## Results

### Oligodendrocytes surviving in the non-demyelinated white matter of NHD brains intensely expressed LC3 immunoreactivity

First, we validated the specificity of anti-LC3 antibody PM036 by western blot analysis of total protein extracted from mouse oligodendrocyte-type 2 astrocyte (O2A) progenitor cells termed OS3 [30], following a 48-hour exposure to rapamycin, a potent inducer of autophagy. This antibody reacted with both LC3-I, the soluble cytosolic form and LC3-II, the autophagy-inducible phosphatidylethanolamine (PE)-conjugated form (Additional file 1: Figure S1a, b, lanes 1, 2). Then, we studied the expression of LC3 in the serial brain sections of five NHD, four MD, and four NC cases by immunohistochemistry using the PM036 antibody. In all cases examined, substantial populations of cortical neurons constitutively expressed LC3 in the cytoplasm at varying intensities. Notably, in all five NHD brains, Nogo-A-positive, cleaved caspase-3 (CASP3)-negative (non-apoptotic) oligodendrocytes surviving in the MBP-positive (non-demyelinated) white matter intensely expressed LC3 with the location in the cytoplasm (Figure 1a-c, Figure 2a-c; Additional file 2: Figure S2a, b). Some LC3-immunolabeled oligodendrocytes showed a morphological feature of swollen cytoplasm (Figure 1d). In contrast, extensively demyelinated white matter, almost totally devoid of oligodendrocytes, was not labeled with anti-LC3 antibody (Figure 1a-c, upper half). Double labeling verified that LC3-expressing cells accumulated in the non-demyelinated white matter of NHD brains coexpressed 2',3'-cyclic nucleotide 3' phosphodiesterase (CNPase), a cell type-specific marker of oligodendrocytes (Figure 2f). The distribution of LC3 immunoreactivity (Figure 2a) was well consistent with the staining pattern of Nogo-A (Figure 2b) but not of GFAP (Figure 2d) or Iba1 (Figure 2e), although some populations of ramified microglia, accumulating macrophages, and reactive astrocytes expressed intensely LC3 in NHD brains

