Truncated CSF3 receptors induce pro-inflammatory responses in severe congenital neutropenia

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Summary
Severe congenital neutropenia (SCN) patients are prone to develop myelodysplastic syndrome (MDS) or acute myeloid leukaemia (AML). Leukaemic progression of SCN is associated with the early acquisition of CSF3R mutations in haematopoietic progenitor cells (HPCs), which truncate the colony-stimulating factor 3 receptor (CSF3R). These mutant clones may arise years before MDS/AML becomes overt. Introduction and activation of CSF3R truncation mutants in normal HPCs causes a clonally dominant myeloproliferative state in mice treated with CSF3. Paradoxically, in SCN patients receiving CSF3 therapy, clonal dominance of CSF3R mutant clones usually occurs only after the acquisition of additional mutations shortly before frank MDS or AML is diagnosed. To seek an explanation for this discrepancy, we introduced a patient-derived CSF3R-truncating mutation in ELANE-SCN and HAX1-SCN derived and control induced pluripotent stem cells and compared the CSF3 responses of HPCs generated from these lines. In contrast to CSF3R-mutant control HPCs, CSF3R-mutant HPCs from SCN patients do not show increased proliferation but display elevated levels of inflammatory signalling. Thus, activation of the truncated CSF3R in SCN-HPCs does not evoke clonal outgrowth but causes a sustained pro-inflammatory state, which has ramifications for how these CSF3R mutants contribute to the leukaemic transformation of SCN.

Keywords
CSF3R mutation, leukaemia, pro-inflammatory signalling, severe congenital neutropenia

Introduction
Severe congenital neutropenia (SCN) is a genetically heterogeneous disease characterized by a promyelocyte maturation arrest and absolute peripheral neutrophil counts below $0.5 \times 10^9/L$. Most SCN patients present with autosomal dominant mutations in ELANE, encoding the granule protein neutrophil elastase (NE), or autosomal recessive mutations in ELANE or HAX1, encoding NE and the NE activator HAX1. The somatic acquisition of additional mutations, which are typically acquired within the bone marrow of these patients, is a prerequisite for the leukaemic transformation of SCN. Understanding the mechanisms by which these mutations promote leukaemic progression is crucial for the development of therapeutic strategies.

Abbreviations: AML, acute myeloid leukaemia; CSF3R, colony-stimulating factor 3 receptor; FDR, false discovery rate; GSEA, gene set enrichment analysis; HPC, haematopoietic progenitor cell; IFN, interferon; iPSC, induced pluripotent stem cell; MDS, myelodysplastic syndrome; NE, neutrophil elastase; NES, normalized enrichment score; SCN, severe congenital neutropenia; TPM, transcripts per million.

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mutations in \( HAX1 \), encoding a multifunctional protein involved in mitochondrial integrity.\(^2\)\(^,\)\(^3\) Treatment with colony stimulating factor 3 (CSF3), also known as G-CSF, alleviates the neutropenia in most (>90%) SCN patients.\(^4\)

Apart from being susceptible to life-threatening bacterial infections, SCN patients also face an increased risk of developing myelodysplastic syndrome (MDS) or acute myeloid leukaemia (AML).\(^5\)\(^,\)\(^6\) Leukaemic progression correlates with somatic mutations in \( CSF3R \), resulting in a truncated CSF3 receptor. These \( CSF3R \) mutations appear during the neutropenic phase of the disease in one or multiple clones, often years before MDS/AML becomes overt.\(^7\) Previous work in mice and cell lines showed that \( CSF3R \)-truncation mutants function abnormally because of defective internalization and lysosomal degradation.\(^8\)\(^,\)\(^9\) Consequently, myeloid progenitors showed increased \( CSF3 \)-induced colony formation and reduced myeloid differentiation.\(^10\)\(^-\)\(^12\)

How \( CSF3R \)-truncating mutants function in SCN-derived haematopoietic progenitor cells (HPCs) is unknown. Studies addressing this were hampered by the lack of suitable models, mainly because \( ELANE \)- or \( HAX1 \)-mutant mice do not develop neutropenia.\(^12\)\(^-\)\(^15\) To overcome this limitation, we generated patient-derived induced pluripotent stem cells (iPSCs), engineered to express a patient specific \( CSF3R \)-Q739* mutation, causing a truncation at amino acid 715 of the mature \( CSF3 \) protein (hereafter named \( CSF3R \)-d715). HPCs derived from these lines showed markedly different \( CSF3 \) responses compared to normal HPCs.

