Elevated granulocyte-colony stimulating factor and hematopoietic stem cell mobilization in Niemann-Pick type C1 disease

Anouk G. Groenen1,†, Anouk M. La Rose1,‡, Mengying Li2, Venetia Bazioti1, Arthur F. Svendsen3, Niels J. Kloosterhuis1, Albertina Ausema1, Alle Pranger1, M. Rebecca Heiner-Fokkema2, Klarý E. Niezen-Koning2, Tom Houben2, Ronit Šírši-Sverdlov2, and Marit Westerterp1,‡

1Department of Pediatrics, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; 2Department of Genetics and Cell Biology, School of Nutrition and Translational Research in Metabolism (NUTRIM), University of Maastricht, Maastricht, The Netherlands; 3European Research Institute for the Biology of Ageing, and 4Department of Laboratory Medicine, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Abstract Niemann-Pick type C1 (NPC1) disease is a progressive lysosomal storage disorder caused by mutations of the NPC1 gene. While neurodegeneration is the most severe symptom, a large proportion of NPC1 patients also present with splenomegaly, which has been attributed to cholesterol and glycosphingolipid accumulation in late endosomes and lysosomes. However, recent data also reveal an increase in the inflammatory monocyte subset in the patients also present with splenomegaly, which has triggered interest in the role of monocytes/macrophages in NPC disease. We transplanted NPC1 disease under conditions of hypercholesterolemia. We transplanted Npc1null (NPC1 null mutation) or Npc1mut bone marrow (BM) into Ldlr−/− mice and fed these mice a cholesterol-rich Western-type diet. At 9 weeks after BM transplant, on a chow diet, the Npc1 null mutation increased plasma granulocyte-colony stimulating factor (G-CSF) by 2-fold and caused mild neutrophilia. At 18 weeks after BM transplant, including 9 weeks of Western-type diet feeding, the Npc1 mutation increased G-csf mRNA levels by ∼5-fold in splenic monocytes/macrophages accompanied by a ∼4-fold increase in splenic neutrophils compared with controls. We also observed ∼5-fold increased long-term and short-term hematopoietic stem cells (HSCs) in the spleen, and a ∼30–75% decrease of these populations in BM, reflecting HSC mobilization, presumably downstream of elevated G-CSF. In line with these data, four patients with NPC1 disease showed higher plasma G-CSF compared with age-matched and gender-matched healthy controls. In conclusion, we show elevated G-CSF levels and HSC mobilization in the setting of an Npc1 null mutation and propose that this contributes to splenomegaly in patients with NPC1 disease.

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Supplementary key words cholesterol/trafficking • storage diseases • neutrophils • macrophages/monocytes • animal models • inflammation • splenomegaly • hematopoietic stem cells

‡These authors contributed equally to this work.
*For correspondence: Marit Westerterp, m.westerterp@umcg.nl.

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Studies using antisense oligonucleotides for Npc1 that mainly cause deletion of this gene in hepatocytes have suggested that Npc1 deficiency causes extramedullary hematopoiesis (21). Extramedullary hematopoiesis is the result of hematopoietic stem and progenitor cell (HSPC) mobilization from the bone marrow (BM) to liver and/or spleen. As a consequence, these organs resume their fetal hematopoietic function, reflected by stem cell proliferation and production of monocytes and neutrophils, which causes hepatosplenomegaly (22). Extramedullary hematopoiesis is the result of hematopoietic stem and progenitor cell (HSPC) mobilization from the bone marrow (BM) to liver and/or spleen. As a consequence, these organs resume their fetal hematopoietic function, reflected by stem cell proliferation and production of monocytes and neutrophils, which causes hepatosplenomegaly (22). 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**MATERIALS AND METHODS**

**Animals**

Niemann-Pick type C1m1 (Npc1m1), also known as Npc1m1/Npc1m1, heterozygous mice on the C57BL/6 background were intercrossed to generate homozygous Npc1m1 and Npc1m1 littermates (28). Mice homozygous for the Npc1m1 null mutation, hereafter referred to as Npc1mut, show similar phenotypes to human carriers of NPC1 mutation including lysosomal cholesterol and sphingolipid accumulation, hepatosplenomegaly, and neurologic impairment (29, 30). Npc1mut and Npc1m1 BM donors were sacrificed at 5 weeks of age, femur and tibia were collected, and BM was harvested. Mice deficient in the LDLR (Ldlr−/−) on the C57BL/6 background (stock no.: 002207) were obtained from Jackson Laboratories (Bar Harbor, ME) and bred inhouse. Mice were housed under standard laboratory conditions with a light cycle of 12 h and ad libitum water and food. Mice were randomly assigned to experimental groups. The number of mice used for each experiment is indicated in the figure legends. All animal studies were approved by the Institutional Animal Care and Use Committee from the University of Groningen under permit number AVD105002015244 and adhered to guidelines set out in the 2010/63/European Union directive.

