +37kb\textsuperscript{WT} donor cell compartment, there was a large contribution of B-cells, as previously observed in other transplantation experiments \textsuperscript{40–42}. Of note, the presence of B-cells and T-cells from the +37kb\textsuperscript{HOM} donors in both the native and the transplanted mice also argues against a strict need for \textit{Cebpa} in LT-HSCs, since they were able to survive and differentiate into the lymphoid lineage.
Transplanted $+37\text{kb}^{\text{HOM}}$ bone marrow causes dysplasia and hypocellularity in recipient mice

Despite the low chimerism, the CD45.2 $+37\text{kb}^{\text{HOM}}$ bone marrow cells persisted and survived for at least 10 months in the CD45.1 recipient, but eventually declined to almost 0% (median = 0.89%) on the day the mice were sacrificed (Figure S5a). Therefore, cells from the blood and bone marrow samples analysed were mostly derived from the recipient mice. We grouped the recipient mice based on their phenotype severity, i.e. $+37\text{kb}^{\text{HOM}}$-mild (n=8) or $+37\text{kb}^{\text{HOM}}$-severe (n=6, namely mice # 2, 5, 7, 9, 12 and 17). The $+37\text{kb}^{\text{HOM}}$-severe mice represented 40% of all the transplanted mice that showed physical weakness (squinting eyes, hunched posture and social isolation), which had to be sacrificed (Figure S5b). One of the mice died before it could be analyzed. Bone marrow hypocellularity and pancytopenia were the primary hallmarks of the $+37\text{kb}^{\text{HOM}}$-severe group (Figures 5a and S5c). Hypocellular bone marrows showed remodeling of blood vessels surrounded by a high degree of immature hematopoietic cells characterized by incomplete differentiation (Figure 5b). The $+37\text{kb}^{\text{HOM}}$-mild group showed lower white blood cell (Figure S6a) and platelet counts (Figure S6c) compared to wild type controls, whereas hemoglobin levels were comparable to those of the $+37\text{kb}^{\text{WT}}$ group (Figure S6b). Bone marrow cellularity in the $+37\text{kb}^{\text{HOM}}$-mild mice varied from normocellular to hypocellular. Both the $+37\text{kb}^{\text{HOM}}$-severe and the $+37\text{kb}^{\text{HOM}}$-mild cohorts showed abnormal megakaryocytes with dysplastic features (Figure 5b). These data are in line with the dysplastic megakaryocytes found in the native $+37\text{kb}$-enhancer-deleted mice (Figure 1e, Figure S7), which might possibly explain the abnormal peripheral blood platelet counts in the recipient mice (Figure S6c). In conclusion, a significant number of mice transplanted with $+37\text{kb}^{\text{HOM}}$ bone marrow exhibit perturbed hematopoiesis featuring hypocellularity and peripheral cytopenia, incomplete differentiation and severe dysplasia.

Acquired neutropenia leading to LT-HSC loss is recapitulated in mice transplanted with $+37\text{kb}^{\text{HOM}}$ bone marrow

Next, we sought to study whether the differences between the $+37\text{kb}^{\text{HOM}}$-severe and the $+37\text{kb}^{\text{HOM}}$-mild phenotypes involve maturation defects of myeloid cells or LT-HSCs in the bone marrow of recipient mice. The $+37\text{kb}^{\text{HOM}}$-severe transplanted mice showed a marked reduction in the numbers of recipient (CD45.1) Mac1+Gr1+ cells (Figure 5c), which was
confirmed by the decrease in S100A8 protein-expressing cells (Figure 5d). In contrast, the 
+37kb\textsuperscript{HOM-mild} mice showed normal neutrophil numbers compared to the wild type controls (Figure 5c). The +37kb\textsuperscript{HOM-severe} mice also showed a reduction in the CD45.1 HSPC and LT-HSC numbers (Figures 5e and 5f), whereas the LT-HSC numbers in the +37kb\textsuperscript{HOM-mild} mice were comparable to those of the +37kb\textsuperscript{WT} group. Thus, the findings in the +37kb\textsuperscript{HOM-severe} support the notion that \textit{Cebpa} null-induced neutropenia triggers LT-HSC loss in a cell non-autonomous manner, as observed in 37kb\textsuperscript{HOM} native mice.