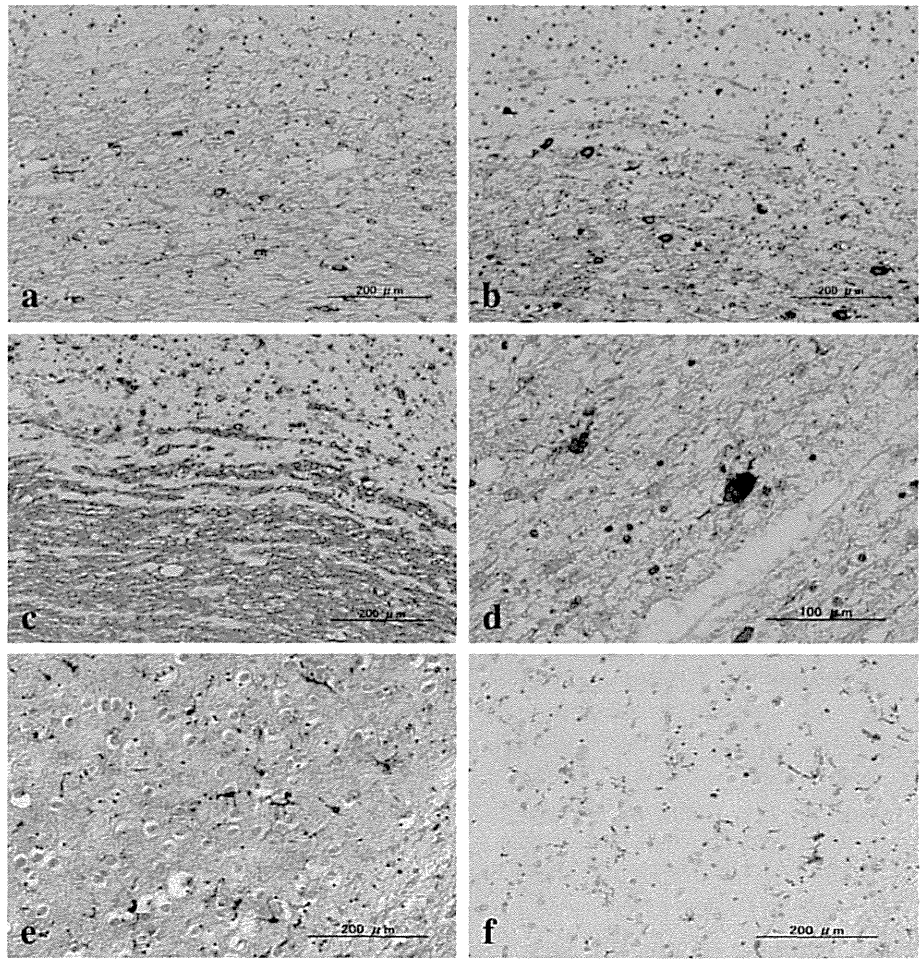


**Figure 1 Surviving oligodendrocytes express LC3 in NHD brains.** The serial brain sections of NHD cases were processed for immunohistochemistry. The panels **(a-f)** represent **(a)** the periventricular white matter, LC3, **(b)** the same field as **(a)**, Nogo-A, **(c)** the same field as **(a)**, MBP, the upper half indicates demyelinated lesions, **(d)** the frontal white matter, LC3, **(e)** the basal ganglia, LC3, and **(f)** the same field as **(e)**, Iba1.