**Patients**

Four patients carrying NPC1 missense mutations and age-matched and gender-matched controls were included in this study (31). The characteristics of these patients and their age-matched and gender-matched controls are shown in Table 1. In two of four patients, filipin staining was performed and positive. Three patients presented with juvenile onset of NPC1 disease and one patient with adult onset (31). Plasma was collected from these patients and their age-matched and

| Patient | Gender | Age at Plasma Investigation (Years) | Control/NPC1 Mutation | Treatment After Plasma Investigation |
|---------|--------|-----------------------------------|------------------------|-------------------------------------|
| 1       | Female | 69                                | Compound heterozygous mutations c.180G>T (p.Gln60His) and c.2849T>G (p.Val950Gly) | None                                |
| 2       | Female | 24                                | Compound heterozygous mutations c.1211G>A (p.Arg404Gln) and c.2861C>T (p.Ser954Leu) | Liver transplantation               |
| 3       | Female | 18                                | Homozygous mutations c.1918G>A (p.Gly640Arg) | Miglustat                           |
| 4       | Male   | 17                                | Compound heterozygous mutations c.346C>T (p.Arg116*) and c.247A>G (p.Thyr825Cys) | None                                |
| 5       | Female | 70                                | Control                | Not applicable                      |
| 6       | Female | 26                                | Control                | Not applicable                      |
| 7       | Female | 17                                | Control                | Not applicable                      |
| 8       | Male   | 17                                | Control                | Not applicable                      |

**TABLE 1. Characteristics of NPC1 patients and controls**

Plasma LDL-cholesterol levels in Ldlr−/− mice fed a WTD. We used plasma from patients with NPC1 missense mutations to assess human relevance.
gender-matched controls during a regular visit at the University Medical Center Groningen. The need for formal ethical review was waived by the local ethics committee of the University Medical Center Groningen, since we made use of blood that was drawn regularly during outpatient visits and leftover from diagnostic investigation. The study design was in accordance with the current revision of the Helsinki Declaration.

**Bone marrow transplantation**

Ldlr<sup>−/−</sup> BM recipients were group-housed in individually ventilated cages and received ciprofloxacin (0.1 mg/ml; Fresenius Kabi, Zeist, The Netherlands) in the drinking water for 10 days, starting 1 day prior to irradiation. At 8 weeks of age, Ldlr<sup>−/−</sup> BM recipients were irradiated with a lethal dose (9 Gy) using the X-rad 320 irradiator (Precision X-Ray, North Branford, CT). The next day, mice were transplanted with 10<sup>6</sup> BM cells. BM donors were 5 weeks of age. Mice were allowed to recover for 3 weeks after bone marrow transplantation (BMT). After the recovery period, mice were transferred to conventional open-top cages. Mice were fed a chow diet for 9 weeks after BMT (catalog no: V1554; Seniff Spezialfutters GmbH, Soest, Germany). Subsequently, mice were fed a WTD (50% carbohydrates, 20% proteins, 25% fat consisting of 20% milk fat and 1% corn oil, 0.15% cholesterol; D12079B; Research Diets, New Brunswick, NJ) for 9 weeks.

**White blood cell counts and flow cytometry**

Blood samples were collected by tail bleeding into EDTA-coated tubes and kept on ice. Total white blood cell (WBC) counts were measured using the Medonic CD620 hematology analyzer (Boule Medical, Sanga, Sweden). For flow cytometry, samples were kept at 4°C for the whole procedure unless stated otherwise. Red blood cells (RBCs) were lysed for 5 min (BD Pharm Lyse; BD Bioscience, Franklin Lakes, NJ), and WBCs were centrifuged, washed, and resuspended in HBSS (0.1% BSA and 0.5 mM EDTA). To assess monocytes, monocyte subsets, and neutrophils, cells were stained with a cocktail of antibodies: CD45-APC-Cy7 (catalog no.: 557659; BD Biosciences, Franklin Lakes, NJ), CD11b-A700 (catalog no.: 101222), CD3-A700 (catalog no.: 100216), CD48-PE (catalog no.: 105808), CD150-PECy7 (catalog no.: 115914), CD48-PE (catalog no.: 105808), CD150-PECy7 (catalog no.: 115914), CD48-PE (catalog no.: 105808), CD150-PECy7 (catalog no.: 115914), and Sca1-BV421 (catalog no.: 108127). For both BM and spleen samples, RBCs were lysed for 3 min in the dark. HSPCs were identified as CD45<sup>hi</sup>CD115<sup>lo</sup>Ly6G<sup>hi</sup>. To assess apoptosis, cells were stained with a solution for 7-ketocholesterol (7-KC)-d7 or cholestanol<sup>d7</sup> and measured in plasma using ELISA kits (for mouse: MCS00; for human: DCS50; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

**Plasma total cholesterol and lipoprotein cholesterol distribution**

Blood samples were collected from mice. Plasma was separated by centrifugation, and cholesterol levels were measured using an enzymatic kit (catalog no: 113009910026; Diasys Diagnostic Systems, Holzheim, Germany) with Cholesterol FS standard (catalog no: 113009910030; Diasys Diagnostic Systems) for the calibration curve. Lipoprotein cholesterol distribution was measured by fast performance liquid chromatography using a system containing a PU-4180 pump with a linear degasser and UV-4075 UV/VIS detectors (Jasco, Tokyo, Japan). Pooled plasma samples (n = 15–16 mice per pool) were injected onto a Supercos 6 Increase 10/300 GL column (GE Healthcare, Hoevelaken, The Netherlands) and eluted at a constant flow rate of 0.31 ml/min in PBS (pH 7.4). Cholesterol was measured in line by addition of cholesterol reagent at a constant flow rate of 0.1 ml/min using an additional PL-1086 infusion pump (Jasco, Tokyo, Japan). Data acquisition and analysis were performed using ChromNav software (version 1.0; Jasco, Tokyo, Japan).