**DISCUSSION**

C/\textit{EBP}\alpha is indispensable for myeloid lineage formation and differentiation\textsuperscript{13,43}. Induced genetic defects in the \textit{Cebpa} locus of mouse models\textsuperscript{9,12,44–46} or \textit{CEBPA} coding mutations detected in human acute myeloid leukemia specimens (AML)\textsuperscript{47,48}, are all associated with myeloid differentiation abnormalities. The expression of \textit{CEBPA} is also frequently repressed in leukemia by transcriptional or post-transcriptional mechanisms\textsuperscript{8}. Notably, \textit{CEBPA} is silenced by oncoproteins targeting the +42 kb enhancer (homologous to +37 kb in mouse), including RUNX1-RUNX1T1\textsuperscript{49} and \textit{EVI1}\textsuperscript{50}. Earlier mouse studies revealed an indispensable role for \textit{Cebpa} in differentiating common myeloid progenitors into granulocyte-monocyte progenitors. The profound neutropenia observed in our +37 kb enhancer-deleted model confirms the absolute requirement for \textit{Cebpa} in myelopoiesis. We observed a 3-fold increase in \textit{Cebpb} expression in cKit+ Sca1- Cd34+ myeloid progenitors, but this was insufficient to compensate for the loss of \textit{Cebpa}. Although C/\textit{EBP}\beta can substitute for C/\textit{EBP}\alpha during hematopoiesis when knocked into the \textit{Cebpa} gene locus\textsuperscript{51}, it cannot fully replace C/\textit{EBP}\alpha when expressed from its native locus.

Several studies hinted towards an intrinsic role for \textit{Cebpa} in myeloid lineage priming of LT-HSCs\textsuperscript{11,12}. However, the bulk-sequencing methods used in these studies do not meet the resolution required to dissect the heterogeneity of the HSPC compartment. Using single cell RNA sequencing, we showed that \textit{Cebpa} is barely detectable in LT-HSCs during steady-state hematopoiesis. In contrast to previous studies, we found that \textit{Cebpa} licenses myeloid-primed HSPCs downstream of LT-HSCs for neutrophil lineage differentiation. This suggests that other transcription factors account for the myeloid lineage bias in LT-HSCs\textsuperscript{52} and activate the neutrophil lineage trajectory through binding the \textit{Cebpa} +37kb enhancer in a
subset of HSPCs\textsuperscript{9,53}. Although Fluidigm C1 exhibits better sensitivity\textsuperscript{54} and fewer dropout events\textsuperscript{55,56} than droplet-based approaches, it remains possible that LT-HSCs with very low Cebp\(\alpha\) levels (below the detection threshold of 0.25 TPM) exist. The physiological relevance of such low transcript levels, which could be attributable to pervasive transcription, is unknown. It is equally possible that the few Cebp\(\alpha\)-expressing LT-HSCs identified are the result of technical noise or phenotypic misclassification.

In the enhancer-deleted models, LT-HSC loss was proportional to the degree of neutropenia, suggesting a causal relationship between them. Although an alternative explanation could be that LT-HSCs are less sensitive to Cebp\(\alpha\) levels than myeloid progenitors, the normal production of lymphoid cells indicates that LT-HSCs remain viable in the absence of Cebp\(\alpha\). This is further supported by the almost complete absence of Cebp\(\alpha\)-expressing LT-HSCs in healthy bone marrow. Critically, LT-HSCs were also lost in mice transplanted with +37 kb\textsuperscript{HOM} bone marrow: 40% of the transplanted mice showed this severe phenotype, while the other 60% showed signs of myeloid differentiation defects and dysplasia, suggesting they were in an early stage of the same pathological process. These effects we can only explain by systemic consequences triggered in the presence of neutropenia. Even in a hybrid model in which a few LT-HSCs express Cebp\(\alpha\), the evidence gathered in this study supports the hypothesis that the major LT-HSC depletion in Cebp\(\alpha\) null mice is an indirect consequence of neutropenia.

