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Toll-like receptor mediated lysozyme expression in Niemann-Pick disease, type C1

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Abstract

Niemann-Pick type C1 (NPC1) is a rare neurodegenerative disease. In NPC1 mouse cerebella, the antibacterial enzyme, lysozyme (*Lyz2*), is significantly increased in multiple cell types. Due to its possible role in toxic fibril deposition, we confirmed *Lyz2* overexpression in culture in different control and NPC1 cell types including human NPC1 fibroblasts. *Lyz2* expression is induced by Toll-like receptors potentially in response to lipid storage but does not play a functional role in NPC disease pathology.

1. Introduction

Niemann-Pick type C (NPC) disease is caused by reduced or loss of function of NPC1 or NPC2 proteins leading to the endolysosomal accumulation of sphingolipids and unesterified cholesterol [1]. In both human and mouse models of NPC, neuroinflammation is a component of the neuropathology, contributing to loss of cerebellar Purkinje neurons and a shorter lifespan [1, 2]. As part of the NPC cerebellar inflammation signature, lysozyme, a highly conserved antibacterial hydrolase, is overexpressed [2–5]. Using single-cell RNAseq we found that *Lyz2* was not only upregulated in microglia, as expected, but expression was also significantly increased in $Npc1^{-/-}$ glial cells and neurons [6]. We proposed that elevated expression of lysozyme protein may functionally contribute to Purkinje neurons loss. This hypothesis was supported by a prior observation in a Sanfilippo syndrome type B mouse model. Although *Lyz2* is ubiquitously expressed, lysozyme protein accumulates in disease sensitive neurons which also accumulate hyperphosphorylated TAU [7]. Aggregation of soluble lysozyme to form oligomers can mimic Aβ amyloid and induce the hyperphosphorylation of TAU [8]. NPC1 is considered a tauopathy and altered tau function

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influences NPC1 pathology [9, 10], thus we investigated the regulation of lysozyme expression and sought to determine if dysregulated lysozyme expression functionally contributes to NPC1 neuropathology.

2. Materials and Methods

2.1. Mouse models

Mouse experiments were approved by the NICHD ACUC. BALB/c-*Npc1*$^{+/-}$ [11] and C57BL/6-*Lyz*$^{-/-}$ mice [12] were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and crossed to obtain *Npc1*$^{+/-}$:*Lyz2*$^{+/-}$ mice. These in turn were intercrossed to obtain control and *Npc1*$^{-/-}$:*Lyz2*$^{-/-}$ mice. Genotype and phenotype characterization *Npc1*$^{-/-}$ mice have previously been described [5, 13].

2.2. Cell culture

Cell lines, culture conditions, TaqMan probes, RNA guides, plasmid and primer sequences are listed in Table S1. Immortalized myeloid (iMM) cells, cerebellar microglia and astrocytes were isolated and used as described previously [13–15]. Cells were treated with 2 μM U18666A (Sigma-Aldrich-Millipore, St-Louis, MO, USA) for 24 hours to inhibit NPC1 function and inhibition was confirmed by increased *Srebf2* expression [16]. Hydroxypropyl-β-cyclodextrin (HPβCD, Kleptose HPB) and miglustat were used as previously described [13, 17]. Toll-like receptor (*Tlr*) 2 or 4 mutations were introduced into Neuro2a (N2a) cells using CRISPR/Cas9 genomic editing (PX458, https://www.addgene.org/crispr/zhang/) and guide RNAs designed with CHOPCHOP [18]. Genomic DNA from individual clones was screened using PCR (Table S1).

2.3. RNA extraction and qPCR

Total RNA was purified with Qiagen RNeasy Mini Columns (Qiagen, Hilden, Germany) and reverse-transcribed (1 μg) to obtain cDNA using a High-Capacity cDNA Archive Kit (ThermoFisher Scientific, Waltham, MA, USA). Probes used for gene expression analysis are listed in Table S1.

2.4. Statistical analysis

Statistical comparisons were performed with GraphPad Prism 5 software (San Diego, CA, USA). Mann-Whitney tests were used to compare 2 groups and two-way ANOVA testing was used when comparing two factors. Box and Whiskers plots display the Tukey post-hoc comparisons, $n > 6$ unless specified. Kaplan-Meier curves were compared using the log-rank test.

