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Niemann-Pick Type C1 deficiency in microglia does not cause neuron death *in vitro*

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ABSTRACT

Niemann-Pick Type C (NPC) disease is an autosomal recessive disorder that results in accumulation of cholesterol and other lipids in late endosomes/lysosomes and leads to progressive neurodegeneration and premature death. The mechanism by which lipid accumulation causes neurodegeneration remains unclear. Inappropriate activation of microglia, the resident immune cells of the central nervous system, has been implicated in several neurodegenerative disorders including NPC disease. Immunohistochemical analysis demonstrates that NPC1 deficiency in mouse brains alters microglial morphology and increases the number of microglia. In primary cultures of microglia from *Npc1*$^{-/-}$ mice cholesterol is sequestered intracellularly, as occurs in other NPC-deficient cells. Activated microglia secrete potentially neurotoxic molecules such as tumor necrosis factor-α (TNFα). However, NPC1 deficiency in isolated microglia did not increase TNFα mRNA or TNFα secretion *in vitro*. In addition, qPCR analysis shows that expression of pro-inflammatory and oxidative stress genes is the same in *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ microglia, whereas the mRNA encoding the anti-inflammatory cytokine, interleukin-10 in *Npc1*$^{-/-}$ microglia is ~60% lower than in *Npc1*$^{+/+}$ microglia. The survival of cultured neurons was not impaired by NPC1 deficiency, nor was death of *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ neurons in microglia–neuron co-cultures increased by NPC1 deficiency in microglia. However, a high concentration of *Npc1*$^{-/-}$ microglia appeared to promote neuron survival. Thus, although microglia exhibit an active morphology in NPC1-deficient brains, lack of NPC1 in microglia does not promote neuron death *in vitro* in microglia–neuron co-cultures, supporting the view that microglial NPC1 deficiency is not the primary cause of neuron death in NPC disease.

1. Introduction

Niemann Pick Type-C (NPC) disease is an autosomal recessive disorder that is characterized by liver disease, lung disease and neurodegeneration that lead to premature death (reviewed in [1]). Mutations in either NPC1 or NPC2 lead to the accumulation of lipids, particularly cholesterol that is derived from endocytosed lipoproteins [2], within late endosomes/lysosomes (LE/L) of all cells [3] including neurons [4] and astrocytes [5]. The two NPC proteins, NPC1 and NPC2, act in tandem in the LE/L to mediate the egress of cholesterol from the LE/L [6].

Neuronal death occurs progressively in NPC disease, with Purkinje neurons of the cerebellum being particularly affected [7,8]. However, the mechanism underlying the neurodegeneration remains unclear. Apoptotic neurons have been detected by TUNEL staining in the cerebral cortex and cerebellum of NPC patients and NPC1-deficient mice [9]. In addition, in the *Npc1*$^{-/-}$ mouse cerebellum, levels of mRNAs encoding apoptotic markers such as caspase-1 and -3 are elevated [8], and the c-Abl/p73 apoptotic pathway is activated [10]. Furthermore, amounts of mRNAs encoding the pro-inflammatory cytokine tumor necrosis factor-α (TNFα) and other components of the TNF death pathway are increased in the cerebellum of NPC1-null mice [8,9,11]. The events underlying the increased apoptosis of neurons in response to NPC deficiency have not been elucidated in detail but might include leakage of lysosomal enzymes and oxidative stress [12]. In addition, dysfunction of non-neuronal cells in the brain, such as microglia and/or astrocytes, has been proposed to contribute to the neurodegeneration that characterizes NPC disease [13,14].

Microglia are resident immune cells of the central nervous system (CNS) and play a crucial role in maintaining health and function of the brain. In a healthy brain, the microglia constantly monitor their surroundings with their ramified processes [15]. When the microglia detect disturbances to the brain environment, such as cell damage or an invading pathogen, the microglia can become activated. Depending upon the activating stimulus, the microglia invoke various responses that include migration to the site of injury [16,17], proliferation [18] and

Abbreviations: CGCs, cerebellar granule cells; CNS, central nervous system; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; Iba1, ionized calcium binding adaptor molecule 1; IL10, interleukin-10; LE/L, late endosomes/lysosomes; MAP2, microtubule-associated protein 2; NPC, Niemann-Pick Type C; PBS, phosphate-buffered saline; TNF, tumor necrosis factor

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phagocytosis of dying cells and debris [19]. Additionally, microglia can generate an inflammatory response through the release of pro-inflammatory cytokines [20,21], glutamate [22], reactive oxygen species [23], and nitric oxide [24]. Although inflammation is a mechanism for protection of the CNS, molecules produced during an inflammatory response are potentially cytotoxic so that the microglia must tightly control the extent of inflammation. Consequently, the microglia also produce anti-inflammatory cytokines and neuroprotective molecules such as growth factors [25–27] that allow the microglia to shift from a classical pro-inflammatory state to a more neuroprotective state so that repair and regeneration of damaged tissue can occur [28]. These wide-ranging capabilities provide the microglia with the first line of defense against a variety of insults to the CNS.

Increasing evidence suggests that chronic activation and/or dysfunction of microglia can contribute to neuron death in neurodegenerative disorders such as Alzheimer disease and Parkinson disease (reviewed in [29]). Moreover, replacement of mutant microglia with wild-type microglia by bone marrow transplantation in mouse models of amyotrophic lateral sclerosis and Sandhoff disease (a lysosomal storage disorder) delayed the onset, and slowed the course, of these diseases [30,31]. On the other hand, microglial defects are not the primary determinants of CNS pathology in Gaucher's disease, another lysosomal lipid storage disease [32]. In the brains of NPC1-deficient mice the number of microglia is markedly increased and these microglia exhibit an activated morphology [13,33,34]. Microglia proliferate in specific regions of the NPC1-deficient brain, such as the thalamus and cerebellum, and the activated microglia eventually spread throughout the brain as the disease progresses [13]. Interestingly, this activation/proliferation of microglia was observed prior to any detectable neurodegeneration [13], suggesting that microglia might play a role in initiating the neurodegeneration in NPC disease. Nevertheless, neuronal damage that was undetectable by the techniques employed might have contributed to activation of the microglia. It is also possible that cholesterol sequestration in NPC1-deficient microglia alters microglial function and renders these cells less able to perform normal neuroprotective functions.