To our knowledge, none of previously reported neutropenia mouse models\textsuperscript{26,57} demonstrated a depletion of LT-HSCs. A likely explanation is that the differentiation block in these other models (such as Sbds\textsuperscript{57} and Gfi1\textsuperscript{58} mutants) often occurs at a late stage of neutrophil differentiation. Niches supporting a late stage of neutrophil differentiation are located distant from LT-HSC niches. Given that the differentiation block in Cebp\(\alpha\) null bone marrow occurs in early progenitors located proximally to LT-HSC niches, the proposed systemic effect of neutropenia onto the LT-HSCs might be specific to this model. Fifteen percent of patients with congenital neutropenia develop LT-HSC clonal bone marrow conditions such as myelodysplasia and AML\textsuperscript{59,60}, suggesting LT-HSC impairment may also occur in human neutropenia. Thus other models of neutropenia are required in order to understand the mechanisms that lead to hematopoietic insufficiency in the presence of
neutropenia, such as Cebpa-\(+37\text{kb}^{\text{HOM}}\)-Enh(f/f);Mx1-Cre mice\(^{12}\). The advantage of those mice with an inducible +37\text{kb} enhancer-deletion system is that one could avoid enhancer-deletion during embryogenesis.

Loss of HSC quiescence is one of the hallmarks identified at the transcriptional level in the +37\text{kb}^{\text{HOM}} enhancer-deleted mice. The activation of compensatory mechanisms forcing neutrophil differentiation in myeloid biased progenitors is a potential underlying cause. For example, increased production of granulocyte colony stimulating factor (G-CSF) in the absence of peripheral neutrophils is seen in patients with neutropenia\(^{61,62}\). G-CSF stimulates granulopoiesis through binding to G-CSF receptor-expressing progenitors\(^{63}\) and activates HSCs through attenuation of the Cxcr4-Cxcl12 retention factors expressed on HSCs and bone marrow stromal cells, respectively\(^{64,65}\). Therefore, the neutropenia-GCSF-HSC activation loop can eventually lead to exhaustion and consumption of the LT-HSC pool. Other potential causes that might lead to LT-HSC quiescence exit include metabolic stress caused by impaired differentiation\(^ {66}\); emergency myelopoiesis due to infections acquired in the absence of neutrophils\(^ {67}\); or the lack of mature neutrophils in the bone marrow niche that support HSC quiescence\(^ {68}\).

Myeloid niches in the bone marrow have been reported to be located spatially distant from niches occupied by HSCs\(^ {69}\) or lymphoid progenitor populations\(^ {70}\). In addition, the dendritic, neutrophil and monocyte lineages are also distantly located from each other and organized in different sinusoid niches. Therefore, it is likely that the neutrophil-primed progenitors derived from both donor and recipient share a common environment that is potentially disturbed upon transplantation of +37\text{kb}^{\text{HOM}} bone marrow. The question that remains to be answered is how transplanted +37\text{kb}^{\text{HOM}} myeloid progenitors impair the differentiation process of the host. Transcriptome analysis shows that myeloid progenitors (cKit+ Sca1-Cd34+) derived from the +37\text{kb} enhancer-deleted model are metabolically reprogrammed and exhibit downregulation of the oxidative phosphorylation (OXPHOS) pathway (Figure S8a, Supplementary Table 6), as compared to normal myeloid progenitors that are dependent on oxidative and mitochondrial metabolism\(^ {71}\). Shutting down OXPHOS eventually activates the glycolytic pathway (known as the Warburg effect), under the control of nuclear factors such as Mycn\(^ {72,73}\), a critical metabolic regulator involved in cell
competition. In line with this, we find Mycn expressed at high levels in the +37kbHOM myeloid progenitors (Figure S8b). Such metabolic changes impact cell-to-cell communication processes required for normal differentiation programs 66, which may partially explain the acquired neutropenia in the recipient when transplanted with +37kbHOM bone marrows.