**METHODS**

**iPSC and patient details**

Bone marrow fibroblasts cultured from SCN patients harboured an \( ELANE \) mutation p.I60F (NC_000019.10:g.852986A>T), \( ELANE \) mutation p.R103L (NC_000019.10:g.853345G>T), or \( HAX1 \) mutation p.W44X (NC_000001.11:g.15427341_15427341insA), and from healthy control bone marrow were reprogrammed as described previously.\(^16\) Introduction of the \( CSF3R \)-d715 mutation (Q739*, NC_000001.11:g.36466653C>T) was done using CRISPR-Cas9 mediated genome editing as described previously.\(^12\) The iPSC lines used showed homozygous (control, \( ELANE \)-R103L, \( HAX1 \)-W44X) or heterozygous (\( ELANE \)-I60F, \( HAX1 \)-W44X) integration of the recombination template. If applicable, the wildtype allele was sequenced to determine the lack of possible additional alterations. Cells were cultured in mTeSR™1 (STEMCELL Technologies) on Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Thermo Fisher Scientific) and were regularly checked for pluripotency, correct karyotype (using Global Screening Array)\(^17\) and their ability to generate haematopoietic progenitor cells and mature neutrophils. The study was performed with the permission of the institutional review boards of the Erasmus Medical Centre, the Netherlands.

**Haematopoietic induction**

Haematopoietic progenitor cells (CD34\(^+\)CD45\(^-\)) were produced with the STEMdiff™ Haematopoietic Kit (STEMCELL Technologies) according to the manufacturer's protocol. Suspension cells were harvested on day 12 and used for further analysis.

**Colony forming unit assays**

Suspension cells were seeded (1 \times 10\(^4\)/ml) in methylcellulose (H4230; STEMCELL Technologies) with the addition of 200 ng/ml CSF3. Colonies were counted after 14 days of culture.

**Flow cytometry and cell sorting**

Haematopoietic induction of iPSCs was assessed using CD34-PE (345802; BD Biosciences) and CD45-FITC (A07782; Beckman Coulter) antibodies. Dead cells were excluded using 7-aminoactinomycin-D (Life Technologies). Flow cytometry was performed using a LSRII flow cytometer (BD Biosciences). Cell sorting was done using a fluorescence-activated cell sorting (FACS) Aria instrument (BD Biosciences). Analyses were performed using FlowJo (TreeStar).

**RNA isolation and sequencing**

RNA was isolated from FACS sorted CD34\(^+\)CD45\(^-\) HPCs using TRIzol (Thermo Fisher Scientific) and GenElute-LPA (Sigma), according to the manufacturer's protocol. The SMARTer Ultra Low Input RNA kit for sequencing (Clontech; version 4) was used to generate cDNA. Sequencing libraries were generated using the TruSeq Nano DNA Sample Preparation kit (Illumina), according to the low sample protocol and run on HiSeq 2500 or Novaseq 6000 instruments (Illumina).

**Bioinformatics**

Demultiplexing was performed using the CASAVA software (Illumina) allowing for one mismatch in the barcodes. Subsequently, SMARTer adapters and poly-A tails were removed (fqtrim; https://ccb.jhu.edu/software/fqtrim/) and quality metrics were estimated (FastQC, Babraham bioinformatics and MultiQC, http://multiqc.info) for all of the resulting fastq files. Reads were then aligned against the Human Transcriptome (Gencode v19)/Genome (hg19) using the STAR aligner,\(^18\) and visualized with the Integrative Genomics Viewer (IGV; https://software.broadinstitute.org/software/igv/).\(^19\) Abundance estimation was performed using Cufflinks\(^20\), and raw counts were measured with the HTSeq-count software set in union mode.\(^21\) Next, the measured raw counts were used to create clustering and principal component plots and to perform differential expression analysis using a combination of
DESeq\textsuperscript{22} and R (https://www.r-project.org/). Gene set enrichment analysis (GSEA) on the curated gene sets C2 and the hallmark pathways H was done using the GSEA software (https://www.gsea-msigdb.org/gsea/msigdb/gene sets.jsp) based on the pre-ranked ASHR log\textsubscript{2} fold change, where a false discovery rate (FDR) of <0.05 was considered significant.\textsuperscript{23,24} Transcripts per million were calculated using StringTie.\textsuperscript{25}