Thus, the role of microglia in NPC disease is controversial. A key question is: do activated NPC1-deficient microglia initiate and/or exacerbate neuronal death, or do microglia become activated in response to cell death in an attempt to prevent further neuronal death? We, therefore, investigated the role of microglia in NPC disease using primary microglia isolated from *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice. Experiments were performed with co-cultures of primary microglia and primary neurons to test the hypothesis that NPC1 deficiency in microglia causes neuron death.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM) high glucose, Ham's F12 medium, Neurobasal medium, fetal bovine serum (FBS), B27 supplement and 0.25% trypsin-EDTA were purchased from Invitrogen. L-glutamine, poly-D-lysine hydrobromide and trypsin (type XII-S from bovine pancreas) were purchased from Sigma, deoxyribonuclease I (DNAse I) was from Worthington Biochemical Corporation (Lakewood, NJ) and U18666A was from Biomol Research Laboratories (Plymouth Meeting, PA). Culture flasks (25-cm^2 and 75-cm^2) and 96-well plates were purchased from BD Falcon (BD Biosciences, Bedford, MA). Bovine serum albumin, saponin and filipin were from Sigma, paraformaldehyde was from Fisher Scientific and Hoechst 33258 pentahydrate (bis-benzimide), Texas Red® goat anti-rabbit IgG secondary antibody and Alexa Fluor® 488-chicken anti-mouse IgG secondary antibody were from Invitrogen. Rabbit anti-Iba1 (ionized calcium binding adaptor molecule 1) antibody was from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and mouse anti-MAP2 (microtubule-associated protein 2) antibody (HM-2, Ab11267) was from Abcam (Cambridge, MA). DNAse I (amplification grade), Oligo(dT)$_{12-18}$ primer, SuperScript® II, Platinum® qPCR SuperMix-UDG, and SYBR Green I nucleic acid gel stain were from Invitrogen, while PCR-grade dNTPs (deoxynucleoside triphosphates) were from Roche (Laval, QC).

2.2. *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice

A breeding colony of Balb/cNctr-*Npc*$^{N/+}$ mice was established at the University of Alberta with breeding pairs obtained from Jackson Laboratories (Bar Harbor, ME). The mice were maintained under temperature-controlled conditions with a 12-h light:12-h dark cycle and were supplied with a 9% fat breeders diet (Purina LabDiet, Richmond, IN) and water *ad libitum*. Mice homozygous and heterozygous for the *Npc1* mutation will be referred to as *Npc1*$^{-/-}$ and *Npc1*$^{+/-}$ mice, respectively, while wild-type mice will be termed *Npc1*$^{+/+}$. Since *Npc1*$^{-/-}$ mice are unable to produce offspring, *Npc1*$^{+/-}$ mice were used for breeding. Prior to dissection of the brains, the *Npc1* genotype of the mouse pups was determined by PCR analysis of genomic DNA isolated from tail clippings using the REDExtract-N-Amp™ Tissue PCR Kit (Sigma), as described [35]. All experiments were approved by the Health Sciences Animal Welfare Committee of the University of Alberta.

2.3. Immunohistochemical staining of mouse brain slices

Samples from brains of 7-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice were prepared according to Amritraj et al. [12]. Briefly, mice were anesthetized with 4% chloral hydrate, then perfused with phosphate-buffered saline (PBS) and subsequently with 4% (w/v) paraformaldehyde. Brains were sectioned (20 μM) on a cryostat and processed using the free-floating procedure [36,37]. Sections were washed with PBS and incubated with anti-Iba1 antibody (1:1000 dilution) overnight at room temperature. Subsequently, sections were washed with PBS and incubated with Texas Red®-conjugated secondary antibody for 2 h at room temperature, then washed with PBS, mounted on slides in glycerol mounting media [1 M Tris-chloride (pH 8.0), water, glycerol (1:4:5)] and imaged using a Leica DM IRE2 fluorescence microscope (Leica Microsystems, Bannockburn, IL). The excitation wavelength was 596 nm. Images were captured using Open Lab 3.1.4 Software (PerkinElmer, Waltham, MA) then processed using Adobe Photoshop CS4 (San Jose, CA).

2.4. Isolation and culture of cortical glia

Glial cells were isolated from cerebral cortices of 1- to 3-day-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice [38]. The cortices were cleaned of meninges and blood vessels, finely chopped, and digested in 0.25% trypsin (Invitrogen) containing 1 mg/mL DNAse I (Worthington). Cells were dissociated by trituration through a Pasteur pipette in DMEM containing 10% FBS. Glial cells were pelleted by centrifugation at 950 rpm, re-suspended in fresh DMEM containing 10% FBS, then plated at a density of two cortices/75-cm^2 flask or one cortex/25-cm^2 flask. Cultures were maintained at 37 °C and 5% CO_2 in DMEM containing 10% FBS. Medium was replaced every 3–5 days.

2.5. Isolation and culture of cortical microglia

Microglia were isolated using the mild trypsinization method [39]. Confluent cultures of mixed glial cells were maintained in flasks for 3 to 4 weeks, then washed with DMEM:Ham's F12 medium (1:1) and incubated at 37 °C with DMEM:Ham's F12 medium (1:1) containing 0.0625% trypsin for 30–60 min until the astrocyte layer lifted. Medium and the astrocyte layer were aspirated leaving microglia attached to the dish. For qPCR analysis, microglia were washed once with DMEM:Ham's F12 medium (1:1) containing 10% FBS, followed by a wash with DMEM:Ham's F12 medium (1:1). Microglia were incubated in the

latter medium at 37 °C and 5% CO_2 for 24 h. In some experiments, after removal of the astrocyte layer, microglia were incubated with 0.25% trypsin at room temperature and subsequently scraped from the dish. DMEM:Ham's F12 medium (1:1) containing 10% FBS was added to the cell suspension, cells were pelleted at 950 rpm and microglia were re-suspended in fresh DMEM:Ham's F12 medium (1:1) containing 10% FBS. Microglia were plated in 96-well plates at a density of 62,500 cells/well. According to immunocytochemical staining for the microglial marker Iba1 and the astrocyte marker, glial fibrillary acidic protein, as well as labeling of nuclei by Hoechst staining, the cultures contained >90% microglia, consistent with ~98% microglia reported previously with this method [39].

