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Gastrointestinal tract pathology in a Balb/c Niemann-Pick disease type C1 null mouse model

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Abstract

Background—Niemann-Pick disease, type C (NPC) is a rare lysosomal storage disorder characterized by progressive neurodegeneration, splenomegaly, hepatomegaly and early death. NPC is caused by mutations in either the *NPC1* or *NPC2* gene. Impaired NPC function leads to defective intracellular transport of unesterified cholesterol and its accumulation in late endosomes and lysosomes. A high frequency of Crohn's disease has been reported in NPC1 patients, suggesting that gastrointestinal tract pathology may become a more prominent clinical issue if effective therapies are developed to slow the neurodegeneration. The *Npc1^{mih}* mouse model on a Balb/c background replicates the hepatic and neurological disease observed in NPC1 patients. Thus we sought to characterize the gastrointestinal tract pathology in this model to determine if it can serve as a model of Crohn's disease in NPC1.

Authors' contribution

AC and MM performed and analyzed the experiment, AC and MM wrote the initial draft of the manuscript. FN participated in the mouse macrophage experiments and provided the method, JI performed the bioinformatics analysis, JP performed the cholesterol analysis, RB organized the pathological analysis and advice about the experimental design, NC, RB, FN, FDP gave essential comments on the manuscripts. All authors read and approved the final manuscript.

Declarations

Availability of data and material

All data reported have been obtained from experiments carried out in author's laboratory.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Methods—We analyzed the gastrointestinal tract and isolated macrophages of Balb/cJ cNctr-Npc1m1N/J (*Npc1*^{−/−}) mouse model to determine if there was any Crohn's-like pathology or inflammatory cell activation. We also evaluated temporal changes in the microbiota by 16S rRNA sequencing of fecal samples to determine if there were changes consistent with Crohn's disease.

Results—Relative to controls, *Npc1* mutant mice demonstrate increased inflammation and crypt abscesses in the gastrointestinal tract; however, the observed pathological changes are significantly less than those observed in other Crohn's disease mouse models. Analysis of *Npc1* mutant macrophages demonstrated an increased response to lipopolysaccharides (LPS) and delayed bactericidal activity; both of which are pathological features of Crohn's disease. Analysis of the bacterial microbiota does not mimic what is reported in Crohn's disease in either human or mouse models. We did observe significant increases in *cyanobacteria* and *epsilon-proteobacteria*. The increase in *epsilon-proteobacteria* may be related to altered cholesterol homeostasis since cholesterol is known to promote growth of this bacterial subgroup.

Conclusions—Although the BALB/c *Npc1*^{−/−} mouse demonstrates macrophage dysfunction similar to that observed in other Crohn's disease models, neither the microbiota changes nor the degree of gastrointestinal track pathology are consistent with this mouse model adequately replicating the Crohn's disease pathology reported in NPC1 patients.

Keywords

Niemann-Pick disease type C; gastrointestinal tract; microbiota; macrophage dysfunction; Crohn's disease

Background

Niemann-Pick disease, type C (NPC) is a rare autosomal recessive disorder characterized by the accumulation of unesterified cholesterol and glycosphingolipids in the viscera and central nervous system [1]. Inherited loss-of-function mutations in genes encoding either *NPC1* or *NPC2* cause Niemann-Pick type C disease [1, 2]. These proteins are involved in the intracellular transport of cholesterol out of the endolysosomal compartment [3]. The endolysosomal accumulation of lipids and cholesterol initiate a pathological cascade that leads to cellular dysfunction and ultimately cell death. This cellular dysfunction and neuronal loss underlies the neurological signs and symptoms observed in patients with either NPC1 or NPC2 deficiencies. NPC1 patients demonstrate a wide phenotypic spectrum, both with respect to age of neurological disease onset and the individual signs/symptoms observed in each patient [1, 4–6]. The pathological mechanisms that contribute to disease progression have not been fully elucidated. One pathological process that likely contributes to the pathological cascade is inflammation. Increased levels of inflammatory biomarkers have been observed in both cerebrospinal fluid [7, 8] and blood [9, 10], suggesting the involvement of an immune response in NPC pathology. Inflammatory markers have also been reported in tissues including the liver [11], lungs [12, 13], and brain [7, 14, 15]. Enteric neuropathology likely contributes to the progressive weight loss that occurs prior to death in the BALB/cJ cNctr-Npc1m1N/J (*Npc1*^{−/−}) mouse [16]; however, the chronic inflammation described in visceral tissues including the gastrointestinal tract may be a contributing factor.