Statistics

Data are presented as mean ± standard error of the mean. Comparison of two groups was performed using an unpaired t-test. DESeq\textsuperscript{2} was used to determine significance based on normalized count data of the RNA-sequencing experiments. A p-value or FDR of <0.05 was considered significant.

RESULTS AND DISCUSSION

The CSF3R-d715 mutation was introduced in iPSCs from a healthy control and SCN patients with an ELANE-R103L, HAX1-W44X, or ELANE-I60F mutation (Figure S1).\textsuperscript{12} ELANE-R103L and HAX1-W44X patients responded favourably to CSF3 treatment, with dosages of 5–10 μg/kg/day being sufficient to alleviate neutropenia (hereafter referred to as CSF3-normoresponsive). The ELANE-I60F patient was hyporesponsive to CSF3, with dosages of 60–70 μg/kg/day leading to a modest increment in neutrophil counts. Introduction of the mutation did not alter the potential of the iPSC lines to generate CD34\textsuperscript{+}CD45\textsuperscript{+} HPCs (data not shown).

Activation of CSF3R-d715 compromises myeloid differentiation in control HPCs, but not in SCN-HPCs

In agreement with previous studies in murine cell lines showing reduced neutrophilic differentiation in response to CSF3,\textsuperscript{10,26,27} activation of CSF3R-d715 in control HPCs induced the expression of various genes associated with immature haematopoietic cells, e.g. CD34 and CD164, while genes associated with more advanced stages of myeloid differentiation, e.g. C/EBPa, MNDA, SRGN and AZU1 are expressed at lower levels compared with CSF3R-wt control HPCs (Figure 2A; Figure S2A). Additionally, gene sets associated with immature haematopoietic cells are upregulated in CSF3R-d715 control-HPCs (e.g. “IVANOVA_HEMATOPOIESIS_EARLY_PROGENITORS” and “RAMALHO_STEMNESS_UP”), while myeloid differentiation gene sets are downregulated (e.g. “REACTOME_TRANSCRIPTIONAL_REGULATION_OF_GRANULOPOIESIS” and “KAMIKUBO_MYELOID_CEBPA_NETWORK”), Figure 2B, https://www.gsea-msigdb.org/gsea/msigdb/gene sets.jsp?collection=C2). In contrast, transcriptomes of CSF3R-d715 SCN-HPCs are suggestive of a myeloid bias within the HPC compartment, where both myeloid gene sets and myeloid genes are increased compared with CSF3R-wt SCN-HPCs (Figure 2A; Figure S2A). Interestingly, ELANE expression is also significantly elevated upon CSF3R-d715 activation in both R103L and I60F ELANE-mutant HPCs, which involves both wildtype and mutant alleles (Figure 2A; Figure S2B). In agreement with the transcriptome data, NE protein levels were increased in CSF3R-d715 ELANE-R103L and -I60F cells, reduced in control and absent in the HAX1-W44X mutant cells compared to cells expressing CSF3R-wt (Figure 2C). These findings suggest that activation of CSF3R-d715 may affect SCN-HPCs by increasing mutant NE levels, thereby possibly aggravating the SCN phenotype. Upon transferring the CSF3-d715 HPCs to CSF3-containing suspension cultures, no gross differences in neutrophil differentiation between control- and CSF3-normoresponsive SCN-derived HPCs are seen (Figure S2C). This is not surprising, given that SCN patients mostly respond well to CSF3 therapy, effectively alleviating the differentiation block, even when major CSF3R mutant clones are present.\textsuperscript{28,29}

CSF3R-d715 exacerbates an inflammatory state of SCN-HPCs

Chronic inflammatory states of HSPCs have recently been linked to myeloid malignancy, in particular myeloproliferative

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neoplasms and MDS, where inflammation has been shown to be responsible for the competitive advantage of mutant clones. How inflammation contributes to the neutropenic state in SCN, expansion of CSF3R mutant clones, and development of myeloid malignancy is unknown. Upon progression to MDS/AML, most SCN patients acquire RUNX1 mutations.