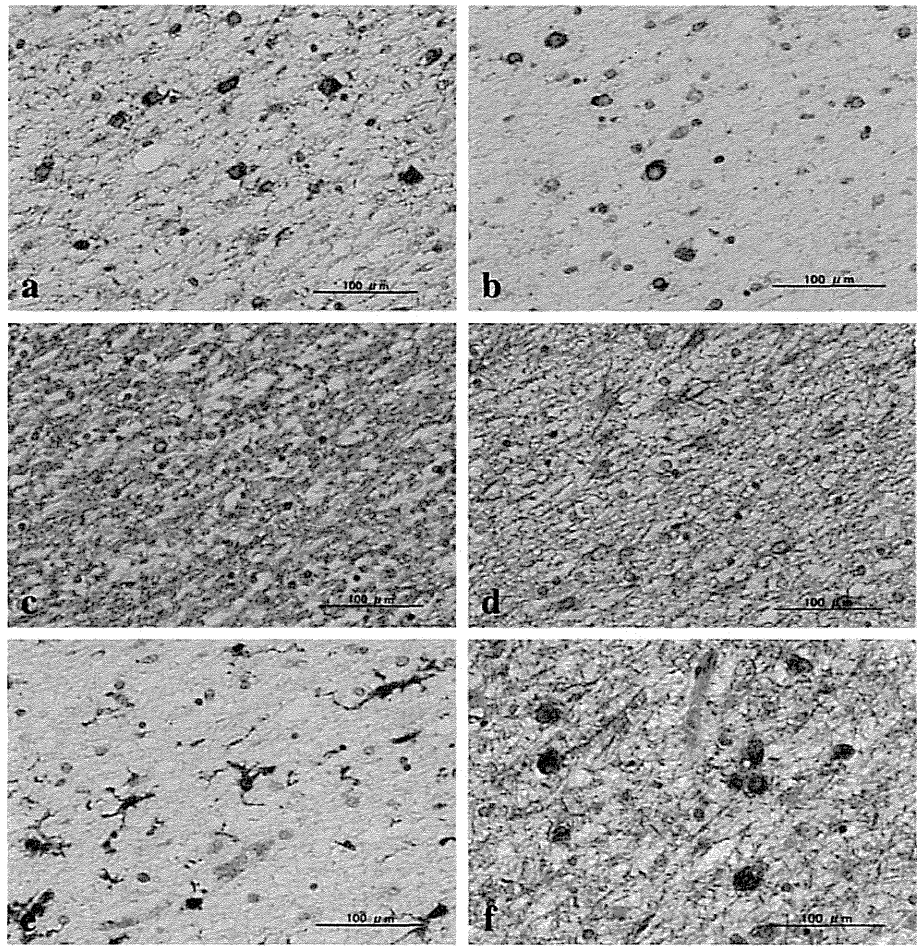


**Figure 2 Surviving oligodendrocytes express LC3 in NHD brains.** The serial brain sections of NHD cases were processed for immunohistochemistry. The panels **(a-f)** represent the identical field of the frontal white matter labeled with **(a)** LC3, **(b)** Nogo-A, **(c)** MBP, **(d)** GFAP, **(e)** Iba1, and **(f)** CNPase (red) and LC3 (brown).

(Figure 1e, f). In NHD brains, substantial numbers of axonal spheroids were also labeled with LC3, along with neurofilament (Figure 3a, b). In contrast, we found no LC3-expressing oligodendrocytes in the white matter of control brains, including NC and MD cases (Figure 3c-f).

### LC3-positive oligodendrocytes did not express p62 or beclin 1 in NHD brains

Next, we studied the expression of a panel of autophagy regulators, such as Beclin 1 (ATG6), p62 or NBR1 in NHD brains. Nogo-A-positive LC3-positive oligodendrocytes did not express either Beclin 1 or p62 (Figure 4a-d), although a subpopulation of LC3-positive oligodendrocytes fairly weakly expressed neighbor of BRCA1 gene 1 (NBR1) (Additional file 2: Figure S2c). In contrast, the majority of LC3-positive oligodendrocytes intensely or moderately expressed histone deacetylase 6 (HDAC6) in the cytoplasm (Additional file 2: Figure S2d). Furthermore, many LC3-positive oligodendrocytes moderately expressed both ubiquitin and ubiquilin-1 (UBQLN1) (Figure 4e, f). These observations suggest that the expression of UPS components is upregulated in surviving oligodendrocytes distributed in the non-demyelinating white matter of NHD brains.

### Oligodendrocytes surviving at the demyelinated lesion edge in MS brains did not express LC3

Finally, to investigate whether oligodendroglial LC3 expression represents a general biological process during demyelination, we studied the expression of LC3 in the cerebral white matter of the brains derived from four MS patients. At the edge of chronic active demyelinated lesions, surviving Nogo-A-positive oligodendrocytes did not express LC3 (Figure 5a, b), whereas infiltrating Iba1-positive macrophages and microglia intensely expressed LC3 in MS lesions (Figure 5e-f). Furthermore, none of Nogo-A-positive oligodendrocytes expressed LC3 in earlier lesions as well as normal-appearing white matter