**Granulocyte colony-stimulating factor ELISAs**

Blood samples were collected from mice or patients carrying NPC1 mutations and their age-matched and gender-matched controls. Plasma was separated by centrifugation, and granulocyte colony-stimulating factor (G-CSF) levels were measured in plasma using ELISA kits (for mouse: MCS00; for humans: DCS50; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

**Plasma oxysterol analysis**

Blood samples were collected from mice, and plasma was separated by centrifugation. Approximately 100 μl internal standard for 7-ketocholesterol (7-KC)-d7 or cholestanol<sup>3β,5α,6β-triol</sup> (C-triol)-d7 and 50 μl butyrylhydroxytoleulene in methanol (40 g/l) were added to 50 μl plasma and mixed for 10 min. Two extractions were performed using methyl tert-butyl ether. Then, 1 ml water was added to a combined sample of two fractions and mixed for 1 min. The two fractions were separated from water and dried using nitrogen. Subsequently, 3 ml hexane and 100 μl Sylon-BTZ (B S:trimethylchlorosilane:N-trimethylsilylimidazole 3:2) were added, and the whole sample was incubated for 10 min, resulting in formation of TMS derivatives from 7-KC or C-triol. These were analyzed with gas chromatography-tandem MS (Agilent 7000B triple quadrupole; 7890A GC) using positive chemical ionization with 5% ammonia in methane as reaction gas and a nonpolar DB-5MS (15 m × 0.250 mm × 1.00 μm) column (Agilent). Approximately 10 μl sample was injected according to the solvent vent approach at 50°C for 0.45 min. Subsequently, the oven temperature increased to 300°C with 600°C/min. The oven temperature was 70°C for 2.64 min and increased to 320°C with 40°C/min and hold time 10.5 min. The pressure in the column was 16 psi. The MS-source temperature is 300°C.
and the quadrupole is 150°C. The selected mass transitions were C-triol precursor m/z 475.4, product m/z 457.4; C-triol-d7 precursor m/z 482.4, product m/z 464.4; 7-KC precursor m/z 473.4, product m/z 383.4; and 7-KC-d7 precursor m/z 480.5 and product m/z 390.5.

**Monocyte assays**

Monocytes were isolated from BM of wild-type (WT) mice using CD11b+ beads and cultured in DEMEM ( Gibco) supplemented with 20% L cell conditioned medium, 10% FCS, and 1% penicillin-streptomycin. After 2 h, 5 μg/ml U18666A (catalog no: 5390-71-2; MilliporeSigma, MO) and 20 μg/ml 7-KC (catalog no: 566-28-9; MilliporeSigma) were added for 17 h. Cells were collected and lysed in RNeasy lysis buffer. RNA was extracted using an RNeasy mini kit (catalog no: 74106; Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized using the transcription universal cDNA master kit (Roche, Basel, Switzerland). G-εf (forward: 5’-GTTCCTCTGTCGCTA-3’; reverse: 5’-TAGGGTGCCACCAACTGTC-3’) mRNA expression levels were assessed by quantitative PCR using QuantiStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA) and corrected for initial variabilities in RNA quantity using the housekeeping genes m36B4 and cyclophilin B.

**Isolation of splenic monocytes and macrophages**

Spleens were isolated and mashed on a 40 μm strainer. RBCs were lysed for 2 min on ice, and WBCs were centrifuged, washed, and resuspended in PBS (0.5% BSA and 2 mM EDTA). First, splenic homogenates were depleted of Ly6G+ cells using Ly6G-coated magnetic beads (catalog no: 130-120-337; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. CD11b+ cells were isolated from splenic homogenates using CD11b-coated magnetic beads (catalog no: 130-049-601; Miltenyi Biotec) according to the manufacturer’s instructions. Cells were lysed in RNeasy lysis buffer, and RNA was extracted using an RNeasy mini kit (catalog no: 74106; Qiagen) according to the manufacturer’s instructions. cDNA was synthesized using the transcription universal cDNA master kit (Roche). G-εf (for primers, see aforementioned), H2Aa (forward: 5′-GGGCAAGTGCTGGTTT-3’; reverse: 5′-GGTGGCACACAACTGTCG-3’), Tnf (forward: 5′-GAGAGCTACAAGAGGATCA-3’; reverse: 5′-GTGTACCCGTGTAGCAAAC-3’), macrophage colony-stimulating factor (M-CSF) (forward: 5′-GGTGGCACACAACTGTCG-3’; reverse: 5′-GTGTACCCGTGTAGCAAAC-3’), and nuclear receptor subfamily 4 group A, member 1 (NRP4) (forward: 5′-TTGAGTTCGCCAAAGCTGTCG-3’; reverse: 5′-GTGTACCAGCTGAGGATTG-3’) mRNA expression levels were assessed by quantitative PCR using QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) and corrected for initial variabilities in RNA quantity using the housekeeping genes m36B4 and cyclophilin B.