Notably, multiple clinical reports have shown an association between NPC1 disease and Crohn's disease [17–21].

Crohn's disease is an inflammatory bowel disease (IBD) with a multifactorial etiology that includes interactions between genetic, environmental and gastrointestinal tract microbiota [22, 23]. Genetic susceptibility occurs in individuals with genetic variants in (*NOD2*, *ATG16L1*, *TLR4*, *STAT3*, *IL23R* [22, 23]). Hypocholesterolemia appears to be associated with IBD, thus potentially implicating cholesterol and lipid metabolism as a contributing factor [24]. Recently, Schwerd *et al.* [21] have shown impaired bacterial clearance in monocytes/macrophages isolated from NPC1 patients due to impaired autophagosome function which inhibits NOD2-mediated bacterial handling. Defective autophagy is a common factor in IBD and may explain the reported cases of Crohn's disease in NPC1 patients. Another similarity between NPC1 and Crohn's disease is the activation of the TLR4 pathway [22, 25–27]. This activation leads to constitutive inflammation that contributes to disease progression in IBD. Impaired autophagy and increased TLR4 signaling thus may underlie the reported clinical association of Crohn's disease in NPC1 patients.

A number of NPC1 mouse models have been characterized. Homozygous BALB/cJ cNctr-Npc1m1N/J (*Npc1*^{−/−}) mice die prematurely with progressive loss of Purkinje cells and a striking decline in body weight after 7 weeks [11, 16]. The degree with which peripheral organ dysfunction contributes to morbidity and mortality is not well defined. Kapur *et al.* [16] have shown that *Npc1*^{−/−} mice exhibit enteric neuronal defects and histologic alterations in intestinal mucosa; however, the degree to which this NPC1 mouse model recapitulates the IBD reported in NPC1 patients has not been investigated until now. Given that gastrointestinal dysfunction may contribute to the weight loss and morbidity, we characterized gastrointestinal track mucosal pathology, macrophage function and the gastrointestinal tract microbiota in the *Npc1*^{−/−} mouse.

Methods

Mouse model

Mice were housed under standard conditions and given free access to food and water. All animal work conformed to the National Institutes of Health guidelines and was approved by the Eunice Kennedy Shriver National Institute of Child Health and Human Development Institutional Animal Care and Use Committee. Heterozygous BALB/cNctr-Npc1m1N/J (Jackson Laboratory, Bar Harbor, ME, USA), *Npc1*^{+/-} mice were intercrossed to obtain control (*Npc1*^{+/+}) and mutant (*Npc1*^{−/−}) littermates. Pups were weaned 3 weeks after birth and subsequently had free access to water and NIH#31 mouse diet (Envigo, Cambridge, UK). PCR genotyping was performed using tail DNA [11]. Mice were sacrificed at 3, 5, 7, and 9 weeks of age.

Western blotting

Proteins extracted from mouse tissues were separated on 4–12% NuPAGE gels (Life Technologies, Carlsbad, CA, USA), dry-transferred to nitrocellulose membranes, blocked

with 2% BSA in PBS-Tween 0.1%, and probed with anti-CD68 (Bio-rad, Hercules, CA, USA), anti-iNOS (Millipore, Darmstadt, Germany), and anti-MPO (R&D, Minneapolis, MN, USA). Secondary antibodies used were HRP anti-mouse, anti-rat, or anti-rabbit (Sigma Aldrich, St-Louis, MO, USA). Proteins were detected by chemiluminescence with the ChemiDocXRSsystem (Bio-Rad Laboratories, Hercules, CA, USA). Densitometric analyses were done using imageLab 4.1 software (Bio-Rad Laboratories, Hercules, CA, USA).

Cholesterol assay

Cholesterol content was determined by extracting sterols from tissue samples as previously described by Picache *et al.* [28]. Sample analysis was done using a Laser diode/thermal desorption source (S-960 series, Phytronix Technologies Inc., Québec, Canada) coupled to an AB-Sciex TripleTOF 5600+ mass spectrometer (Redwood City, CA, USA).