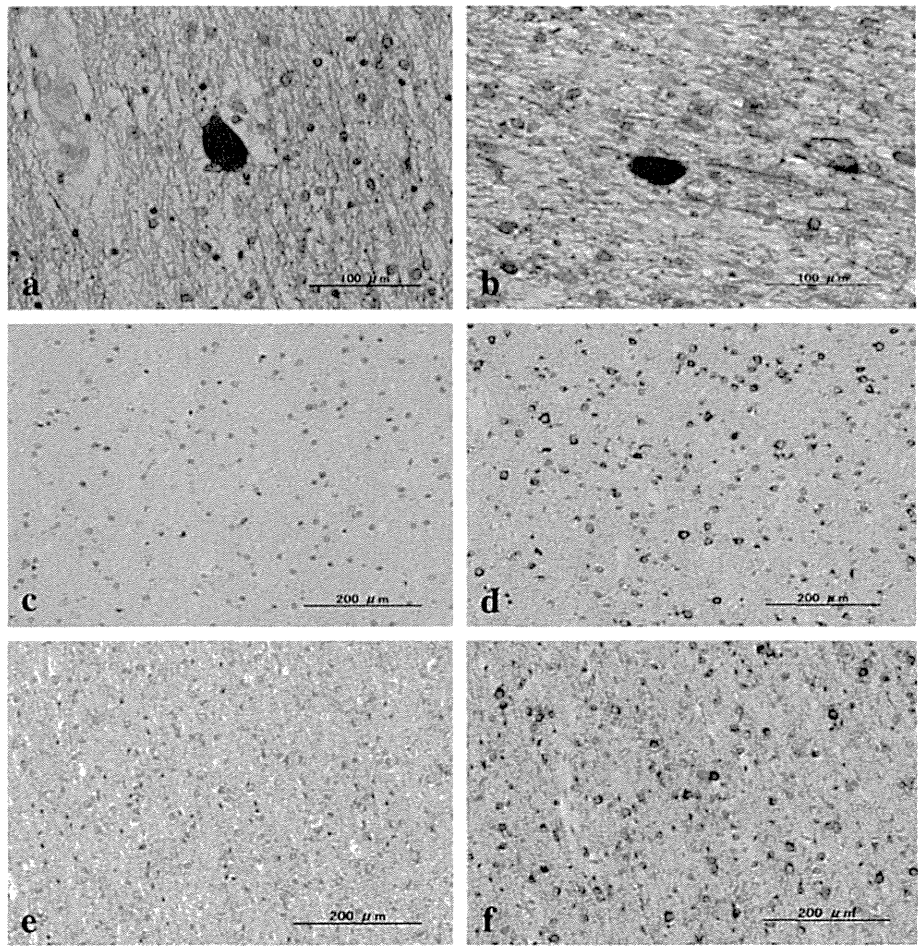


**Figure 3 Oligodendrocytes do not express LC3 in control brains.** The serial brain sections of NHD, myotonic dystrophy (MD), and non-neurological control (NC) cases were processed for immunohistochemistry. The panels **(a-f)** represent **(a)** NHD, the basal ganglia, LC3, **(b)** NHD, the frontal white matter, neurofilament protein (red) and LC3 (brown), **(c)** NC, the frontal white matter, LC3, **(d)** the same field as **(c)**, Nogo-A, **(e)** MD, the frontal white matter, LC3, and **(f)** the same field as **(e)**, Nogo-A.

(NAWM) of MS brains (Additional file 3: Figure S3a, b). These observations suggest that oligodendroglial expression of LC3 is not unique to demyelinating events.

## Discussion

Here, we found that oligodendrocytes surviving in the non-demyelinated white matter of NHD brains but not in the white matter of control brains intensely expressed LC3, the most reliable *in vivo* marker of autophagosomes. LC3-positive oligodendrocytes also expressed ubiquitin, ubiqulin-1 and HDAC6, whereas they marginally expressed NBR1 and did not express Beclin 1 or p62. Furthermore, a subset of axonal spheroids expressed LC3 in NHD brains. Since oligodendrocytes support axonal function by continuously supplying energy metabolites to axons [31], a functional relationship might exist between LC3-expressing oligodendrocytes and axonal spheroids. In contrast, surviving oligodendrocytes located at the demyelinated lesion edge of MS did not express LC3, indicating that different molecular mechanisms might be involved in demyelinating processes between NHD and MS. It is well known that demyelinated lesions in MS brains have a well-demarcated border but the lesions are ill-defined and diffuse in the white matter of NHD brains [4]. Cortical demyelination is common in MS brains [32], while the architecture of the cerebral cortex is well preserved in NHD brains [33]. Demyelinated lesions of MS are often accompanied by perivascular infiltration of numerous T lymphocytes, while NHD brain lesions contain a limited number of CD3-positive T cells [19], supporting the general view that MS is a T cell-mediated autoimmune disease affecting the central nervous system white matter, whereas autoimmune mechanisms are unlikely to play a central role in the pathogenesis of NHD. Previously, we found that the levels of expression of a guanine nucleotide exchanger for Rap termed RAPGEF4,