**Statistical analysis**

All data are presented as mean ± SEM. The two-tailed unpaired t-test was used to compare two datasets. The one-way ANOVA with a Bonferroni multiple comparison post-test was used to compare four groups. To assess differences between NPC1 mutation carriers and their age-matched and gender-matched controls, one-tailed paired t-test was used, taking into account data clustering based on age and gender. Group size and statistical test are reported in the figure legend. The criterion for significance was set at P < 0.05. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc, San Diego, CA).

**RESULTS**

**Effects of hematopoietic Npc1 loss-of-function on myeloid cells in Ldlr−/− mice**

Splenomegaly in patients with NPC1 disease can be due to various mechanisms that are presumably regulated via its role in intracellular cholesterol transport in hematopoietic cells. To investigate the role of hematopoietic Npc1 in splenomegaly under conditions of high LDL-cholesterol, we transplanted BM from mice with a homozygous Npc1 null mutation (Npc1mut) or from Npc1 WT (Npc1+/-) mice (controls) into Ldlr−/− mice. At 5 weeks after BMT, when mice were fed a chow diet, we observed no effects of hematopoietic Npc1 mutation on total WBC counts (Fig. 1A), suggesting no difference in BM reconstitution between the groups of mice, which was similar to a previous study using the exact same approach (28). Hematopoietic Npc1 loss-of-function did not affect total blood monocytes in Ldlr−/− mice but decreased Ly6Clo monocytes by ~55% and increased proinflammatory Ly6Chi monocytes by ~38% and blood neutrophils by ~41% (Fig. 1B, D). While effects of the Npc1 mutation on blood neutrophils have not been reported previously, the observations on blood monocytes were in line with previous findings and already observed in mice with the same Npc1 mutation at 5 weeks of age, the same age of the BM donors (32).

WTD feeding increased blood monocytes in Npc1mut BMT Ldlr−/− mice, in line with previous findings (33, 34), but, unexpectedly, not in Npc1mut WT Ldlr−/− mice (Fig. 2A and supplemental Fig. S1). Surprisingly, the Npc1 mutation also did not longer affect blood neutrophil levels in Ldlr−/− mice fed WTD (Fig. 2A and supplemental Fig. S1). The ~48% decrease in blood monocytes in WTD-fed Npc1mut BMT compared with Npc1mut BMT Ldlr−/− mice was reflected by a decrease in the Ly6Clo monocyte subset (Fig. 2A and supplemental Fig. S1). In addition, hematopoietic Npc1 loss-of-function mutation decreased plasma cholesterol levels, mainly in LDL-cholesterol in WTD-fed Ldlr−/− mice (Table 2), in line with previous studies (28, 35). The decrease in plasma cholesterol was reflected by a reduction in the VLDL-cholesterol and LDL-cholesterol in Ldlr−/− mice fed a WTD for 9 weeks (supplemental Fig. S2). Similar to findings in blood, also in BM of WTD-fed Npc1mut BMT Ldlr−/− mice, Ly6Clo monocytes were decreased, whereas Ly6Chih monocytes and neutrophils were not affected compared with WTD-fed Npc1mut BMT Ldlr−/− mice (Fig. 2B, C). In spleen, we observed a similar reduction in Ly6Clo monocytes of WTD-fed Npc1mut BMT Ldlr−/− mice,
whereas Ly6C<sup>hi</sup> monocytes and neutrophils were increased compared with WTD-fed Npc1<sup>wt</sup> BMT Ldlr<sup>−/−</sup> mice (Fig. 2D, E). The increase in neutrophils was ∼4-fold (Fig. 2D, E).

Collectively, the Npc1 mutation in hematopoietic cells decreased blood, BM, and splenic Ly6C<sup>lo</sup> monocytes in Ldlr<sup>−/−</sup> mice fed chow diet or WTD. The Npc1 mutation increased blood Ly6C<sup>hi</sup> monocytes and neutrophils only in chow diet, but not WTD-fed Ldlr<sup>−/−</sup> mice, while increasing splenic Ly6C<sup>lo</sup> monocytes and neutrophils in WTD-fed Ldlr<sup>−/−</sup> mice. Of all changes, the decrease in Ly6C<sup>lo</sup> monocytes (∼74%) and the increase in splenic neutrophils were most pronounced (∼4-fold).

**Effects of the Npc1 mutation in hematopoietic cells on inflammatory gene expression in splenic monocytes and macrophages of WTD-fed Ldlr<sup>−/−</sup> mice**

Because of the pronounced effects of the hematopoietic Npc1 loss-of-function on splenic neutrophils, we characterized the splenic myeloid cell population further. G-CSF production in monocytes and macrophages acts on the granulocyte macrophage progenitors (GMPs) to stimulate neutrophil production, while M-CSF acts on GMPs to stimulate the production of monocytes (36–38). We thus isolated splenic monocytes and macrophages (Ly6G<sup>−</sup>CD11b<sup>+</sup> cells) and measured G-csf and M-csf mRNA expression, as well as expression of other proinflammatory cytokines that may be increased in NPC1 disease (28, 39–41). The Npc1 mutation increased G-csf mRNA expression by ∼5-fold, while not affecting M-csf, and increasing Tnfα and Mcp-1 (Fig. 3A). While the increased G-csf mRNA expression likely explains the expansion of the splenic neutrophil population, the increase in Tnfα and Mcp-1 suggests an overall increase in inflammation, in line with previous studies (39, 41, 42), and potentially contributing to the increase in Ly6C<sup>hi</sup> monocytes (43).