RNA extraction and RT-qPCR

Total RNA was extracted from mouse tissue using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), followed by purification with Qiagen RNeasy Mini Columns (Qiagen, Hilden, Germany). For gene expression analysis by real-time qPCR, the quality and quantity of RNA were assessed using NanoDrop (Thermo Scientific, Inc., Pittsburgh, PA, USA). 1 µg of total RNA was reverse-transcribed to obtain cDNA using a high-capacity cDNA archive kit, according to the manufacturer's instructions (Thermo Scientific, Inc., Pittsburgh, PA, USA). The following TaqMan assays were used to assess the expression level of: *Tnf* (Mm00443258_m1), *Il-1b* (Mm00434228_m1), *TnfRI* (Mm00441883_g1) and *Actb* (4352341E). Amplifications were performed in 96-well plates with an Applied Biosystems 7300 real-time PCR system. Each sample was analyzed in triplicate, using 50 ng of total cDNA for each reaction.

Microbiota analysis

The stool samples were collected from individually housed mice at 3, 5, 7, and 9 weeks of age from *Npc1^{+/+}* or *Npc1^{-/-}* mice and stored at -80°C until used. Microbial DNA was isolated using the QiAmp DNA stool kit (Qiagen, Hilden, Germany) as described by the manufacturer. Multiple replicate PCRs were performed using the fecal DNA sample. Each 100 µL reaction contained 100 ng of template DNA, 0.3 µM of the forward universal bacterial primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3'), 0.3 µM of the reverse universal primer 1525r (5'-AAGGAGGTGWTCCARCC-3'), 1U Taq DNA Polymerase (Roche, Basel, Switzerland), 1X amplification buffer, and 0.3 mM of each dNTP. Cycling conditions were 94°C for 2 minutes, followed by 20 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 2 minutes, and a final elongation step at 72°C for 10 minutes. Replicate PCRs were pooled and purified using the QiAmp DNA mini kit (Qiagen, Hilden, Germany). All sequence reads with less than 97% similarity with the known organisms in the Greengenes database [29] were not considered for further analysis. Representative sequences for each Operative Taxonomic Unit were assigned to different bacterial taxonomic levels by using Greengenes database as previously described using QIIME [30].

Macrophage and *in vitro* infection.

Peritoneal macrophage were isolated as previously described [31] and maintained in RPMI (Life Technology, Carlsbad, CA, USA), L-glutamine (4 mM), and sodium pyruvate (110 µg/ml) (Life Technologies, Carlsbad, CA, USA) and supplemented with 10% heat-inactivated, low-endotoxin-unit FBS (Omega Scientific, Offenburg, Germany) prior to infection. Cells were detached from plates, counted, and plated (onto a 12-well plate at a density of 105 cells per well). The following day, *E. coli*, grown to an optical density (OD_{600nm}) of 0.3 were used to infect the cells or purified MDP or LPS was used to stimulate the cells according to standard procedures. Immediately before infection, cells were washed twice with PBS and the medium containing the bacteria was added. Macrophages and bacteria were incubated for 60 min and then washed twice, after which medium containing gentamicin (20 µg/ml) was added for 1 h. Cells were washed and either lysed with 1% Triton X100-PBS (t=2, 4, 8, or 16 h). Bacteria in lysis buffer were serially diluted in PBS and plated onto LB agar plates. Growth was recorded 48 to 72 h later by direct counting of the bacteria colonies.

Macrophage were grown in 8-well TC Lab-Tek Chamber Slides (Thermo Fischer Scientific, MA, USA) for 24 hours. Cells were then washed with PBS, fixed with 4% paraformaldehyde for 10 min at +4°C and permeabilized with BD Perm/Wash buffer (BD Bioscience, San Diego, CA, USA). Non-specific binding was blocked with 10% normal goat serum diluted 2% BSA/PBS for 30 minutes at room temperature. Anti-NLRP3-488 (1:50, Biolegend, San Diego, CA, USA), anti-Cleaved caspase 1 (1:100, Biolegend, San Diego, CA, USA), anti-Iba1 (1:200, Wako, Richmond, VA, USA), in 10% normal goat serum diluted 2% BSA/PBS were applied and kept for 30 min at 37°C followed by overnight incubation at +4°C. Secondary antibodies were diluted 1:1000 10% normal goat serum diluted 2% BSA/PBS and kept for 1 hour at room temperature in the dark. After washing samples were mounted with Mowiol (EMD-Millipore, Darmstadt, Germany). Images were recorded using a Zeiss Axioplan microscope and the ZEN2 Zeiss application software (Zeiss, Oberkochen, Germany).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 software (San Diego, San Diego, CA, USA). *P*-values <0.05 were considered statistically significant.