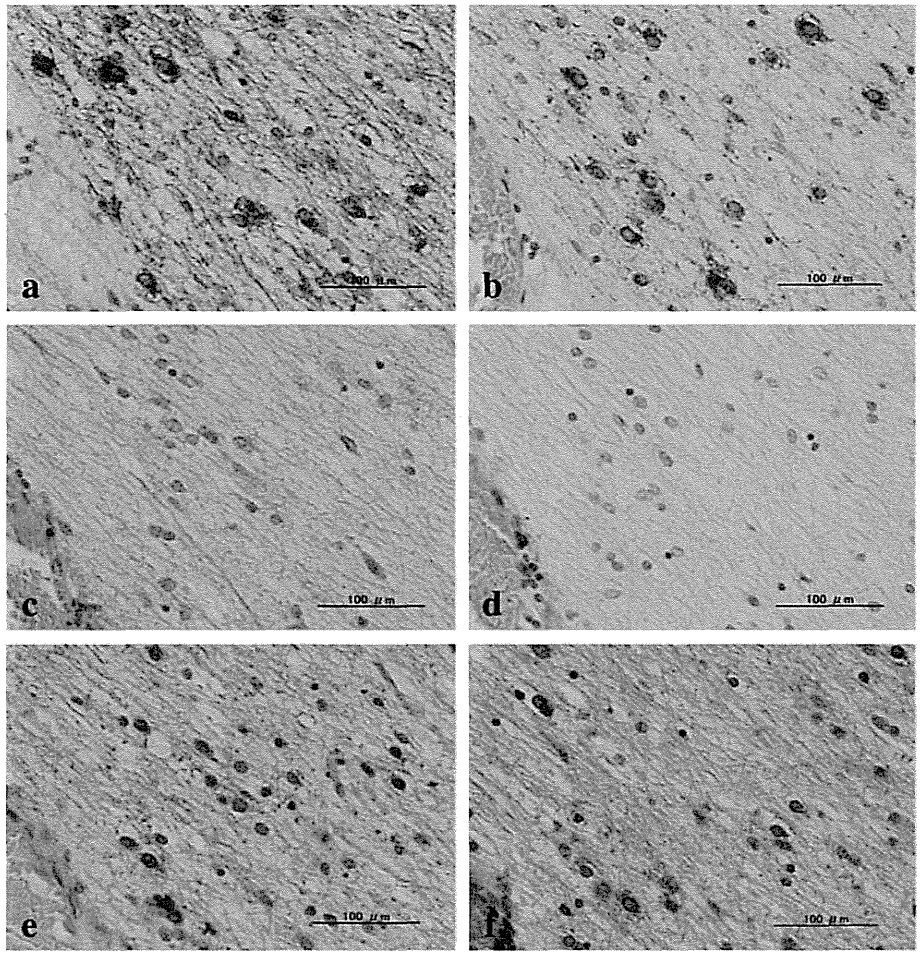


**Figure 4 Surviving oligodendrocytes do not express Beclin 1 or p62 in NHD brains.** The serial brain sections of NHD cases were processed for immunohistochemistry. The panels **(a-f)** represent the identical field of the frontal white matter labeled with **(a)** LC3, **(b)** Nogo-A, **(c)** Beclin 1, **(d)** p62, **(e)** ubiquitin, and **(f)** UBQLN1.

which plays a role in the inhibition of autophagy [34], are greatly reduced in NHD brains [35]. All of these observations suggest the hypothesis that aberrant regulation of autophagy might induce oligodendrogliopathy causative of leukoencephalopathy in NHD brains.

Autophagy is mediated by the molecular machinery that involves numerous regulatory proteins [20,21]. Recently, more than 400 interacting proteins that constitute the basal autophagy network have been identified in human cells, representing the extreme complexity of autophagy [36]. LC3 (ATG8), synthesized as a precursor form, is cleaved at its C-terminus by the cysteine protease ATG4B, which generates the cytosolic isoform termed LC3-I [20]. During the phagophore elongation, LC3-I is conjugated to PE via a reaction that involves ATG7 and ATG3 to form LC3-II that is specifically targeted to the elongating autophagosomal membranes. Following the fusion of autophagosomes with lysosomes, LC3-II located on the cytoplasmic face of autolysosomes is delipidated by ATG4 and processed for recycling, while LC3-II on the internal surface of autophagosomes is processed for degradation by lysosomal enzymes of autolysosomes [20]. All currently available anti-LC3 antibodies, including the PM036 antibody utilized in the present study, recognize both LC3-I and LC3-II. When autophagosomes are accumulated in the cell extremely in number due to excessive induction or reduced completion of autophagy, LC3 intensities are elevated chiefly by an increase in LC3-II expression on autophagosomal membranes.