In addition, we observed that the Npc1 mutation
increased interleukin-23a (IL-23a) mRNA expression (Fig. 3A). IL-23 regulates G-CSF levels, by activating the differentiation of naïve T-cells into T h17 cells that stimulate production of G-CSF (36). The increase in IL-23 may thus have contributed to the elevated G-csf mRNA expression in mice with the Npc1 mutation. We also observed that the Npc1 mutation decreased mRNA expression of Nr4a1 (Nur77 or NGFIB) in splenic monocytes/macrophages (Fig. 3B). Since Nr4a1 is crucial for Ly6C<sup>lo</sup> monocyte survival (44, 45), this decrease could explain the decrease in the Ly6C<sup>lo</sup> monocyte population. We further assessed this by measuring pro-apoptotic cleaved caspase-3/7 in Ly6C<sup>lo</sup> monocytes. We found that the Npc1 mutation in hematopoietic cells increased cleaved caspase-3/7 in blood Ly6C<sup>lo</sup> monocytes (Fig. 3C, D), suggestive of increased apoptosis. This observation is consistent with a decrease in the Ly6C<sup>lo</sup> monocyte population.
Collectively, the increase in splenic neutrophils in Ldlr−/− mice with the Npc1 mutation in hematopoietic cells may be the consequence of increased G-csf mRNA expression in splenic monocytes/macrophages, whereas the decrease in Nr4a1 mRNA expression likely accounts for the decrease in Ly6Clo monocytes. The increase in caspase-3/7 suggests this is downstream of apoptosis.

NPCI inhibition increases G-csf mRNA expression in monocytes stimulated with 7-KC

We then investigated the mechanism for the increase in G-csf mRNA in splenic monocytes/macrophages of Ldlr−/− mice with the Npc1 mutation in hematopoietic cells. Previous studies have shown that hematopoietic Npc1 loss-of-function increases plasma 7-KC and C-atriol levels in WTD-fed Ldlr−/− mice, similar to findings in NPC1 disease patients (35, 46). We replicated this finding in our study (Fig. 3E). The increase in inflammation in NPC1 disease has been attributed to increased oxidative stress downstream of 7-KC accumulation (35), presumably mediated by reactive oxygen species and NF-kB activation (47–49). We investigated whether 7-KC could account for the increase in G-csf mRNA in monocytes and macrophages with Npc1 loss-of-function. In the unstimulated condition, G-csf mRNA expression in BM monocytes was ~20-fold higher than in BM-derived macrophages (results not shown). We thus used BM monocytes for our experiments and incubated these with 7-KC, while inhibiting NPC1 using the U18666A compound. 7-KC increased G-csf mRNA expression in monocytes treated with U18666A (Fig. 3F). These data suggest that the increase in plasma 7-KC may account for the increased G-csf mRNA expression in splenic CD11b+ monocytes in WTD-fed Ldlr−/− mice with hematopoietic Npc1 loss-of-function.

Npc1 mutations increase plasma G-CSF levels in Ldlr−/− mice fed chow diet

We then investigated whether an increase in splenic monocyte/macrophage G-csf mRNA expression would translate into an increase in plasma G-CSF levels in the setting of the Npc1 mutation. While this was indeed the case in Ldlr−/− mice fed chow diet, hematopoietic Npc1 loss of function did not affect plasma G-CSF levels in Ldlr−/− mice fed WTD (Fig. 3G, H). Since monocytes produce G-CSF, we attribute this seeming discrepancy to the decrease in blood monocytes in WTD-fed, but not in chow diet-fed Npc1mut BMT Ldlr−/− mice (Figs. 1, 2A). Consistently, the Npc1 mutation increased blood neutrophil levels in chow diet, but not WTD-fed Ldlr−/− mice (Figs. 1, 2A).

Hematopoietic Npc1 loss-of-function enhances hematopoietic stem cell mobilization in WTD-fed Ldlr−/− mice

G-CSF increases mobilization of stem cells from BM to spleen (26). We thus assessed splenic and BM stem cell populations. The hematopoietic Npc1 mutation increased LT-HSCs and ST-HSCs in spleen and tended to increase MPPs (Fig. 4A, B), which was accompanied by a 2-fold increase in spleen weight (Fig. 4C). These data are suggestive of HSC mobilization. We also assessed BM stem cell populations. The Npc1 mutation decreased BM LT-HSC, ST-HSC, and MPP populations (Fig. 4D, E), further supporting HSC mobilization. Together, these data suggest that the Npc1 mutation in hematopoietic cells induces mobilization of HSCs from BM to spleen. Given that the Npc1 mutation increases G-csf mRNA expression in monocytes and macrophages, and increases plasma G-CSF in chow diet-fed Ldlr−/− mice, these effects are most likely dependent on G-CSF.