Results

Gastrointestinal tract pathology

In order to evaluate the potential clinical association between NPC1 and Crohn's disease, we characterized the degree of inflammatory pathology found in the digestive tract of *Npc1*^{-/-} mice. Hematoxylin/eosin stained sagittal sections of the digestive tract demonstrated disruption of normal gastric architecture and intracellular storage in all layers of the gastric epithelium in *Npc1* mutant mice relative to control mice (Figure 1A–F). In the *Npc1*^{-/-} mice, vesicles were visible along the width of the gastric epithelium but not in the muscular layer, suggesting that gastric myoblasts are less affected by the loss of NPC1 than the epithelial cells. Parietal cell and chief cell sizes were not affected and no obvious

intracellular storage was noted; however, a disorganization of the mucosal layer was observed. The observed storage is associated with increased levels of cholesterol measured in the stomach tissue of *Npc1*^{-/-} mice (10.93 ± 1.28 vs. 20.24 ± 1.56 μ g of total cholesterol per mg of protein in *Npc1*^{+/+} mice compared to *Npc1*^{-/-} mice, respectively, $p=0.01$). None of the analyzed inflammation makers were significantly increased in stomach tissue (Figure 1G).

Consistent with what has been reported in other mouse models with gastrointestinal inflammation [32, 33], the *Npc1*^{-/-} mice exhibited a significantly shorter gastrointestinal tract length than the control mice at 9 weeks of age, when normalized for mouse mass (Figure 2A (ileum) and 2B (colon)). These changes were not observed at 5 and 7 weeks of age. At 9 weeks of age we also observed a significant increase in the expression of inflammatory proteins CD68, MPO iNOS (Figure 2C), and increased mRNA expression of *TnfaR1* (Figure 2D) in *Npc1*^{-/-} gastrointestinal tract tissue. We did not observe altered gene expression of either *Tnfa* or *Il-1b*, two other inflammatory markers (Figure 2D).

Given that no inflammation markers and no change in size were measured at earlier time points, we used the scoring system developed by Erben *et al.* [34] to quantify the degree of pathological changes in the gastrointestinal tract of 9 week old *Npc1*^{-/-}. This evaluation includes characterization of inflammatory cell infiltrates (severity and extent), epithelial change (hyperplasia, loss of goblet cell, cryptitis and crypt abscess formation) and mucosal architecture (erosion, ulceration, granulation tissue, irregular crypts, crypt loss, villous blunting). Data comparing control and mutant mice are provided in Table 1. Minimal abnormalities were observed in *Npc1*^{+/+} animals. These included mucosal inflammation and leukocyte depletion, in a small number of animals. A similar degree of mucosal inflammation and leukocyte depletion were observed in tissue from *Npc1* mutant mice. In contrast, the frequency of crypt abscesses (Figure 3A, Table 1) was significantly increased in both ileum and colonic tissue from *Npc1*^{-/-} mice. Specifically, 6/14 and 5/14 *Npc1*^{-/-} mice had crypt abscesses in the ileum and colon respectively ($p<0.05$). Crypt abscesses were observed in both the ileum and colon in two mutant animals. Although the total score was elevated in *Npc1*^{-/-} mice for both the ileum (0.73 ± 0.28 vs. 2.79 ± 0.70) and colon (0.27 ± 0.21 vs. 1.07 ± 0.40), only the ileum difference was significant ($p=0.02$ and $p=0.15$, respectively). In an acute Crohn's disease mouse model, such as Dextran Sulfate Sodium (DSS) or in genetic models such as the *Tnf*^{ARE/ARE} or SAMP1/Yit [35], a major erosion of the mucosal architecture is observed in association to the crypt abscesses and ulceration with a 100% penetrance [36]. The observed pathological changes observed in the *Npc1* mutant mice are substantially less than these mouse models of Crohn disease.