2.6. Isolation and culture of neurons

Cerebellar granule cells (CGCs) were cultured as described [40] with minor modifications. Briefly, cerebella were dissected from 7- to 8-day-old mice, cleaned of meninges and blood vessels, finely chopped, and digested in 0.25% trypsin containing 1 mg/mL DNAse I. Cells were dissociated by trituration through a fire-polished Pasteur pipette in Neurobasal medium containing 10% heat-inactivated FBS. Dissociated cells were pelleted by centrifugation at 950 rpm, re-suspended in Neurobasal medium supplemented with 2% B27, 0.5 mM glutamine and 20 mM potassium chloride, then passed through a 40 μm nylon cell strainer (BD Biosciences, Bedford, MA) to remove clumped cells. Trypan blue-excluding cells were counted using a hemocytometer and plated at a density of 31,250 cells/well in 96-well plates coated with 10 μg/mL poly-D-lysine hydrobromide. Cells were maintained at 37 °C and 5% CO_2 without re-feeding for 7 days until used for experiments.

Cortical neurons were isolated from the cortex of one-day-old mice by the same protocol that was used for CGCs [40]. The neurons were maintained in Neurobasal A medium (Invitrogen) supplemented with 2% B27 and 0.5 mM glutamine at 37 °C and 5% CO_2 without re-feeding for 3–4 days until used for experiments.

2.7. Filipin staining

Npc1$^{+/+}$ and *Npc1*$^{-/-}$ microglia were isolated as described earlier, plated in 96-well plates and allowed to rest overnight. The microglia were washed with PBS, fixed for 15 min in 4% (w/v) paraformaldehyde and stained for 1.5 h with filipin (0.15 mg/mL) at room temperature. The cells were then washed with PBS and examined with a Leica DM IRE2 fluorescence microscope (Leica Microsystems, Bannockburn, IL) at an excitation wavelength of 351 nm. Images were captured using Open Lab 3.1.4 Software (PerkinElmer, Waltham, MA) and processed using Adobe Photoshop CS4 (San Jose, CA).

2.8. Measurement of TNFα secretion

Npc1$^{+/+}$ and *Npc1*$^{-/-}$ microglia were isolated as described earlier, plated in 96-well plates, and allowed to rest overnight. Medium was removed and the cells were incubated for 24 h with fresh DMEM/Ham's F12 medium (1:1) containing 10% FBS. Media were collected and stored at −20 °C. Amounts of TNFα secreted into the culture medium were determined by ELISA with a standard curve (Biosource, Camarillo, CA), according to manufacturer's instructions.

2.9. RNA isolation and quantitative real-time PCR analysis

Npc1$^{+/+}$ and *Npc1*$^{-/-}$ microglia were cultured in 75-cm^2 flasks for 24 h in DMEM:Ham's F12 medium (1:1) then washed with ice-cold PBS. Total RNA was isolated using the RNeasy Mini Kit (Invitrogen) and stored at −80 °C. RNA was treated with DNAse I (amplification grade) to prevent DNA contamination and cDNA was synthesized from 0.5 μg total RNA using oligo(dT)$_{12–18}$ random primers and Superscript II reverse transcriptase according to manufacturer's instructions. qPCR reactions were performed using Platinum® Quantitation PCR supermix, SYBR Green I and 250 nmol of gene-specific primers in a total volume of 25 μL in 0.2 mL tubes (Axygen, Union City, CA). Transcripts were detected by qPCR with a Rotor-Gene 3000 instrument (Montreal Biotech, Montreal, QC) and data were analyzed with Rotor-Gene 6.0.19 software. Amounts of transcripts were determined using the standard curve method. Intron-spanning primers were designed using OLIGO 6.71 software (Cascade, CO) and specificity was confirmed with the NCBI BLAST nucleotide query tool, by melt curve analysis, and by agarose gel electrophoresis. All primers were synthesized by IDT Technologies (San Diego, CA) (sequences shown in Table 1). Data were normalized to levels of control mRNA encoding glyceraldehyde-3-phosphate dehydrogenase.

2.10. Microglia–neuron co-cultures

Npc1$^{+/+}$ and *Npc1*$^{-/-}$ CGCs were cultured in 96-well plates for 7 days. *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ microglia were isolated as described earlier and plated directly on top of the CGC cultures at densities that were either 25% or 50% of the CGC density (7800 cells/well or 15,600 cells/well, respectively). The co-cultures were given Neurobasal medium supplemented with 2% B27, 0.5 mM glutamine and 20 mM potassium chloride. After 24 h, cells were fixed with 4% (w/v) paraformaldehyde then washed with PBS and permeabilized for 1 h at room temperature with PBS containing 1% bovine serum albumin and 0.05% saponin. Subsequently, the cells were incubated with anti-MAP-2 (1:500) and anti-Iba1 (1:500) antibodies diluted in PBS containing 1% bovine serum albumin and 0.05% saponin for 1 h at room temperature. The cells were washed with PBS then incubated with Alexa Fluor® 488- and Texas Red®-conjugated secondary antibodies (1:200) for 45 min at room temperature in the dark. Cells were rinsed with PBS, stained for 12 min with 0.5 μg/mL Hoechst 33258, and examined using a Leica DM IRE2 fluorescence microscope (Leica Microsystems, Bannockburn, IL). The excitation wavelength was 495 nm for Alexa Fluor® 488, 596 nm for Texas Red® and 351 nm for Hoechst stain. Images were captured using Open Lab 3.1.4 Software (PerkinElmer, Waltham, MA) and the number of apoptotic cells stained by the neuronal marker MAP2 was quantified.

2.11. Phagocytosis assay

Npc1$^{+/+}$ and *Npc1*$^{-/-}$ microglia were plated in 96-well plates and allowed to rest overnight after which medium was replaced with fresh

Table 1
qPCR primers designed to quantify mRNA levels of mouse genes involved in microglial functions.

Gene	Primer sequence
Tumor necrosis factor-α (TNFα)	F: 5′-GTCTACTGAACTTCGGGGTGA-3′ R: 5′-CACTTGGTGGTTTGCTACGAC-3′
Interleukin-1β (IL1β)	F: 5′-GAAGTTGACGGACCCCAAAA-3′ R: 5′-CCACGGGAAAGACACAGGTAG-3′
Inducible nitric oxide synthase (iNOS)	F: 5′-AAGCCCCGCTACTACTCCATC-3′ R: 5′-GCCACTGACACTTCGCACAA-3′
NADPH oxidase (NADPHox)	F: 5′-GACTGGACGGAGGGGCTAT-3′ R: 5′-ACTTGAGAATGGAGGCAAAGG-3′
Interleukin-10 (IL10)	F: 5′-GCTGGACAACATACTGCTAAC-3′ R: 5′-CCGCATCCTGAGGGTCTTC-3′
Transforming growth factor-β (TGFβ)	F: 5′-CGCCATCTATGAGAAAACCA-3′ R: 5′-CCAAGGTAACGCCAGGAAT-3′
Glutaminase (GLTase)	F: 5′-GTTTGCCGCATACACTGGAG-3′ R: 5′-ACATGGAGGGCTGTTCTGGA-3′
Macrophage colony-stimulating factor receptor (M-CSFR)	F: 5′-CAAGATTGGGGACTTTGGACT-3′ R: 5′-AGAGGAGGATGCCGTAGGA-3′
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	F: 5′-GAGCCAAACGGGTCATCATC-3′ R: 5′-CATCACGCCACAGCTTTCCA-3′