NPCI patients show high plasma G-CSF levels

We then evaluated translational relevance of our findings in NPC1 patients. NPC1 mutations and patient characteristics are listed in Table 1. Plasma samples were drawn from NPC1 patients before any intervention for NPC1 disease. Interestingly, patients carrying mutations in NPC1 also showed high G-CSF plasma levels compared with gender-matched and age-matched controls (Fig. 5), suggesting that the findings on G-CSF in mice with the Npc1 mutation may be relevant for NPC1 disease in humans. Although patient populations were small, it is of interest that the two patients with the highest plasma G-CSF concentration were the ones that eventually received an intervention, either being miglustat, a sphingolipid synthesis inhibitor that has been approved as the first drug specifically targeted for NPC1 disease in 2009 (in Europe), and delays disease progression and improves neurological symptoms (50, 51), or a liver transplantation. These observations perhaps suggest that these patients with the highest plasma G-CSF were the most affected in terms of NPC1 disease.
G-CSF levels were also higher in patients with NPC1 missense mutations. It has been well established that G-CSF induces splenomegaly because of HSC mobilization in mice or humans (18). We thus propose that the increase in G-CSF and HSC mobilization accounts for the splenomegaly in patients with NPC1 disease.

We observed that the Npc1 loss-of-function mutation increased plasma G-CSF in Ldlr−/− mice fed chow diet.
but not WTD. However, on WTD, Npc1 loss-of-function increased G-csf mRNA levels by 5-fold in splenic Ly6G<sup>-</sup>CD11b<sup>+</sup> monocytes/macrophages. G-CSF has been reported to skew GMPs toward neutrophil production (37). Indeed, the Npc1 mutation led to a splenic neutrophil expansion of ~4-fold in WTD-fed Ldlr<sup>−/−</sup> mice. In addition, we observed an increase in splenic Ly6Chi monocytes, which we attribute to augmented inflammation (43). Mirroring the findings in spleen, the Npc1 loss-of-function mutation induced neutrophilia and an increase in Ly6G<sup>hi</sup> monocytes in blood of chow diet-fed Ldlr<sup>−/−</sup> mice. These data suggest that similar mechanisms occur during hematopoiesis early after BMT (in BM) and early extramedullary hematopoiesis in the spleen. 

While we cannot exclude an effect of WTD, we propose that plasma G-CSF and blood neutrophils not being elevated by the Npc1 mutation in Ldlr<sup>−/−</sup> mice fed WTD is rather the consequence of the timing of the measurement after BMT than the WTD feeding itself. 

At 18 weeks after BMT, the Npc1 mutation decreased blood monocytes by ~50%, whereas at 9 weeks after BMT, blood monocytes were not affected. Since monocytes produce G-CSF, plasma G-CSF levels no longer being elevated at 18 weeks after BMT may simply have been the consequence of a decrease in blood monocytes. This has likely been preceded by an increase in neutrophil production at the expense of monocyte production by GMPs in mice with the Npc1 loss-of-function mutation, as shown previously in conditions of high G-CSF (37). Thus, after BMT, high G-CSF in mice with the Npc1 loss-of-function mutation induced neutrophilia and subsequently a decrease in blood monocytes. The latter resulted in plasma G-CSF no longer being elevated. As a consequence, later after BMT, blood neutrophils were no longer increased. 

In addition, we observed that mice with the Npc1 loss-of-function mutation showed a decrease in Ly6C<sup>lo</sup> monocytes at all time points after BMT, in blood, BM, and spleen. We attribute this to a decrease in monocyte...
was due to an increase in IL-23 leading to T h17 inflammation in NPC1 disease has been attributed to loss-of-function mutations on \( \text{Npc1} \) carrying the \( \text{Npc1} \) loss-of-function mutation, including \( \text{Abca1} \) and \( \text{Abcg1} \) deficiency or patients with myeloproliferative diseases that are also characterized by a decrease in VLDL/LDL-cholesterol, accompanied by HSPC mobilization and splenomegaly. It has been proposed that the VLDL/LDL-cholesterol uptake by hematopoietic cells drives HSPC proliferation in the spleen, as we recently reviewed (55).

In addition, we found that, similar to \( \text{Ldlr}^{-/-} \) mice carrying the \( \text{Npc1} \) mutation in hematopoietic cells on the chow diet, plasma G-CSF was higher in patients with \( \text{NPC1} \) loss-of-function mutations, suggesting human relevance. No abnormalities on WBC populations have been reported in NPC1 disease, except for platelet dysfunction and rare cases of thrombocytopenia (56). It would be of interest to investigate effects of \( \text{NPC1} \) mutations on monocyte subsets, as we observed in mice, in patients with NPC1 disease. This would not necessarily reflect a change in total monocyte numbers.