Figure 1: Activation of CSF3R-d715 results in hyperproliferation in control, but not severe congenital neutropenia-haematopoietic progenitor cells (SCN-HPCs). Gene set enrichment analysis (GSEA) hallmark pathways showing the expression of E2F targets, G2M checkpoint and MYC targets which are (A) induced in CSF3R-d715 control HPCs compared to their CSF3R-wt counterpart, and (B) reduced or not consistently altered in CSF3R-d715 SCN-HPCs compared to their isogenic control. Data are combined from two independent experiments. Additionally, CSF3R-d715 HAX1-W44X data are derived from two independent clones (homozygous and heterozygous CSF3R-d715 clones) and two independent experiments. (C) CSF3-induced colony formation is significantly increased after introduction of the CSF3R-d715 in control, but not SCN-HPCs. Data are from three independent experiments (biological replicates), performed in triplicate (technical replicates), derived from two independent CSF3R-d715 control clones, two independent CSF3R-d715 HAX1-W44X clones and one CSF3R-d715 ELANE-R103L clone. FDR, false discovery rate; NES, normalized enrichment score; ns, not significant, ****p < 0.0001. CSF3-normoresponsive: patient responding favourably to relatively low (5–10 μg per kg body weight per day) dosages of CSF3; CSF3-hyporesponsive: patient responding poorly, even to high CSF3-dosages.
in clones already harbouring CSF3R mutations. Leukaemic progression of SCN in a Csfsr-d715/RUNX1 mutant mouse model revealed increased pro-inflammatory signalling in HPCs, which was further aggravated by the downregulation of TET2. We recently reported that non-leukaemic HSPCs from an ELANE-SCN patient who progressed to AML

FIGURE 2 Activation of CSF3R-d715 results in reduced differentiation in control, but not severe congenital neutropenia-haematopoietic progenitor cells (SCN-HPCs). (A) Genes expressed at more advanced stages of myeloid differentiation are downregulated in CSF3R-d715 control HPCs and upregulated in CSF3R-d715 SCN-HPCs compared to their isogenic control. Datapoints represent biological replicates. Additionally, CSF3R-d715 HAX1-W44X data are derived from two independent clones and two independent experiments. (B) Gene set enrichment analysis showing the expression of more immature HPC pathways (e.g. early haematopoietic progenitors and stemness) and mature myeloid pathways (e.g. C/EBPα network and transcriptional regulation of granulopoiesis), where CSF3R-d715 control HPCs show a more immature phenotype, while CSF3R-d715 SCN-HPCs show a more differentiated phenotype. Data are combined from two independent experiments and depict the comparison of CSF3R-wt and CSF3R-d715 HPCs. Additionally, CSF3R-d715 HAX1-W44X data are derived from two independent clones and two independent experiments. (C) Immunoblots showing neutrophil elastase (NE) protein abundance, which is increased in CSF3R-d715 ELANE-R103L and -I60F HPCs, decreased in CSF3R-d715 control HPCs and absent in HAX1-W44X HPCs, compared to their isogenic control. Actin was used as loading control. TPM, transcript per million. p-values are determined with DESeq2 based on the normalized count data; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. FDR, false discovery rate; NES, normalized enrichment score; ns, not significant. CSF3-normoresponsive: patient who responds good to relatively low dosages of CSF3; CSF3-hyporesponsive: patient who responds poorly to high CSF3-dosages.
already showed upregulation of interferon (IFN)-response genes before leukaemic transformation.\(^{13,14}\) In line with a potentially important role for inflammatory signalling in leukaemic transformation, IFN-response genes, e.g. \(IRF7, IRF9\) and \(MX1\), are upregulated by CSF3R-d715 in SCN-HPCs (Figure 3A). In addition, activation of the CSF3R-d715 in iPSC-derived cells enhances the expression of IFN-response gene sets of normoresponsive SCN-HPCs, but not of control HPCs (Figure 3B). Interestingly, IFN-response genes were also induced, albeit less strongly, in CSF3R-d715 SCN-HPCs derived from the hyporesponsive \(ELANE\)-I60F patient (Figure 3A,B), suggesting that this may occur irrespective of