medium containing 1.0 μm fluorescent carboxylate-modified polystyrene latex beads (Sigma, L4655) at a concentration of 3.1×10^6 beads/well (50 beads/microglia). Microglia were incubated with the beads at 37 °C for 2 h, then washed 3 times with ice-cold PBS. Microglia were examined using a Leica DM IRE2 fluorescence microscope (Leica Microsystems, Bannockburn, IL). The excitation wavelength for the beads was 505 nm. Phase and fluorescent images were captured using Open Lab 3.1.4 Software (PerkinElmer, Waltham, MA) and the number of beads associated with the microglia was quantified. As a control for beads that had bound to microglia without being internalized, parallel experiments were performed at 4 °C to block phagocytosis. The number of beads associated with microglia at 4 °C was subtracted from the number of beads associated with microglia at 37 °C.

2.12. Statistical analysis

The statistical significance of differences was determined using the Student's *t* test. Differences were considered significant for $P<0.05$.

3. Results

3.1. Microglia with an active morphology accumulate in *Npc1*$^{-/-}$ mouse brains

Since microglia have been implicated in several neurodegenerative disorders, including NPC disease, we investigated the involvement of microglia in neuronal death in NPC disease. We first performed immunohistochemical analysis of brain slices from the cerebellum and cerebral cortex of *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. Sections were taken from 7-week-old mice and immunostained for the microglial marker Iba1. Mice of this age were selected since overt signs of NPC neurological disease are apparent in 7-week-old mice. In the *Npc1*$^{+/+}$ cerebellum (Fig. 1A) and cortex (Fig. 1C) microglia were primarily detected in a ramified, resting morphology. In the *Npc1*$^{-/-}$ cerebellum (Fig. 1B) and cortex (Fig. 1D), however, the number of microglia was markedly increased, and the *Npc1*$^{-/-}$ microglia had swollen cell bodies and shortened processes, indicating that the microglia were activated. Thus, NPC1 deficiency in the brain markedly increases the number of microglia, and the morphology of the microglia shifts to a more activated phenotype.

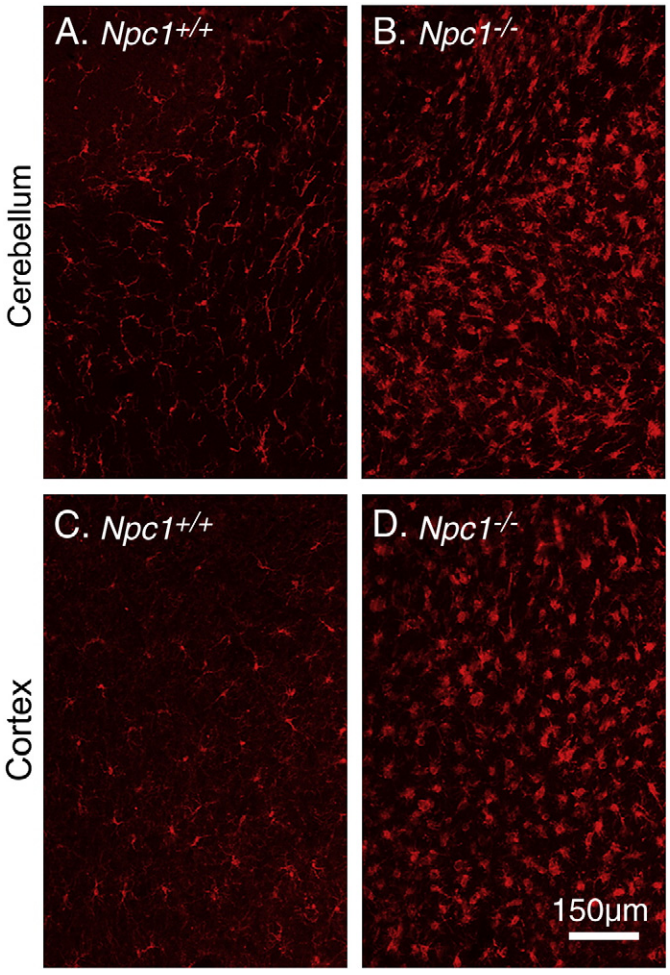

Fig. 1. Active microglia accumulate in the cerebellum and cortex of *Npc1*$^{-/-}$ mice. Immunohistochemical staining of microglia in brains of 7-week-old *Npc1*$^{+/+}$ (*panels A, C*) and *Npc1*$^{-/-}$ (*panels B, D*) mice. Brains were fixed. Cerebella (*panels A, B*) and cerebral cortices (*panels C, D*) were sectioned then labeled with fluorescent antibodies raised against Iba1, a microglial marker. Shown are representative images from independent preparations from 3 mice.

3.2. Intracellular cholesterol distribution is altered in *Npc1*$^{-/-}$ microglia

The compound U18666A induces cholesterol sequestration in LE/Ls of cells, similar to the cholesterol trafficking defect in NPC1-deficient cells [41]. In preliminary experiments we incubated primary cultures of *Npc1*$^{+/+}$ microglia with U18666A (3 μM) for 6 h and examined the intracellular distribution of unesterified cholesterol by filipin staining. As expected, U18666A induced a bright punctate staining pattern in *Npc1*$^{+/+}$ microglia upon filipin staining (data not shown). This intracellular sequestration of cholesterol is typical of that exhibited by NPC-deficient cells such as fibroblasts [3], sympathetic neurons [4] and astrocytes [5]. We next used filipin staining to compare the distribution of cholesterol in primary microglia isolated from *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. The *Npc1*$^{+/+}$ microglia showed a faint, diffuse intracellular filipin staining for cholesterol (Fig. 2A) that is likely attributable to cholesterol in the endosomal recycling compartment, a region of the cell that is enriched in cholesterol [42]. In contrast, *Npc1*$^{-/-}$ microglia showed markedly increased intracellular filipin staining in a punctate pattern characteristic of that of other NPC-deficient cells in which cholesterol becomes sequestered in the LE/L [3,4] (Fig. 2B). These data show that NPC1 deficiency in the microglia causes the intracellular sequestration of cholesterol.