On the C57BL/6 background, the average life span of mice carrying this \( \text{Npc1} \) mutation is 48.1 ± 5.1 days (57), illustrating the early onset of NPC1 disease. Based on the WBCs after BMT being similar between the groups after BM reconstitution, we anticipate no adverse effects of early onset NPC1 disease to the BM donors. This is also supported by the finding that the decrease in blood Ly6C\(^{\text{lo}} \) and the increase in Ly6C\(^{\text{hi}} \) monocyte populations at 5 weeks after BMT is similar to those of the BM donors at 5 weeks of age (32).

In sum, we here elucidate a mechanism that may account for splenomegaly in NPC1 disease, involving increased G-CSF and HSC mobilization. While elevated G-CSF may be disadvantageous in terms of its contribution to splenomegaly, G-CSF stimulates neurogenesis (58), which could counteract the neurodegenerative disease in NPC1 patients. Recent studies have shown that stanol supplementation reduces inflammation in mice with the \( \text{Npc1} \) loss-of-function mutation, including a decrease in inflammatory blood monocytes and hepatic neutrophil infiltration (32, 59), perhaps because of reducing 7-KC. Injections with 2-hydroxypropyl-\( \beta \)-cyclodextrin are currently in phase 2/3 clinical trials for NPC1 disease (NCT02534844) and improve liver function, delay neurodegeneration, and increase life span of \( \text{Npe1}^{-/-} \) mice (60–62) but do not decrease splenomegaly in WTD-fed \( \text{Ldlr}^{-/-} \) mice transplanted with BM from mice with \( \text{Npc1} \) loss-of-function (63). A combination therapy of miglustat, 2-hydroxypropyl-\( \beta \)-cyclodextrin, and allopregnanolone decreases splenic T cells, splenic macrophages, and splenic lipid accumulation but does not affect splenomegaly in \( \text{Npc1}^{-/-} \) mice.
mice on the Balb/c background (20). Effects of miglustat on splenomegaly of Npc1−/− mice have not been reported (64, 65). Together, these studies suggest that cyclodextrin or miglustat, while delaying neurodegeneration, may not affect splenomegaly in NPC1 disease. We found that 7-KC, which is increased in plasma of NPC1 disease patients, induced G-csf mRNA expression upon Npc1 inhibition in monocytes. 7-KC is a main constituent of oxLDL and oxLDL neutralization by increasing plasma IgM antibodies of the E06 idiotype strongly trended toward a decrease in splenomegaly (P = 0.06) in a previous study employing the same mouse model of NPC1 disease, where this treatment also improved hepatic inflammation (28). These data indicate a link between oxLDL, 7-KC, G-CSF production, and splenomegaly and suggest E06 as a potential therapy for the peripheral symptoms of NPC1 disease, mainly with regard to the hepatosplenomegaly.

**Data availability**

All data are included in the article.

**Supplemental data**

This article contains supplemental data.

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**Author contributions**

A. G. G., A. M. L. R., and M. W. conceptualization; A. G. G., A. M. L. R., M. W. methodology; A. G. G., A. M. L. R., M. W. investigation; M. R. H.-F., K. E. N.-K., T. H., and R. S.-S. resources; A. G. G., A. M. L. R., and M. W. writing—original draft; M. R. H.-F., K. E. N.-K., T. H., R. S.-S., and M. W. writing—review & editing; M. W. supervision; M. W. funding acquisition.

**Author ORCIDs**

Anouk C. Groenen [https://orcid.org/0000-0002-2573-8701](https://orcid.org/0000-0002-2573-8701)

Anouk M. La Rose [https://orcid.org/0000-0002-9092-4316](https://orcid.org/0000-0002-9092-4316)

Marla Rebecca Heinier-Fokkema [https://orcid.org/0000-0002-3477-0770](https://orcid.org/0000-0002-3477-0770)

Klary E. Niezen-Koning [https://orcid.org/0000-0002-9584-4354](https://orcid.org/0000-0002-9584-4354)

Tom Houben [https://orcid.org/0000-0002-0441-3166](https://orcid.org/0000-0002-0441-3166)

Marit Westerterp [https://orcid.org/0000-0003-2230-1659](https://orcid.org/0000-0003-2230-1659)

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**Conflict of interest**

The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**

7-KC, 7-ketocholesterol; BM, bone marrow; BMT, bone marrow transplantation; C-trioi, cholesterol-3β,α,β-trioi; cDNA, complementary DNA; cK-it, cKit-positive; G-CSF, granulocyte-colony stimulating factor; GMP, granulocyte macrophage progenitor; HSC, hematopoietic stem cell; HSPC, hematopoietic stem and progenitor cell; IL-23A, interleukin-23a; LDLR, LDL receptor; Lin−, lineage-negative; LT-HSC, long-term hematopoietic stem cell; M-CSF, macrophage-colony stimulating factor; Mcp-1, monocyte chemoattractant protein-1; MPP, multipotential progenitor; Npc1, Niemann-Pick type C1; Npc1−/−, Niemann-Pick type C1−/−, Nr4a1, nuclear receptor subfamily 4, group A, member 1; oxLDL, oxidized LDL; RBC, red blood cell; Sca1+, stem cells antigen-1-positive; ST-HSC, short-term hematopoietic stem cell; WBC, white blood cell; WT, wild-type; WTD, Western-type diet.
10. Liscum, L., and Sturley, S. L. (2004) Intracellular trafficking of Niemann-Pick C proteins 1 and 2: obligate components of subcellular lipid transport. Biochim. Biophys. Acta 1685, 22–27