Our study suggests that prolonged neutropenia induced perturbations in localized myeloid niches and further caused systemic bone marrow changes resulting in LT-HSC loss, bone marrow hypocellularity and severe dysplasia. Although the underlying mechanisms remain unclear, we hypothesize that Cebpa null progenitors acquire metabolic reprogramming that impairs differentiation and disturbs HSC quiescence. Functional studies are required to investigate the intracellular role of Cebpa in controlling the metabolic pathways related to neutrophil differentiation, and further elucidate how Cebpa null progenitors are metabolically reprogrammed to inflict systemic changes in the bone marrow. From a clinical perspective, this phenomenon may explain how metabolic stress on HSCs might give rise to bone marrow clonal disorders in a subset of congenital neutropenia patients. Therefore, our study sets a paradigm about the underlying mechanisms involved in the progression of neutropenia to clonal bone marrow disorders.

DATA SHARING STATEMENT

Sequencing data generated for this study have been deposited in the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo), under accession number GSE189332. Single cell sequencing data from previous studies were downloaded from GEO (GSE70245, GSE141472, and GSE81682).

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AUTHOR CONTRIBUTIONS
R.A., R.M-L and RD designed the research and wrote the paper. R.A. M.H, L.S, N.S., E.B performed Research, R.A, R.M, R.H, N.S, R.K.S, L.G analysed data.

CONFLICT OF INTEREST

None of the authors has a relevant conflict of interest.

FIGURE LEGENDS

Figure 1. Cebpa enhancer deletion causes neutropenia and reduction in LT-HSCs of +37kbHOM bone marrows. (a-b) Representative flow cytometry plots showing Mac1+Gr1+ myeloid cell populations in peripheral blood (a) and bone marrow (b) of +37kbWT (blue), +37kbHET (green) and +37kbHOM (red) mice; (c) Absolute numbers of Mac1+Gr1+ cells calculated from total peripheral blood counts of 37kbWT (blue), +37kbHET (green) and +37kbHOM (red) mice; (d) Relative numbers of total Mac1+Gr1+ cells calculated from total bone marrow cell count from 1 femur, corrected for body weight in grams of each mouse. (e) Hematoxylin and Eosin (H&E) staining of representative cross sections showing bone marrow architecture (left), identifying megakaryocytes [right (arrows)] in +37kbWT and +37kbHOM mice. (f) S100A8 Immunohistochemical staining of representative bone marrow cross sections from 37kbWT and +37kbHOM mice. (g) Representative flow cytometry plots of LK, LSK, LT-HSC, ST-HSC, MPP3, MPP2 cell populations in bone marrow of 37kbWT, +37kbHET and +37kbHOM mice. (h) Absolute numbers of CD150+CD48− LT-HSCs calculated from total bone marrow cell counts from 1 femur. All data are represented as mean +/- SD. Statistical significance was calculated using a Student’s t-test: N.S. = not significant; pvalue <0.05 (*); pvalue <0.005 (**); pvalue <0.0005 (**); SD = standard deviation. LK: Lineage-, cKit+; LSK: Lineage- Scal+ cKit+; LT-HSC: long-term hematopoietic stem cell; ST-HSC: short-term hematopoietic stem cell; MPP: multipotent progenitor; +37kbWT: wild type +37kb enhancer; +37kbHET: heterozygous +37kb enhancer deletion; +37kbHOM homozygous +37kb enhancer deletion.