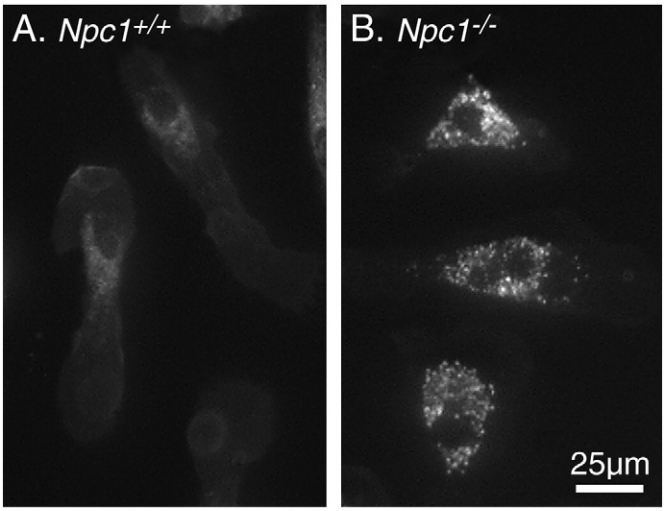

Fig. 2. Altered intracellular distribution of unesterified cholesterol in *Npc1*$^{-/-}$ microglia. Microglia were isolated from mixed glial cultures from *Npc1*$^{+/+}$ (*panel A*) and *Npc1*$^{-/-}$ (*panel B*) mice. The microglia were allowed to rest overnight then stained with filipin for detection of unesterified cholesterol. Shown are representative images from 3 independent microglial preparations.

3.3. Pro- and anti-inflammatory cytokines and oxidative stress genes in NPC1-deficient microglia

Microglia produce pro- and anti-inflammatory cytokines as well as molecules involved in oxidative stress and cell proliferation. To determine if NPC1 deficiency altered the expression of these factors, we quantified levels of mRNAs encoding some of these proteins in primary microglia isolated from *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice. The amounts of mRNAs encoding the pro-inflammatory cytokines TNFα and interleukin-1β (IL1β) were not significantly different between *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ microglia (Fig. 3A). Nor did NPC1 deficiency increase the amount of TNFα secreted into the medium by microglia during a 24 h period (Fig. 4). Moreover, levels of mRNAs encoding markers of oxidative stress, such as inducible nitric oxide synthase (iNOS, which produces nitric oxide [43]), and NADPH oxidase (NADPHox, which produces superoxide [44], were similar in *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ microglia (Fig. 3B). Thus, NPC1 deficiency does not appear to increase the expression of genes involved in the generation of reactive oxygen species in microglia. However, the amount of mRNA encoding the anti-inflammatory cytokine interleukin-10 (IL10) was ~60% lower in *Npc1*$^{-/-}$ microglia than in *Npc1*$^{+/+}$ microglia (Fig. 3C), although the level of mRNA encoding another anti-inflammatory cytokine, transforming growth factor-β (TGFβ), was not different between *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ microglia (Fig. 3C). Additionally, we quantified the mRNAs encoding glutaminase (which produces glutamate that can lead to excitotoxic death [45]) and the macrophage colony-stimulating factor receptor (which is involved in microglial proliferation and activation [46]). No significant differences in expression of these genes were observed between *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ microglia (Fig. 3D). These results indicate that although NPC1 deficiency in the CNS induces an activated morphology in microglia, the production of pro-inflammatory cytokines and markers of oxidative stress was not increased in primary cultures of *Npc1*$^{-/-}$ microglia. On the other hand, since the level of IL10 mRNA was lower

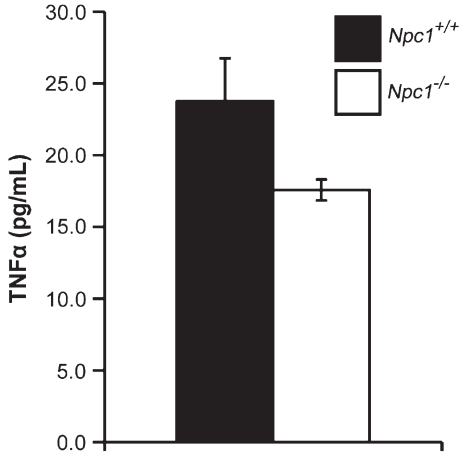

Fig. 4. *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ microglia secrete similar amounts of TNFα *in vitro*. Primary cultures of *Npc1*$^{+/+}$ (black bars) and *Npc1*$^{-/-}$ (white bars) microglia were established. The microglia were incubated with fresh medium for 24 h after which the amount of TNFα secreted into the medium was measured by ELISA. Values are means ± S.E. of 3 independent microglial preparations, each analyzed in duplicate. $P > 0.05$ (Student's *t* test).