3. Results

3.1 Lysozyme 2 expression in NPC1 is dependent on lipid storage and Toll-like receptor activation

Microglia and astrocytes isolated from *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice cerebella, N2a neurons, human fibroblasts and iMM cells all show increased expression of lysozyme when NPC1 function is decreased or absent (Fig. 1A–E). We and others have previously shown increased

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lysozyme protein expression in *Npc1*$^{-/-}$ cerebellar tissue [6, 19]. Increased lysozyme expression was prevented by treating microglia with HPβCD and reduced with miglustat (Fig. 1A). Miglustat inhibits glycosphingolipid synthesis and HPβCD reduces unesterified cholesterol storage in NPC1 cells. These data suggest that increased lysozyme expression is a cellular response to abnormal lipid storage. Lysozyme expression increases in response to Toll-like Receptor (TLR) activation [20], notably activation of TLR2 and 4. In the absence of *Tlr4*, U18666A induced expression of *Lyz2* was abolished and in the absence of *Tlr1* and *Tlr2*, induction of *Lyz2* expression was attenuated (Fig. 1D). In contrast, disruption of *Tlr6* had no effect on *Lyz2* expression (Fig. 1D). Involvement of *Tlr2* and *Tlr4* was confirmed in N2a cells (Fig. 1C). TLR receptor signaling through MYD88 but not TRIF was confirmed using *Mvd88* and *Trif* mutant cell lines (Fig. 1D).

3.2 Increased lysozyme expression does not functionally contribute to NPC1 neuropathology

Characteristic NPC1 histopathological findings related to both neuroinflammation and neurodegeneration were indistinguishable between the tested mouse genotypes: *Npc1*$^{-/-}$:*Lyz2*$^{+/+}$, *Npc1*$^{-/-}$:*Lyz2*$^{+/-}$ and *Npc1*$^{-/-}$:*Lyz2*$^{-/-}$ (Fig. 1F, S1). *Lyz2* gene loss did not appear to play a role in the mice phenotype as it did not alter NPC disease progression (Fig. 1G) or survival (Fig. 1H).

4. Discussion

Neuroinflammation in NPC disease has been extensively characterized with *Lyz2* identified as a gene commonly found to be overexpressed [4, 13, 19]. The present results suggest LYZ2, an antibacterial glycosyl-hydrolase, is overexpressed in several cell types of the cerebellum of NPC mice, including neurons and astrocytes. Our results show that *Lyz2* is upregulated following TLR1/2 and TLR4 activation through MYD88. Based on substrate reduction using HPβCD and miglustat we propose that increased *Lyz2* expression may be secondary to host-generated lipids accumulating in the endolysosomal compartment. As these TLRs have overlapping target genes, the inhibition of both TLR2 and TLR4 signaling may be needed for a protective effect on NPC1 disease progression [21]. Given that the NPC1 mouse phenotype is not appreciably altered in *Npc1*$^{-/-}$:*Lyz2*$^{-/-}$ double mutant mice, increased lysozyme expression does not appear to play a direct role in NPC1 pathology.

However, lysozyme may serve as a useful biomarker that could provide insight into pathological mechanisms or a pharmacodynamic marker to support efficacy of therapeutic interventions. Our current work indicates that increased lysozyme expression is dependent on lipid accumulation, thus it may serve as a tool in future studies to identify specific sterols, lipids or glycolipids contributing to the neuroinflammatory state. Increased lysozyme has previously been identified as a biomarker of neuroinflammation [19]. Our current data extends this observation and shows that lysozyme is an indicator of abnormal TLR activation. Prior work by Suzuki et al. implicated endosomal accumulation and activation of TLR4 as a potential mechanism contributing to cell-autonomous microglial activation in NPC1 [21]. This would be consistent with observations that implicate microglial activation

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as an early pathological process preceding neuronal loss in NPC1 [6, 13], and underscores the potential of lysozyme to be used as a biomarker in assessing early disease progression.