Macrophage antibacterial function

Impaired antibacterial autophagy has been implicated in Crohn's disease [22, 23, 37, 38] and impaired bacterial autophagy and bacterial killing has been reported in macrophages isolated from NPC1 subjects [21]. Thus, we evaluated the bactericidal activity of macrophages isolated from *Npc1*^{-/-} mice. We isolated peritoneal macrophages at 5, 7, and 9 weeks of age and evaluated the expression of two key inflammation markers, caspase 1 and NLRP3 by Western blot and indirect immunofluorescence (ICC). Peritoneal macrophages deficient for

Npc1 showed increased levels of active caspase 1 as early as 5 weeks of age, followed by increased levels of NLRP3 at 7 and 9 weeks when compared to age matched controls (Figure 4A). An increase in the expression of NLRP3 and cleaved caspase 1 in *Npc1*^{-/-} macrophages as assessed by ICC compared to *Npc1*^{+/+} (Figure 4B and C), was also observed. We elected to perform further experimental analyses on macrophages isolated from mice at 7 weeks of age, the earliest time macrophages showed activation directly after isolation.

To characterize the immune response towards bacterial products of the mouse macrophages, macrophages isolated from 7 week old *Npc1*^{+/+} or *Npc1*^{-/-} mice peritoneal lavage (PLM) extracts showed no significant difference in *Tnf* expression levels at the basal level (values Pplm = 0.48) or in response to purified muramyl-dipeptide (MDP) (1.67 ± 0.61 vs. 6.82 ± 3.71 , Pplm = 0.16, n=6) (Figure 4D). This is consistent with the observation made in human macrophages where no difference after MDP stimulation was observed [21]. This also suggests that the environment where the macrophages come from does not modulate the ability to respond to bacterial stimuli. However, a significant increase in *Tnf* expression was seen in macrophages from *Npc1*^{-/-} mice in response to lipopolysaccharides (LPS) compared to macrophages from *Npc1*^{+/+} mice, (1451.46 ± 21.51 vs. 1996.00 ± 133.2 , Pplm = 0.03, n=6/group) (Figure 4D); a finding that is also consistent with what has been previously reported for human cells [21, 26]. Hence, macrophages from *Npc1*^{-/-} mice appear to behave similarly to human macrophages from NPC1 patients with respect to stimulation of their immune response.

Assessments of phagocytic activity using the non-pathogenic *Escherichia coli* was then tested in peritoneal macrophages. These cells showed a similar phagocytic activity independent of the genotype (Figure 4E). However, the intracellular bacterial survival was significantly increased in the *Npc1*^{-/-} cells compared to *Npc1*^{+/+} at 8 and 16 hours (Figure 4E). These results, in addition to the well characterized autophagy defect in NPC1 [25, 39], highlight an abnormal innate immune response and correlates with the chronic inflammation reported in NPC1 patients with Crohn's disease and the inflammation in the *Npc1*^{-/-} mice [7, 11]. Taken together, these results recapitulate the observations made using human cells [21] and validate the use of these mice to study the Crohn's disease-like phenotype with the *Npc1*^{-/-} background specifically in macrophages.

Intestinal microbiota

Altered intestinal microbiota is known to play a role in Crohn's disease and cholesterol is a known microbiota modifier [40–42]. In addition, since the defective bacterial clearance in Crohn's disease has been associated with modification of the intestinal microbiota, we then evaluated the microbiota over the course of the disease in the mouse model of NPC1 [43]. 16S rRNA from the stool samples of *Npc1*^{-/-} and *Npc1*^{+/+} at 3, 5, 7, and 9 weeks of age (n=4/group) were analyzed. No significant differences were seen between the bacterial groups independent of age or genotype of the mice (Figure 5A). In addition, no significant diversity change, were seen over time between the *Npc1*^{+/+} or *Npc1*^{-/-} mice. The two main populations, *firmicutes* and *bacteroidetes* do not change significantly over the course of the disease, however, there was a trend toward enrichment of *proteobacteria* (p3w=0.69,