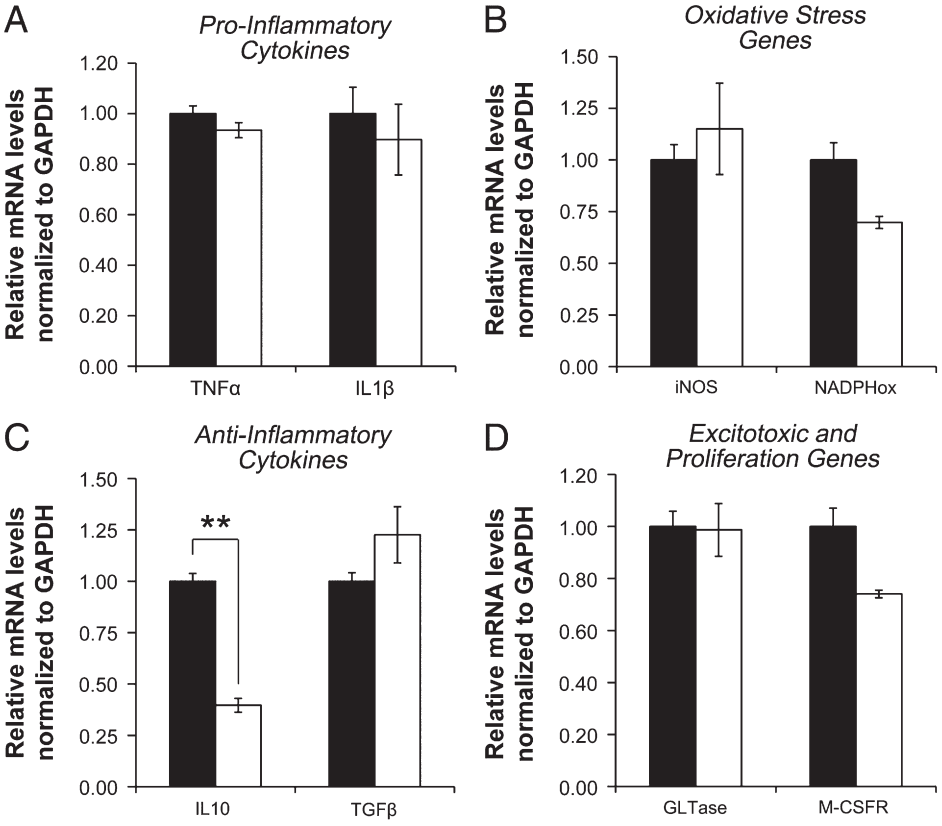

Fig. 3. Expression of mRNAs encoding pro- and anti-inflammatory cytokines and mRNAs associated with oxidative stress, excitotoxicity and proliferation. Primary cultures of *Npc1*$^{+/+}$ (black bars) and *Npc1*$^{-/-}$ (white bars) mouse microglia were isolated. qPCR mRNA analysis was performed for: *panel A*, pro-inflammatory cytokines [TNFα and interleukin-1ß (IL1ß)]; *panel B*, oxidative stress genes [inducible nitric oxide synthase (iNOS) and NADPH oxidase (NADPHox)]; *panel C*, anti-inflammatory cytokines [interleukin-10 (IL10) and transforming growth factor-ß (TGFß)]; and *panel D*, excitotoxic [glutaminase (GLTase)] and proliferative [macrophage colony-stimulating factor (M-CSFR)] genes. Amounts of mRNAs were normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Data are means ± S.E. from 3 independent microglial preparations, each of which was analyzed in triplicate. **, $P < 0.01$ according to Student's *t* test.

in *Npc1*$^{-/-}$ microglia than in *Npc1*$^{+/+}$ microglia, specific protective functions, or the suppression of an inflammatory response, might be impaired in *Npc1*$^{-/-}$ microglia.

3.4. Neuronal apoptosis is not increased by NPC1 deficiency in microglia–neuron co-cultures

Since NPC disease is characterized by extensive neurodegeneration, we determined whether or not NPC1 deficiency in isolated neurons compromised neuronal survival. We cultured cerebellar granule cells (CGCs) from *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice for 7 days, then quantified the extent of neuronal death by staining the cells with Hoechst stain to detect condensed nuclei that are typical markers of apoptotic cell death [47]. The percentage of apoptotic neurons in the CGC cultures was ~20% and was independent of *Npc1* genotype (Fig. 5). Similar results were obtained in preliminary experiments with primary cultures of cortical neurons, as well as sympathetic neurons, isolated from the superior cervical ganglion of *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. Thus, NPC1 deficiency does not decrease survival of primary cultures of these types of neurons.

To determine if the intracellular sequestration of cholesterol that we observed in *Npc1*$^{-/-}$ microglia impaired neuron survival, for example by inducing the secretion of factors that are toxic to neurons, we performed several types of co-culture experiments. First, we incubated *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ CGCs for 24 h with culture medium that had been conditioned for 24 h by *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ microglia. Cell death was assessed by Hoechst staining. The extent of cell death was the same (25—30%) for *Npc1*$^{+/+}$ and for *Npc1*$^{-/-}$ neurons incubated for 24 h with medium conditioned by *Npc1*$^{+/+}$ or *Npc1*$^{-/-}$ microglia.

We also established co-cultures of neurons (CGCs) with microglia. The microglia were plated at either a low density (25% of the number of neurons) or a high density (50% of the number of neurons), directly on top of the CGC cultures. Different combinations of *Npc1* genotypes of neurons and microglia were plated together so that co-cultures of *Npc1*$^{+/+}$ or *Npc1*$^{-/-}$ CGCs were established with either *Npc1*$^{+/+}$ or *Npc1*$^{-/-}$ microglia. After 24 h, the cells were fixed and stained for specific neuronal (MAP2) and microglial (Iba1) marker proteins. The percentage of *Npc1*$^{-/-}$ neurons that were apoptotic, according to Hoechst staining, was similar (20–23%) when the neurons were co-cultured with a low density of either *Npc1*$^{+/+}$ or *Npc1*$^{-/-}$ microglia (Fig. 6A). In addition, when *Npc1*$^{+/+}$ CGCs were cultured with a low density of microglia the extent of neuronal death (18–22%) was independent of *Npc1* genotype of the microglia (Fig. 6A). Similarly, when *Npc1*$^{-/-}$ neurons were cultured with a high concentration of microglia, the number of neurons that were apoptotic (12–17%) was independent of the *Npc1* genotype of the microglia (Fig. 6B). On the other hand, the number of apoptotic *Npc1*$^{+/+}$ neurons was significantly lower when the neurons were cultured in the presence of a high concentration of *Npc1*$^{-/-}$ microglia compared to *Npc1*$^{+/+}$ microglia (10% *versus* 19%, $P<0.001$; Fig. 6B). In a physiological context, in the brains of *Npc1*$^{-/-}$ mice, *Npc1*$^{-/-}$ microglia would interact with *Npc1*$^{-/-}$, but not *Npc1*$^{+/+}$, neurons, whereas in *Npc1*$^{+/+}$ mice *Npc1*$^{+/+}$ microglia would interact with *Npc1*$^{+/+}$, but not *Npc1*$^{-/-}$, neurons. When the data in Fig. 6 are compared in this way, the number of apoptotic neurons was significantly lower ($P<0.01$) when *Npc1*$^{-/-}$ neurons were cultured in the presence of a high concentration of *Npc1*$^{-/-}$ microglia ($12.3 \pm 1.7\%$) than when *Npc1*$^{+/+}$ neurons were cultured with a high concentration of *Npc1*$^{+/+}$ microglia ($19.8 \pm 1.5\%$); however, no difference in apoptosis was apparent when the neurons were co-cultured with low concentrations of microglia. The reason why high concentrations of *Npc1*$^{-/-}$ microglia appear to protect CGCs is not clear although under all culture conditions neuronal death was very low and survival of the CGCs was 85-90%. Thus, *Npc1*$^{-/-}$ microglia do not induce neuron death in co-cultures of microglia and neurons, supporting the view that *Npc1*$^{-/-}$ microglia do not directly cause neuronal death.

The conditions we used for culture of neurons were selected for maximal survival. We, therefore, considered the possibility that the optimal amounts of growth factors present in the neuronal culture medium had prevented factors secreted by the microglia from inducing neuronal apoptosis. Consequently, we performed experiments with microglia and cortical neurons co-cultured in the presence of Neurobasal A medium lacking any growth supplements. Under these conditions, neuronal apoptosis was ~50% after 48 h and was independent of *Npc1* genotype of both neurons and microglia.