Supplementary Material

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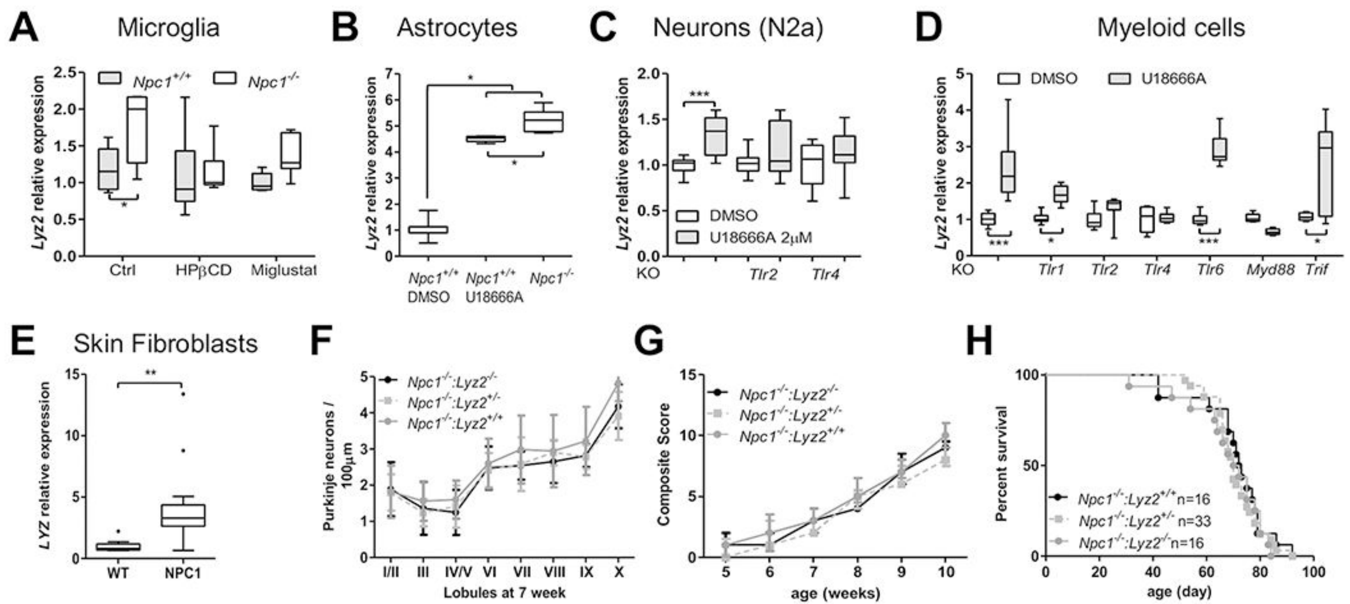

Figure 1. Lysozyme in NPC1 pathology.

A) Relative expression by qPCR analysis of Lyz2 in *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ microglia. Treating *Npc1*$^{-/-}$ microglia for 24 hours with either 100 μM miglustat or 100 μM HPβCD significantly decreased *Lyz2* expression toward normal levels. **B)** Increased expression of *Lyz2* in *Npc1*$^{-/-}$ astrocytes from 7-week old mice. Pharmacological inhibition of NPC1 function with 2 μM U18666A increased expression of *Lyz2* in *Npc1*$^{+/+}$ astrocytes. DMSO is a vehicle control **C)** Increased expression of *Lyz2* in a neuronal cell line (N2a) when treated with U18666A to inhibit NPC1 function. Increased expression of *Lyz2* is abrogated in *Tlr2* and *Tlr4* knockout (KO) lines. **D)** Increased *Lyz2* expression in myeloid cell lines (control, *Tlr1*$^{-/-}$, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$ or *Trif*$^{-/-}$) treated with DMSO (vehicle control) or U18666A to inhibit NPC1 function. **E)** Increased *LYZ* expression in NPC1 patient skin fibroblasts. Genotypes are provided in table S1. **F)** Purkinje neuron density is not altered in cerebella from 7-week *Npc1* mutant mice heterozygous or mutant for *Lyz2* (n 6). **G)** Phenotypic severity is similar in *Npc1* mutant mice irrespective of the *Lyz2* genotype (n 6). **H)** Kaplan-Meier survival curves. *Lyz2* genotype does not alter survival of *Npc1*$^{-/-}$ mice. Median survival was 71 and 72 days for *Npc1*$^{-/-}$:*Lyz2*$^{+/+}$ and *Npc1*$^{-/-}$:*Lyz2*$^{-/-}$mice, respectively (p=0.59). Statistical analysis used a log-rank test. Two-way ANOVA was used to analyze data in panels A, D , F and G. Mann-Whitney test was used to analyze data in panels B, C and E, and a log-rank test was used for data in panel H. For all panels: *$p< 0.05$, **$p<0.01$ and ***$p<0.001$.