Loss-of-function mutations in *CSF3R* cause moderate neutropenia with fully mature neutrophils: two novel pedigrees

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To the Editor:

The growth factor granulocyte colony-stimulating factor (G-CSF) has been described as a key regulator of neutrophil development by inducing proliferation, differentiation, and survival of myeloid progenitors. In case of infection, G-CSF stimulates emergency granulopoiesis (Panopoulos and Watowich 2008). The receptor for G-CSF (G-CSF-R), encoded by CSF3R, is a member of the type I cytokine receptor superfamily. One of the major pathways activated upon G-CSF-R stimulation is the Janus kinase (JAK)-signal transducer and activators of transcription (STAT) pathway, leading to phosphorylation of STAT1, 3 and 5, which subsequently can induce transcription of their target genes (Dong et al., 1998).

To date, 8 families with 11 (inherited) loss-of-function mutations in CSF3R in 10 patients have been reported (supplementary Table SI) (Dror et al., 2000; Druhan et al., 2005; Klimiankou et al., 2015; Sinha et al., 2003; Triot et al., 2014; Ward et al., 1999; Yilmaz Karapinar et al., 2019; Yilmaz Karapinar et al., 2018). These patients suffer from congenital neutropenia and cannot be treated with G-CSF due to refractoriness. The consequence of G-CSF-R deficiency for cellular differentiation, effector functions and signal transduction in human primary neutrophils have not been studied in detail.

Genetic testing on the first patient with moderate-to-mild neutropenia was performed at the age of 40 (Fig 1A-B; panel I). She had been admitted with several infectious episodes for which intravenous antibiotics were prescribed. A bone marrow aspirate showed a left shift in granulopoiesis. Upon treatment with G-CSF (before genetic analysis was performed) no improvement in neutrophil count was repeatedly noticed (for patient history, see Supporting Information). Genetic analysis identified compound heterozygous mutations in CSF3R (NM_000760.3) with a novel conserved missense variant (c.359G>A, p.(G120D); CADD score 29) and a nonsense variant with a population frequency of approximately 1:5000 (gnomAD AF = 0.0003; BRIDGE/GEL AF = 0.0007 at c.1640G>A, p.(W547*)).

A second case, from a consanguineous family, had mild neutropenia and suffered from unexplained febrile episodes for about 5 years (Fig 1A-B; panel II). Genetic analysis identified a homozygous missense mutation in CSF3R (c.1318C>T, p.(R440*))). Out of 4 other siblings, one was also homozygous (patient 3), one a heterozygous carrier, and one did not have the genetic variant. Whilst being mildly neutropenic, patients 2 and 3 were clinically not severely affected (Fig 1B; Supporting Information).

All reported inherited loss-of-function mutations in CSF3R are present in the extracellular domain of CSF3R, of which 4 are localized near or in the conserved WSXWS motif (Fig 1C). The mutations in
patient 1 were localized in the so-called CRH region and third FNIII domain, respectively (Fig 1C). The mutation in the FNIII domain leading to a stop-codon (p.W547*) has been previously described (Klimiankou et al., 2015). The homozygous mutations in patients 2 and 3 are localized in the second FNIII domain and lead to a premature stop.

Circulating neutrophils demonstrated a normal morphology and segmented nuclei (Fig 1D). Neutrophils from the UK samples showed some apoptotic cells, which can be explained by the shipment of blood samples to our laboratory. Flow cytometric analysis did not reveal differences in expression of the neutrophil maturation marker EGF module-containing mucin-like hormone receptor 3 (EMR3) (Matmati et al., 2007) between control and patient neutrophils (Figure S1).

Label-free quantitative mass spectrometry-based proteomics was performed on neutrophils of patients, patients’ family members, and controls. No large differences between granule proteins and components of the NADPH-oxidase complex were found, indicating intact neutrophil development at all stages (Hoogendijk et al., 2019) (Figure S2, A-B). Of the 2,849 proteins which were identified and quantified, 10 proteins showed altered expression between patients and controls (Figure S3). Altered expressed proteins include proteoglycan 3 (PRG3), eosinophil cationic protein (RNASE3) and eosinophil peroxidase (EPX), indicative of some eosinophil contamination which was also observed by cytospin (i.e. 5.3 ± 3.3% in controls vs. 10.3 ± 2.6% in patients).

Immunostaining of G-CSF-R showed greatly reduced expression on patient neutrophils. In the second pedigree, flow cytometry showed intermediate expression on the heterozygous family members, while the unaffected sibling had G-CSF-R surface staining comparable to unrelated healthy controls (Fig 2A and Figure S4). Intermediate G-CSF-R expression did not result in reduced neutrophil counts (data not shown).

G-CSF is important for neutrophil survival. Although granulocyte-macrophage colony-stimulating factor (GM-CSF) has overlapping functions with G-CSF, it signals via a different receptor (GM-CSF-R) (Mehta et al., 2015). When comparing the response to both growth factors, G-CSF failed to induce survival of patient neutrophils (Fig 2B and Figure S5). Additionally, we tested the priming capacity of G-CSF by formyl-Met-Leu-Phe-induced reactive oxygen species (ROS) production in neutrophils. Again and in contrast to control cells, patient neutrophils did not show an enhanced ROS production upon pre-incubation with G-CSF while being responsive to GM-CSF (Fig 2C).
To confirm the absence of G-CSF-R signalling, STAT3 phosphorylation was evaluated upon incubation with GM-CSF or G-CSF. Both control and patient neutrophils showed STAT3 phosphorylation upon GM-CSF stimulation, whereas only control neutrophils demonstrated STAT3 phosphorylation after G-CSF stimulation (Figure 2D and Figure S6). These data show that the mutations in CSF3R in these patients resulted in lack of G-CSF-R expression and function.