We conclude that the survival of CGCs and cortical neurons cultured *in vitro* does not appear to be inherently reduced by NPC1 deficiency.

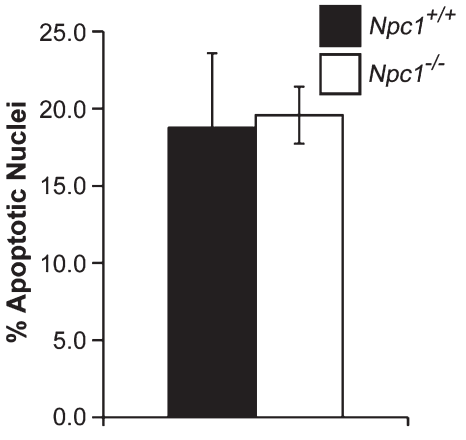

Fig. 5. Survival of cerebellar granule neurons is not impaired by NPC1 deficiency. Cerebellar granule neurons (CGCs) from *Npc1*$^{+/+}$ (black bars) and *Npc1*$^{-/-}$ (white bars) mice were cultured for 7 days, fixed with paraformaldehyde and nuclei were stained with Hoechst stain. The number of apoptotic nuclei from >500 cells in each preparation is given as a percentage of total number of neurons. Data are means ± S.E. from 3 independent preparations of CGCs, each of which was analyzed in duplicate. $P>0.05$ (Student's *t* test).

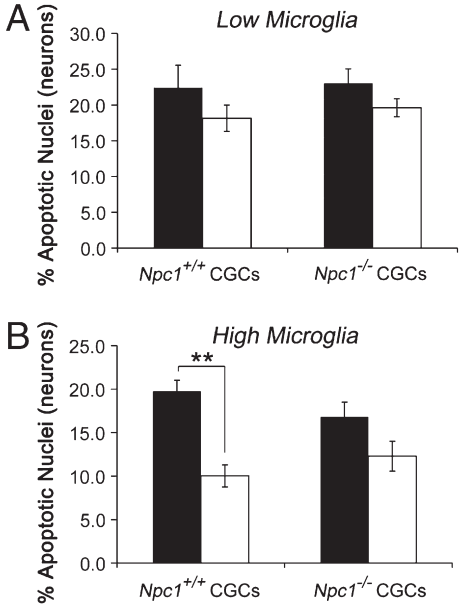

Fig. 6. NPC1 deficiency in microglia does not cause neuron death in microglia–neuron co-cultures. Primary cultures of *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ CGCs were co-cultured with *Npc1*$^{+/+}$ (black bars) or *Npc1*$^{-/-}$ (white bars) microglia. The microglia were plated at densities that were either low (*panel A*: 25% of the number of neurons) or high (*panel B*: 50% of the number of neurons) in co-culture with 7-day-old neuronal cultures. After 24 h, the cells were fixed and stained with anti-MAP2 antibodies (marker of neurons), anti-Iba1 antibodies (marker of microglia) and Hoechst stain (marker of nuclei). Data are means ± S.E. of the number of apoptotic nuclei in neurons as a percentage of total number of neurons, for >1000 neurons from 3 independent microglia preparations and 2 independent CGC preparations. **, $P<0.001$ (Student's *t* test).

Moreover, *Npc1*$^{-/-}$ microglia do not compromise neuronal survival when these microglia are co-cultured with either *Npc1*$^{-/-}$ or *Npc1*$^{+/+}$ neurons.

3.5. Phagocytosis is not impaired in *Npc1*$^{-/-}$ microglia

Microglia are phagocytic cells of the CNS that engulf degenerating cells and other debris [19]. Therefore, to determine if the sequestration of cholesterol within *Npc1*$^{-/-}$ microglia altered their phagocytic capability, microglia were incubated for 2 h with fluorescent latex beads, then extensively washed and the number of beads that were internalized by the microglia was quantified. As a control for beads that had attached to the cells, but had not been internalized, parallel cultures were incubated at 4 °C in order to block phagocytosis. The number of cell-associated beads at 4 °C was subtracted from the number of beads associated with the cells at 37 °C. Fig. 7 shows that the number of latex beads phagocytosed by *Npc1*$^{-/-}$ microglia and *Npc1*$^{+/+}$ microglia was not significantly different. Thus, according to this criterion of microglial activity, phagocytosis by microglia is not impaired by NPC1 deficiency.

4. Discussion

4.1. Accumulation of active microglia in NPC1-deficient brains

Inappropriate activation of microglia has been implicated in several neurodegenerative disorders including NPC disease [13,33,34]. Consistent with the findings of others [13,48], we observed a marked accumulation of microglia with activated morphology in *Npc1*$^{-/-}$ mouse brains. As in other NPC1-deficient cells, filipin staining revealed a pronounced intracellular sequestration of unesterified cholesterol in *Npc1*$^{-/-}$ microglia. However, the levels of mRNAs encoding pro-inflammatory cytokines and oxidative stress proteins were not increased by NPC1 deficiency in primary cultures of microglia. These data suggest that the microglia that accumulate in NPC1-deficient mouse brains are not activated solely by NPC1 deficiency in the microglia, but by an additional insult, such as neuron death. Evidence is accumulating that macrophages can cross the blood-brain barrier from the circulation, particularly in states of disease and inflammation [49,50]. Thus, the proliferation of Iba1-positive cells in NPC1-deficient mouse brains might also be due, at least in part, to an infiltration of macrophages since Iba1 is expressed in both microglia and macrophages [51,52].

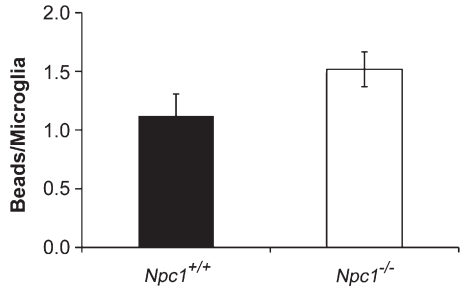

Fig. 7. Phagocytosis of latex beads is not impaired in *Npc1*$^{-/-}$ microglia. Microglia were isolated from *Npc1*$^{+/+}$ (black bar) or *Npc1*$^{-/-}$ (white bar) mice and allowed to rest overnight. Carboxylate-modified poylstyrene latex beads were added to the culture medium and incubated with the microglia for 2 h at 37 °C. The cells were extensively washed and the number of beads per microglial cell was quantified. As a control for binding of beads in the absence of uptake, microglia were incubated with beads for 2 h at 4 °C, and these values were subtracted from the total number of beads associated with the microglia at 37 °C. Values are means ± S.E. from >600 microglia in 3 independent experiments. $P > 0.05$ (Student's *t* test).