Under physiological conditions, UPS mainly regulates degradation of short-lived polyubiquitinated proteins, while autophagy predominantly degrades long-lived proteins having higher-ordered structures inaccessible to the narrow pore of the barrel structure of the proteasome, although functionally redundant interactions exist between the two systems [37]. A battery of autophagic receptors/adaptors that connect the UPS and autophagy, such as p62, NBR1, UBQLN1, optineurin (OPTN), and HDAC6,

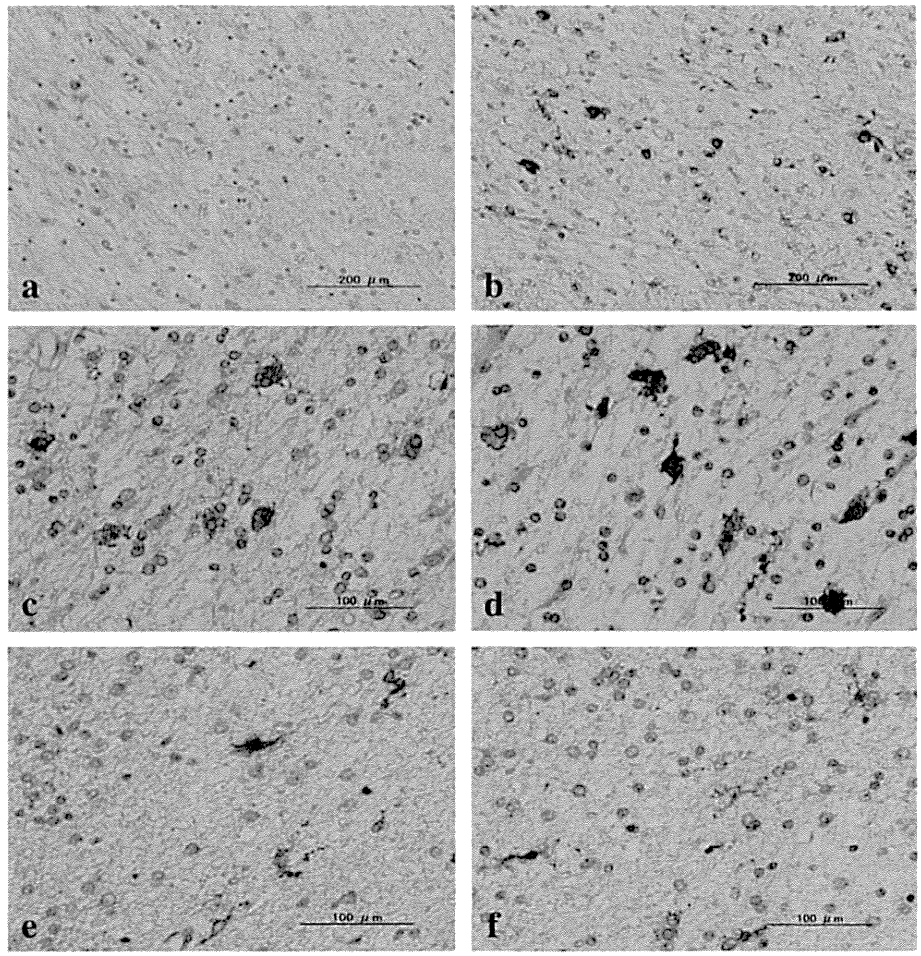


**Figure 5 Macrophages and microglia but not oligodendrocytes express LC3 in MS brains.** The serial brain sections of multiple sclerosis (MS) cases were processed for immunohistochemistry. The panels **(a-f)** represent **(a)** the edge of demyelinated lesions in the parietal white matter, LC3, **(b)** the same field of **(a)**, Nogo-A, **(c)** the edge of demyelinated lesions in the frontal white matter, LC3, **(d)** the same field as **(c)**, Iba1, **(e)** the edge of demyelinated lesions in the frontal white matter, LC3, and **(f)** the same field as **(e)**, Iba1.