Figure 2. Single cell RNA sequencing data in wild type bone marrows exclude the presence of Cebpa-expressing LT-HSCs. (a) Bar plot of single cell expression of Cebpa in different progenitor populations, alongside genes involved in LT-HSC quiescence (Mecom, Hlf) or myeloid differentiation (Elane, Mpo) (b) Scatterplots of single cell gene expression showing that Mecom and Cebpa are mutually exclusive, whereas Hlf and Cebpa are occasionally co-expressed and Mecom frequently co-occurs with Hlf (c) Scatterplots of single cell gene expression showing co-expression of Cebpa with Cd48 (a marker not found in LT-HSCs) and the myeloid differentiation markers Elane and Mpo. All gene expression data are presented as transcripts per million (TPM). Cells are color coded by the population they belong to. Pearson correlation coefficients and the related p-values for the pairwise gene combinations are depicted in the scatter plots.

Figure 3. Transcriptome analysis reveals loss of HSPC quiescence in +37kbHOM mice. (a) Volcano plot showing genes differentially expressed in HSPCs of +37kbHOM (n = 3) compared to +37kbWT mice (n = 3). Differentially expressed genes are represented as log2 fold change
(X-axis) and log10 p-value (Y-axis). Significantly upregulated genes (log2 fold change > 1.5, p-value < 0.05) are shown in red, downregulated genes (log2 fold change < -1.5, p-value < 0.05) are shown in blue, and genes without significant differences are shown in grey. (b) Mean and SD of Cebpa expression in +37kb\textsuperscript{WT} and +37kb\textsuperscript{HOM} HSPCs compared to +37kb\textsuperscript{WT} HSPCs, presented as log2 fold change. (c) Bar plot showing the top downregulated neutrophil-associated genes in +37kb\textsuperscript{HOM} HSPCs compared to +37kb\textsuperscript{WT} HSPCs, presented as log2 fold change. (d) GSEA enrichment plot (left) showing upregulation (NES= 2.22; FDR <0.05) of the early myeloid pathway in +37kb\textsuperscript{HOM} HSPCs. Heatmap (right) showing significant differentially expressed genes of this pathway in +37kb\textsuperscript{HOM} vs +37kb\textsuperscript{WT} HSPCs. (e) Bar plot showing downregulation of LT-HSC quiescence-related pathways in +37kb\textsuperscript{HOM} vs +37kb\textsuperscript{WT} HSPCs, expressed as -log2 transformation of FDR. (f) GSEA enrichment plot (left) showing downregulation (NES = -2.52; FDR <0.05) of the HSC quiescence pathway from +37kb\textsuperscript{HOM} vs +37kb\textsuperscript{WT} HSPCs. Heatmap (right) of the significant differentially expressed genes in this dataset. (g) GSEA enrichment plot (left) showing downregulation (NES = -2.22; FDR <0.05) of the ribosome pathway and heatmap (right) of differentially expressed ribosomal Rsp and Rlp genes. (h) Mean and SD of Mycn expression in +37kb\textsuperscript{WT} and +37kb\textsuperscript{HOM} HSPCs, expressed as FPKM values. HSPC: hematopoietic stem and progenitor cells; SD: standard deviation; FDR: False discovery rate; FPKM: Fragments Per Kilobase of transcript per Million. The experiment was done in triplicates for each condition, +37kb\textsuperscript{WT} (n=3) and +37kb\textsuperscript{HOM} (n=3) and the heatmap values were calculated using Z-scores.