**FIGURE 3** Activation of CSF3R-d715 induces interferon responses in severe congenital neutropenia-haematopoietic progenitor cells (SCN-HPCs). (A) Expression of various interferon-\(\alpha\) and -\(\gamma\) response genes in transcript per million (TPM). Datapoints represent biological replicates. Additionally, CSF3R-d715 HAX1-W44X data are derived from two independent clones (containing either a heterozygous or a homozygous CSF3R-d715) and two independent experiments. (B) Gene set enrichment analysis (GSEA) showing expression of interferon-\(\alpha\) response genes in transcript per million (TPM). Datapoints represent biological replicates. Additionally, CSF3R-d715 HAX1-W44X data are derived from two independent clones and two independent experiments. (C) Schematic overview of the proposed model based on data presented here combined with previous studies.\(^{13,14}\) FDR, false discovery rate; NES, normalized enrichment score. \(p\)-values are determined with DESeq2 based on the normalized count data; \(*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.\) CSF3-normoresponsive: patient who responds good to relatively low dosages of CSF3; CSF3-hyporesponsive: patient who responds poorly to high CSF3-dosages.
a favourable response to CSF3 treatment. Thus, SCN-HPCs that are already rewired towards an inflammatory state endure a further increase in inflammatory signalling upon acquisition of a CSF3R mutation. How CSF3R-d715 and the SCN-causing mutations in ELANE or HAX1 cooperate in enhancing IFN responses, rather than proliferation of HPCs, is not yet clear, but it explains why a strong dominance of CSF3R mutant clones does not occur in patients undergoing CSF3 treatment.28,35 A possible explanation could be that the increased oxidative stress levels observed in SCN-HPCs play a role in CSF3R-d715 signalling, but this merits further studies.36

In conclusion, data presented here combined with previous studies fit into a scenario where increased inflammation of HPCs accompanies disease progression of SCN, creating a vulnerability for mutations leading to malignant transformation (Figure 3C). This differs from how CSF3R truncation mutants function in HPCs without SCN mutations, where their activation merely resulted in a hyper-proliferative response and more immature HPCs (Figures 1 and 2).10-12 In SCN-HPCs, activation of the truncated CSF3R aggravates the inflammatory state initiated by the disease-causing mutation,13 as characterized by the increased expression of IFN-α and IFN-γ response genes (Figure 3). When the HPCs undergo transformation to AML, additional inflammatory pathways (e.g. characterized by upregulation of TNF-α/NF-κB and interleukin-6 response genes) are activated, fuelled by the loss of TET2 activity.12 Because wild-type RUNX1 also inhibits its inflammatory cytokine production by neutrophils, RUNX1 mutations may further enhance the inflamed state of SCN-HPCs, possibly via a feed-back loop involving the more mature progeny of these HPCs.37

**AUTHOR CONTRIBUTIONS**

Conceptualization: Patricia A. Olofsen and Ivo P. Touw. Methodology: Patricia A. Olofsen and Hans W.J. de Looper. Formal Analysis: Patricia A. Olofsen and Remco M. Hoogenboezem. Investigation: Patricia A. Olofsen, Dennis A. Bosch, Hans W.J. de Looper, Paulina M.H. van Strien, Vincent H.J. van der Velden and Eric M.J. Bindels. Data Curation: Remco M. Hoogenboezem. Writing – Original Draft: Patricia A. Olofsen and Ivo P. Touw. Writing – Reviewing and Editin: Eric M.J. Bindels, Emma M. de Pater. Visualization: Patricia A. Olofsen. Supervision: Ivo P. Touw. Funding Acquisition: Ivo P. Touw.

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**CONFLICT OF INTEREST**

None of the authors have conflicts of interest to disclose.

**DATA AVAILABILITY STATEMENT**

The RNA sequencing data are available at ArrayExpress: E-MTAB-10288. For additional original data, please contact i.touw@erasusmc.nl.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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