4.2. Consequences of NPC1 deficiency on cytokine production by microglia

Microglia are the major source of TNFα in the CNS [53] and TNFα is often associated with inflammation-mediated cell death. Since increased levels of mRNAs encoding TNFα (and other genes involved in the TNFα death pathway) have been detected in brains [9] and livers [54,55] of *Npc1*$^{-/-}$ mice, we compared the level of TNFα mRNA in *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ primary microglia. Neither the amount of TNFα mRNA in microglia, nor the amount of TNFα secreted by the microglia, was increased by NPC1 deficiency. In addition, immunoblotting for TNFα in *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ primary microglia revealed no difference in the amount of intracellular TNFα. It is likely, therefore, that the amount of TNFα mRNA [9] is increased in *Npc1*$^{-/-}$ mouse brains upon activation of the microglia in response to neuron death in the intact brain. In addition, the increased amounts of TNFα mRNA in NPC1-deficient brains might be, at least in part, a consequence of the increased number of activated microglia in the brain. Our data show that in primary cultures of microglia NPC1 deficiency does not increase the production of pro-inflammatory cytokines, such as TNFα. These observations are consistent with the recent studies of Lopez et al. in which the inducible expression of NPC1 in specific types of neurons in the brains of *Npc1*$^{-/-}$ mice rescued the neurodegeneration despite the presence of *Npc1*$^{-/-}$ microglia [56]. Furthermore, the highest concentration of activated microglia in *Npc1*$^{-/-}$ mouse brains was detected in areas of the brain in which neurodegeneration was most pronounced [56].

Ultimately, the role of microglia is to maintain brain health. Although microglia are capable of exerting cytotoxic effects, microglia can also be neuroprotective [25–27]. For example, microglia secrete IL10, an anti-inflammatory cytokine that decreases the synthesis of pro-inflammatory cytokines, such as TNFα and interleukin-1β, as well as reactive oxygen species, both *in vitro* and *in vivo*[57–60]. Following lipopolysaccharide-induced inflammation in the rat cerebral cortex, IL10 immunostaining was primarily detected in activated microglia [61]. When IL10 activity was abrogated with anti-IL10 antibodies, neuronal death increased, as did the amounts of reactive oxygen species and pro-inflammatory cytokines [61]. IL10 can also prevent excitotoxic death of neurons, apparently via inhibition of pro-apoptotic proteins such as caspase-3 [62,63]. Our studies revealed that NPC1 deficiency in primary cultures of microglia significantly reduced the level of the mRNA encoding IL10. It is possible that the decrease in the amount of IL10 mRNA that we observed in *Npc1*$^{-/-}$ microglia attenuates the ability of the microglia to prevent neuronal death. Thus, the reduction in IL10 production might lead to chronic activation of *Npc1*$^{-/-}$ microglia and the prolonged production of pro-inflammatory cytokines and other neurotoxic molecules that would eventually contribute to neuron death in the CNS.

4.3. Microglia–neuron co-cultures

Active microglia have been detected in NPC1-deficient mouse brains prior to any detectable neurodegeneration [13], suggesting that NPC1-deficient microglia might initiate neuron death in NPC disease. Our experiments with microglia–neuron co-cultures demonstrate that the survival of CGCs is not compromised when the neurons are cultured in the presence of *Npc1*$^{-/-}$, instead of *Npc1*$^{+/+}$, microglia. This observation is consistent with the idea that NPC1 deficiency in microglia does not initiate neuron death. Interestingly, in the presence of high concentrations of microglia, less neuronal apoptosis occurred when *Npc1*$^{-/-}$ neurons were cultured with *Npc1*$^{-/-}$ microglia, than when *Npc1*$^{+/+}$ neurons were cultured with *Npc1*$^{+/+}$ microglia. Whether or not this neuroprotective effect would be physiologically relevant is not clear since the number of apoptotic neurons was only 10–20% under all culture conditions. Thus, our results support *in vivo* studies in which Purkinje cell death in NPC disease was shown to be cell autonomous [48,56,64]. For example, NPC1 deficiency in Purkinje cells alone caused Purkinje cell death despite other cells of the brain, including microglia,

having the *Npc1*$^{+/+}$ genotype. Furthermore, when NPC1 was inducibly expressed specifically in neurons in different regions of the brain of *Npc1*$^{-/-}$ mice, such as the thalamus, cholesterol accumulation in the neurons was eliminated, neurodegeneration was prevented, and glial activity was reduced [56]. Our results expand on these findings by showing that in microglia–neuron co-cultures, NPC1 deficiency in microglia does not cause neuronal death.

Nevertheless, it is important to note that our findings do not preclude the involvement of microglia in NPC disease progression since extensive interactions occur among the different cell types within the intact brain. Thus, although NPC1 deficiency in neurons appears to be the primary cause of neuronal death in NPC-deficient brains, chronic microglial activation and inflammation in the CNS might occur in response to neuron death which might exacerbate the neurodegeneration. Indeed, anti-inflammatory therapy delayed the onset of neurological symptoms and prolonged the lifespan of *Npc1*$^{-/-}$ mice [65]. Thus, our data support the view that the loss of NPC1 in microglia is not the primary cause of CNS pathogenesis in NPC disease.

5. Conclusions

We show that *Npc1*$^{-/-}$ microglia sequester cholesterol intracellularly. Although microglia with an activated morphology proliferate in NPC1-deficient mouse brains, NPC1 deficiency in primary cultures of microglia does not increase the secretion of the pro-inflammatory cytokine, TNFα, or the level of mRNAs encoding oxidative stress genes in microglia. On the other hand, the level of mRNA encoding IL10, an anti-inflammatory cytokine, was lower in *Npc1*$^{-/-}$ microglia than in *Npc1*$^{+/+}$ microglia. Our data also show that the survival of primary cultures of neurons was not impaired by NPC1 deficiency. Furthermore, *Npc1*$^{-/-}$ microglia did not induce neuron death in microglia–neuron co-cultures. Thus, while *Npc1*$^{-/-}$ microglia might exacerbate the neurodegeneration in *Npc1*$^{-/-}$ brains, NPC1 deficiency in microglia alone appears to be insufficient to initiate neuron death.

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