11. Chang, T. Y., Reid, P. C., Sugii, S., Ohgami, N., Cruz, J. C., and Chang, G. C. Y. (2005) Niemann-Pick type C disease and intracellular cholesterol trafficking. J. Biol. Chem. 280, 20917–20920

12. Colombo, A., Dinkel, L., Müller, S. A., Sebastian Monasor, L., Cremers, S., Levine, R. L., Tall, A. R., and Yvan-Charvet, L. (2012) Identification of a genetic locus modulating splenomegaly in mice. Blood 120, 1384–1390

13. Patterson, M. C., Manglez, M. J., Crooke, R. M., and Liscum, L. (2004) Intracellular trafficking of cellular cholesterol trafficking. Blood 103, 350–356

14. Imrie, J., and Wraith, J. E. (2001) Isolated splenomegaly as the presenting feature of Niemann-Pick disease type C. Mod. Pathol. 14, 1550–1556

15. Colombo, A., Dinkel, L., Müller, S. A., Sebastian Monasor, L., Cremers, S., Levine, R. L., Tall, A. R., and Yvan-Charvet, L. (2012) Identification of a genetic locus modulating splenomegaly in mice. Blood 120, 1384–1390

16. Toruner, C. R., Barnes, J. G., Kogan, S., and Seargeant, E. L. (2015) A rare case of Niemann-Pick disease type C without neurological involvement in a 66-year-old patient. Mol. Genet. Metab. Rep. 3, 18–20

17. Dike, C. R., Bernat, J., Bishop, W., and DeGeeter, C. (2019) Niemann-Pick disease type C presenting as very early onset inflammatory bowel disease. BMJ Case Rep. 12, 1–3

18. Roberts, A. W., Hasegawa, M., Metcalf, D., and Foote, S. J. (2000) Bone marrow NR4A expression is not a dominant factor in the development of atherosclerosis or macrophage polarization in mice. Atherosclerosis 151, 839–849

19. Arbel, A. M., Engelen, R., Engelmann, R., Müller-Hilke, B., Frank, M., Burstein, C., Rolls, A., Neidhardt, J., Wree, A., Witt, M., and Bräuer, A. U. (2019) A therapy with miglustat, 2-hydroxypropyl-ß-cyclodextrin and allopregnanolone restores splenic cholesterol homeostasis in Niemann-Pick disease type C. Lipids Health Dis. 18, 146

20. Parra, J., Klein, A. D., Castro, J., Morales, M. G., Mosqueira, M., Valencia, I., Coronado, E., y, Gómez, J. M., and Arenas, J. (2018) ApoE deficiency in LDLr−/− mice, a genetic background enhances Niemann-Pick disease type C spleen pathology. Biochem. Biophys. Res. Commun. 413, 400–406

21. Neißlauer, A. M., Gisler, A., Grüler, M., Engelmann, R., Müller-Hilke, B., Frank, M., Burstein, C., Rolls, A., Neidhardt, J., Wree, A., Witt, M., and Bräuer, A. U. (2019) A therapy with miglustat, 2-hydroxypropyl-ß-cyclodextrin and allopregnanolone restores splenic cholesterol homeostasis in Niemann-Pick disease type C. Lipids Health Dis. 18, 146

22. Picardi, M., De Rosa, G., Selleri, C., Scarpato, N., Soscia, E., Martinelli, V., Gancia, R., and Rotoli, B. (2003) Spleen enlargement following recombining human granulocyte colony-stimulating factor administration for peripheral blood stem cell mobilization. Haematologica 88, 794–800

23. Westerterp, M., Murphy, A. J., Löffler, M., Gourion-Arsiquaud, S., Murphy, A. J., Shih, A., Cremers, S., Levine, R. L., Tall, A. R., and Yvan-Charvet, L. (2012) Regulation of hematopoietic stem and progenitor cell mobilization by cholesterol efflux pathways. Cell Stem Cell. 11, 195–206

24. Jeuressen, M. J. J., Walenbergh, S. M. A., Houben, T., Gijbels, M. J. J., Li, J., Hendrixs, T., Oligschlaeger, Y., van Gorp, P. J., Binder, C. J., Donners, M. P. C., and Shiri-Sverdlov, R. (2016) Presence of oxLDL uptake leads to decreased atherosclerosis in hematopoietic NPC-deficient LDLr−/− mice. Atherosclerosis 255, 59–65

25. Houben, T., Oligschlaeger, Y., Bittorina, A. V., Hendriks, T., Walenbergh, S. M. A., Lenders, M. H., Gijbels, M. J. J., Verheyen, L., Lüütjohann, D., Hofker, M. H., Binder, C. J., and Shiri-Sverdlov, R. (2017) Blood-derived macrophages prone to accumulate lysosomal lipids trigger oxLDL-dependent murine hepatic inflammation. Sci. Rep. 7, 12556

26. Liscum, L., and Sturley, S. L. (2004) Intracellular trafficking of cellular cholesterol trafficking. Blood 103, 350–356