p5w=0.3, p7w =0.34, p9w =0.2; Figure 5A and B) and *cyanobacteria* (p3w=1, p5W=1, p7W =0.34, p9w =0.06; Figure 5A and B). Analysis at the class level revealed that the increase in *proteobacteria* was mainly due to the increase in *epsilon-proteobacteria* (Figure 5C) with no significant variation in the levels of *alpha*-, *beta*-, *delta*- or *gamma-proteobacteria*. *Epsilon-proteobacteria* present in our analysis was assigned to two main organisms: *Helicobacter* and *Campylobacter* (Figure 5D). Analysis at the species level suggests *Helicobacter* enrichment is driven by *H. hepaticus* since no change in *H. pylori* was measured. *H. bilis*, *H. rodentium*, *H. typhlonius* and *H. trogonum* were not identified in the 16S reads. Hence, the significantly increased level of cholesterol in the tissues may explain the increase in these two closely related *epsilon-proteobacteria*. The other bacterial population that exhibited a trend to increase over time in *Npc1*^{-/-} mice was *Cyanobacteria* (Figure 5B). To date, these bacteria have not been associated with inflammatory bowel diseases or lysosomal storage diseases; and although they have been associated with gastroenteritis after consumption of contaminated water [44].

Discussion

The *Npc1*^{-/-} mouse model has proven to be an extremely useful tool for studying the pathology of NPC1 and developing therapeutic interventions. It recapitulates many aspects of the disorder and in particular neurodegeneration. We were interested in determining if the null *Npc1* mutant mouse would also be useful in understanding the reported association between NPC1 and Crohn's disease [17–19, 21]. Although we were able to demonstrate several pathological features that are consistent with Crohn's disease, the *Npc1*^{-/-} gastrointestinal track pathology is minimal and does not adequately recapitulate Crohn's disease. The only notable pathology consistent with Crohn's disease that we observed was an increase in the number of animals with crypt abscesses. However, we did not observe immune cell infiltration that is typically observed in other Crohn's disease models. We also did not observe microbiota changes that have been associated with Crohn's disease. One limitation of this study was the use of BALB/c mice, since these mice are more resistant to colitis than C57/BL6 mice; commonly used in mouse IBD models [36]. C57/BL6 *Npc1*^{-/-} mice manifest severe liver and spleen pathology that results in an early non-neurological death [45]. Pathological analysis of the gastrointestinal tract has not been reported in this model.

Although histopathological changes were minimal and microbiota changes were not significant, we did observe increased expression of inflammatory biomarkers similar to what has been reported in chemically induced or genetic mouse models of Crohn's disease [23, 34, 36, 46–50]. We also demonstrate that *Npc1*^{-/-} peritoneal macrophages, similar to NPC1 patient macrophages or in genetic models of Crohn's disease [20, 24, 54–56], show an enhanced response to TLR4 induction and a defect in antibacterial autophagy and clearance. Given the multifactorial nature of Crohn's disease, it is possible that additional genetic or environmental factors are necessary for manifestation of Crohn's disease in this *Npc1* mutant mouse model.

Treatment of mice with DSS is frequently used to model Crohn's disease [36] and treatment of *Npc1* mutant mice with DSS could potentially provide data showing that NPC1 disease

predisposes to the development of Crohn's disease. However, the short lifespan of the *Npc1* mutant mice does not allow sufficient time for DSS induction of Crohn disease [36]. Consideration should be given to evaluating DSS sensitivity in less severe NPC1 mouse models, such as with D1005G [51] or I1061T [52] missense mutations on the C57bl6 background.

To our knowledge, this is the first study evaluating microbiota in *Npc1*^{-/-} mice. We observed increases in *epsilon-proteobacteria*, specifically *Helicobacter* and *Campylobacter*. These two bacterial classes are well documented as pro-inflammatory organisms in mice and humans [53–56]; however, at the species level we found that the *Helicobacter* enrichment was due to an increase in *H. hepaticus* rather than *H. pylori*. In addition it has been reported that cholesterol favors the growth of *Helicobacter* *in vitro* and could participate to the enrichment in those populations [57]. The other bacterial population that exhibited a trend to increase over time in *Npc1*^{-/-} mice was *Cyanobacteria*. To our knowledge, these *Cyanobacteria* have not been associated with inflammatory bowel diseases or lysosomal storage diseases. Whether this finding has pathological implications is unknown.

This work suggests that the association of Crohn's disease with NPC1 may not simply be due to loss of NPC1 function, but rather may represent a more complicated interaction with other genetic factors and the environment. The high incidence (3–7%) of Crohn's disease reported in NPC1 patients from the United Kingdom is not apparent in a cohort of 102 NPC1 patients followed at the National Institutes of Health (FDP unpublished observation). These discordant observations support the hypothesis that additional genetic or environmental factors contribute to the development of Crohn's disease in NPC1 patients. Additional work will be needed to elucidate these contributing factors.