**Figure 4**: Transplanted +37kb enhancer-deleted bone marrow cells show low chimerism and are neutropenic. (a) Transplantation scheme representing donor (CD45.2) wild type, donor (CD45.2) with homozygous 1.2kb deletion and donor (CD45.2) with homozygous 1.15kb deletion in (CD45.1) recipients. Eight recipient mice per condition (n=24) were used in the experiment. One mouse transplanted with 1.2kb enhancer-deleted bone marrow cells died before it could be analyzed. (b) Bar chart showing percentage of CD45.1 and CD45.2 cell chimerism in peripheral blood twelve weeks after transplantation of wild type and homozygous bone marrow. (c) Flow cytometry contour plot of bone marrow after transplantation with +37kb\textsuperscript{WT} cells: Mac1+Gr1+ myeloid cells, CD220+ B-cells and CD3+ T-cell populations were gated from CD45.1 and CD45.2 fractions. (d) Proportion of +37kb\textsuperscript{WT} Mac1+Gr1+ myeloid cells, B220+ B-cells and CD3+ T-cell populations from recipient CD45.1 (up) and donor CD45.2 (lower). (e) Flow cytometry contour plots of bone marrow after transplantation with +37kb\textsuperscript{HOM} cells: Mac1+Gr1+ myeloid cells, B220+ B-cells and CD3+ T-cell populations were gated from CD45.1 and CD45.2 fractions. (f) Proportion of +37kb\textsuperscript{HOM} Mac1+Gr1+ myeloid cells B220+ B-cells and CD3+ T-cell populations from recipient CD45.1 (upper) and donor CD45.2 (lower). Experiments were conducted on peripheral blood samples of +37kb\textsuperscript{WT} (n=8) and +37kb\textsuperscript{HOM} (n=15), drawn 12 weeks post-transplantation.

**Figure 5**: Systemic bone marrow perturbations impair myeloid differentiation and deplete the LT-HSC pool of recipient mice. (a) Total bone marrow cellularity per femur, corrected for body weight in grams of each mouse. The mice transplanted with homozygous bone marrow cells were divided into two subgroups based on disease severity: homozygous mild and homozygous severe. Mice transplanted with wild type bone marrows were used as controls. (b) Histological examination using hematoxylin and eosin (H&E) on processed paraffin bone marrow sections of recipient CD45.1 transplanted mice. (c) Absolute numbers of Mac1+Gr1+ neutrophils from bone marrow of CD45.1 recipient mice. (d)
Immunohistochemistry of S100A8 protein expression on histological bone marrow sections of recipient CD45.1 transplanted mice (e) HSPC absolute numbers in bone marrows of CD45.1 recipient mice, calculated from LSK/lineage negative/live cells (7AAD) and corrected for total cellularity per femur and body weight (in grams) of each mouse. (f) LT-HSC absolute numbers in bone marrow of CD45.1 recipient mice, calculated from CD48-Cd150+/LSK/lineage negative/live cells (7AAD) and corrected for total cellularity per femur and body weight in grams of each mouse. All data are represented as mean +/- SD. Statistical significance was calculated using a Student’s t-test: N.S. = not significant; pvalue <0.05 (*); pvalue <0.005 (**); pvalue <0.0005 (***); SD = standard deviation.

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Figure 1

1a

Peripheral Blood

Counts (x10^3/μL)

**

*WT HOM HET

MAC1+ GR1+

Bone Marrow

1b

% of MAC1+ GR1+

***

** WT HOM HET

Bone Marrow

1c

Peripheral Blood

Graph showing statistics

1d

Bone Marrow

Graph showing percentages

1e

+37kb^WT

+37kb^HET

+37kb^HOM

Images showing morphological differences

1f

+37kb^WT

+37kb^HET

+37kb^HOM

Images showing cell distribution

1g

+37kb^WT

+37kb^HET

+37kb^HOM

Images showing cell distribution

1h

Bone Marrow (Absolute numbers)

Graph showing absolute numbers

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Figure 2

2b. $r = -0.13$  
$p-value = 2.02E-07$

2c. $r = 0.27$  
$p-value = 4.10E-29$
Figure 3

3a. 

Myeloid Cell Development
(Brown et al., 2006)

3b. 

Differential expression:
+37kbWT (n=3) vs +37kbHOM (n=3)

3c. 

Neutrophil Associated genes
(+37kbHOM vs +37kbWT)

3d. 

3e. 

Downregulated HSC gene sets
(HSC quiescence)

3f. 

HSC quiescence
(Giladi et al., 2018)

3g. 

Kegg Ribosome pathway

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