Although the missense variant (c.359G>A, p.(G120D)) in patient 1 might result in residual expression of G-CSF-R, functional analysis on primary neutrophils and in-vitro and in-vivo refractoriness to G-CSF proves that this missense variant has a deleterious impact on receptor signalling.

Despite lack of G-CSF-R expression and signalling in neutrophils, the neutropenia in these patients is moderate-to-mild. This finding implies that the myeloid progenitors are able to generate a pool of normal differentiated and functionally mature neutrophils. This is reminiscent of G-CSF-R knockout models in mice, in which neutrophil counts were decreased for 50% at most (Liu et al., 1996). Bone marrow samples of patients were not available for functional analysis. Still, there is a lack of immediate response to G-CSF-induced release of neutrophils from the bone marrow upon infection or exogenous administration, as exemplified in the first patient. In healthy controls, there is low expression of G-CSF-R on bone marrow neutrophils compared to circulating neutrophils as assessed by flow cytometry (Figure S7). These results suggest that G-CSF-R is present on committed progenitors for accelerating neutrophil production required for ‘emergency neutrophilia’ at the final stage of segmented neutrophils and immediate release of the bone marrow reserve pool. G-CSF-induced mobilization of hematopoietic progenitor cells coincides with cleavage of C-X-C Motif Chemokine Receptor 4 (CXCR4) and its ligand (stromal cell-derived factor 1, SDF-1) by neutrophil proteases in the bone marrow (Levesque et al., 2003). As the CXCR4/SDF-1 axis is also implicated in neutrophil retention (Kawai and Malech 2009), it is expected that the same mechanism applies for the egress of mature neutrophils. The increase of G-CSF-R expression at the final stage of neutrophil development would also be compatible with the strong pro-survival effect of G-CSF on circulating and bone marrow neutrophils (data not shown).

In the absence of G-CSF-R, neutrophil differentiation in the bone marrow can still proceed under the influence of other factors like GM-CSF, interleukin-6 and as yet unidentified factors (Mehta et al., 2015). However, these factors combined do not seem to fully compensate for G-CSF deficiency because neutrophil production remains quantitatively impaired in individuals which lack GCSF-R expression.
In sum, our data support the notion that the major role of G-CSF in neutrophil expansion and release may be during inflammatory responses when a larger bone marrow output and prolonged survival is required to cope with serious infections but not for neutrophil differentiation per se.

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Authorship contributions

Contribution: E.G.G.S., S.O.B. and T.W.K. wrote the manuscript; E.G.G.S., A.T.J.T., and T.W.K. designed the experiments. E.G.G.S., and A.T.J.T. performed experiments and analyzed the data; S.O.B. and T.W.K. analyzed clinical data; NIHR BioResource, J.M. and K.v.L. performed DNA analysis; I.C.K. and F.P.J.v.A. performed mass spectrometry analysis; and all authors read, revised and approved the manuscript.

Conflict of interest

The authors declare that they have no relevant conflicts of interest.

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Supporting information

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

References


**Figure Legends**

**Fig 1.** *CSF3R* mutations and family pedigrees. (A) Pedigree of the UK patient (panel I) and NL patients (panel II). (B) Absolute neutrophil counts (ANC) of the UK patient during G-CSF treatment (panel I) and the NL patients (no treatment; panel II). Dashed horizontal line represents normal ANC counts. (C) Schematic representation of G-CSF-R showing the various structural domains and their amino acid positions. The zoomed panel shows the conserved WSXWS motif located in the CRH region. Mutations in the extracellular domain of G-CSF-R described in congenital neutropenia patients are displayed above the diagram, novel mutations below. Underlined mutations represent homozygous mutations. Boxes with identical color represent mutations in same patient/family. aa = amino acid (D) Cytospins of isolated neutrophils from controls and patients (original magnification x400; May-Grünwald/Giemsa stain). ¥ = apoptotic cell, * = eosinophil.

**Fig 2.** Functional characteristics of G-CSF-R-deficient primary neutrophils. (A) G-CSF-R expression on neutrophils was assessed by flow cytometry. (B) Viability upon overnight incubation with GM-CSF (20 ng/mL) or G-CSF (20 ng/mL) was assessed by flow cytometry (mean + range, n = 2 of patient 1 and 2, n = 1 of patient 3). (C) Priming capacity of neutrophils was assessed upon 30 minute pre-incubation with GM-CSF or G-CSF and subsequent stimulation with fMLF (1 μM). Extracellular H₂O₂ was measured by Amplex Red assay (mean + range, n = 2 of patient 1 and 2, n = 1 of patient 3). (D) Phosphorylation of STAT-3 (p-STAT3) in neutrophils upon stimulation with GM-CSF and G-CSF (time of stimulation shown in minutes) was assessed by Western blot. GAPDH was used as loading control. Western blot of patient 3 is shown as representative blot.
A

B

C

D

- Controls
- Patient 1 - UK
- Patient 2 - NL
- Patient 3 - NL

% AnnexinV negative PMNs

- GM-CSF
- G-CSF

fMLF-induced Amplex Red
(nMol H2O2/ min/ 1x10⁶ PMNs)

- Control
- Patient 3
- G-CSF

- GM-CSF

minutes
0 5 10 20

p-STAT3
STAT3
GAPDH

- Control
- Patient 3
- G-CSF

- GM-CSF