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Abbreviations

NPC1	Niemann-Pick type C1
IBD	Inflammatory Bowel Disease
E. coli	Escherichia coli

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Figure 1:
 Histological analysis of *Npc1*^{-/-} and control mouse stomach. A-C) *Npc1*^{+/+} mice D-F) *Npc1*^{-/-} mice at 5 (A, D) and 9 (B, C, E, F), week respectively. * indicate lipid accumulation, black arrowhead indicate vesicular storage. PC: parietal cell; CC: Chief cell
 G) RT-qPCR analysis of inflammation markers, *Tnf*, *Il1β* and *TnfaR1*, n>6.

Figure 2:

Inflammation marker analysis in *Npc1*^{+/+} and *Npc1*^{-/-} gastrointestinal tracts. Length measurements of *Npc1*^{-/-} and control mice A) ileum and B) colon, over the course of the disease normalized to mouse mass, n=6; U-test comparison of age matched animals *p<0.01. C) Western blot analysis of inflammation markers iNOS, CD68, ZO-1 and MPO. d) RT-qPCR analysis of inflammation markers *Tnf*, *Il1β* and *TnfaR1*, n>6; U-test, *p<0.01.

Figure 3:
Pathological analysis of mouse gastrointestinal tract. A-C) *Npc1*^{-/-} mice ileal transverse sections stained with hematoxylin and eosin. Green arrows show crypts abscesses, Green arrowhead show crypt loss and black arrows show enlarged crypts. D) Cumulative inflammation score, from the ileum and colon pathological analysis summarized in Table 1 n>6; U-test, *p<0.01.

Figure 4: Macrophage characterization. A) Western blot analysis of cleaved caspase1 and NLRP3 expression levels at 5, 7, and 9 weeks in *Npc1*^{+/+} and *Npc1*^{-/-} peritoneal macrophages. B) upper two panels; NLRP3 (Alexa-488; Green) and B lower two panels; cleaved caspase 1 (Alexa-488; Green) staining and fluorescence quantification in *Npc1*^{+/+} and *Npc1*^{-/-} peritoneal macrophages at 7 weeks counterstaining used Hoechst3342 (Blue) and IBA1 (Alexa 594; Red). Scale bar is for 20 μ m. C) Mean fluorescence intensity per cell for NLRP3 (Upper graph) and Cleaved caspase 1 (lower graph). D) *Tnf* expression levels in peritoneal macrophage at basal level and following MDP or LPS stimulation. E) *Escherichia coli* phagocytosis and survival assay measure at 2, 4, 8, 16, and 24 hours post-infection of *Npc1*^{+/+} and *Npc1*^{-/-} macrophages.

Figure 5:

16S rRNA analysis of fecal microbiota in *Npc1*^{-/-} and control mice over the course of the disease. A) Mean abundance of the 10 most represented taxa (n=4). B) Relative abundance at 3, 5, 7 and 9 week of *firmicutes*, *bacteroidetes*, *proteobacteria* and *cyanobacteria*. C) Frequency analysis of the main bacteria within the *proteobacteria*. D) Frequency analysis of the *epsilon-proteobacteria* *Helicobacter* and *campylobacter*. Dashed line are for *Npc1*^{-/-} solid line *Npc1*^{+/+}.

Table 1:

Pathological analysis score of *Npc1*^{+/+} and *Npc1*^{-/-} mice ileum and colon.

Defect	Ileum		Colon	
	<i>Npc1</i> ^{+/+}	<i>Npc1</i> ^{-/-}	<i>Npc1</i> ^{+/+}	<i>Npc1</i> ^{-/-}
Mucosal inflammation	26.6% (4/15)	42.9% (6/14)	6.7% (1/15)	0%
Cryptus inflammation	0%	14.3% (2/14)	0%	0%
Cryptus abscess	0%	42.9% (6/14) *	0%	35.7% (5/14) *
Cryptus loss	0%	7.1% (1/14)	0%	0%
Erosion	0%	7.1% (1/14)	6.7% (1/15)	0%
Leucocyte depletion	20% (2/10)	77.8% (7/9)	20% (2/10)	66.7% (6/9)

* Indicates significance between *Npc1*^{+/+} v *Npc1*^{-/-} (Chi-square)