recognize ubiquitinated target proteins and promote their degradation by autophagy [38]. Importantly, p62, NBR1, UBQLN1, and OPTN have a capacity to bind directly to LC3 [39,40]. We found that LC3-positive oligodendrocytes intensely express UBQLN1 and HDAC6, both of which play a pivotal role in the aggresome formation [41,42]. In contrast, LC3-positive oligodendrocytes did not much express either p62 or NBR1. It is worthy to note that p62 knockout mice do not show a defect in bulk autophagy, suggesting that p62 is primarily dispensable for the clearance of autophagic substrates [43].

Under stressful conditions, autophagy serves as a protective mechanism for the cell to prevent the accumulation of cytotoxic protein aggregates and damaged organelles [20,21]. Actually, increased levels of autophagy promote survival of oligodendrocytes in a myelin-deficient rat [44]. However, uncontrolled activation of autophagy often induces cell death. During ischemia-reperfusion injury of the myocardium, early activation of autophagy upon ischemia is protective, while delayed and robust activation of autophagy during reperfusion is detrimental for cell survival [45]. Furthermore, a tight linkage is found between autophagy and apoptosis. The autophagy inhibitor 3-methyladenine (3-MA) inhibits apoptotic cell death of TNFα-treated T lymphoblastic leukemia cells and NGF-deprived sympathetic neurons [46,47]. The prototype anti-apoptotic regulator Bcl-2 inhibits starvation-induced autophagy by directly interacting with Beclin 1 [48].

At present, the precise mechanism remains unknown how microglial dysfunction termed microgliopathy caused by the genetic defect of DAP12 or TREM2 induces oligodendrogliopathy characterized by enhanced LC3 expression on oligodendrocytes in NHD brains. It is possible that microglia persistently deregulated in NHD brains produce excessive amounts of reactive oxygen species (ROS) that potentially activate autophagy in

oligodendrocytes. In turn, autophagy itself controls inflammation through regulatory interactions with innate immune signaling pathways [40]. By gene expression profiling, we recently identified 324 DEGs expressed in frozen brain tissues of a NHD patient with a splicing mutation of TREM2 [35]. Among them, the set of 136 genes involved in inflammatory response and immune cell trafficking are upregulated, while the set of 188 genes including a battery of GABA receptor subunits and synaptic proteins are downregulated in NHD brains. These observations suggested that both neuroinflammatory and neurodegenerative events proceed concurrently in NHD brains. Notably, the expression of a set of macrophage/microglia markers, such as CD163, MSR1, and CD68, is greatly elevated in NHD brains [35].

Upregulation of LC3 is attributable to increased autophagic flux or decreased autophagic substrate clearance, or both. Diverse stress-inducing stimuli, including exposure to ROS and deprivation of nutrients, growth factors, or adenosine triphosphate (ATP), all turns on autophagy by inhibiting the mammalian target of rapamycin complex 1 (mTORC1) [20]. Notably, rapamycin, a relatively selective inhibitor of mTORC1, ameliorates neurodegeneration in mouse models of AD, PD, and frontotemporal lobar degeneration (FTLD), where neuronal cell death is attributable to a defect in autophagy [49,50]. On the contrary, activation of mTORC1 and mTORC2 is pivotal for oligodendrocytes differentiation at the stage of transition from the late progenitors to immature oligodendrocytes [51]. All of these observations suggest that delicate regulation of cellular autophagy levels plays a decisive role in neural cell survival or cell death.

## Conclusions

We for the first time found that LC3 is expressed on surviving oligodendrocytes in the non-demyelinated white matter of NHD brains but not in the white matter of control brains. These observations propose a novel hypothesis that aberrant regulation of autophagy might induce oligodendrogliopathy causative of leukoencephalopathy in NHD brains.

## Additional files

**Additional file 1: Figure S1.** Validation of the specificity of anti-LC3 antibody. Total protein extracted from oligodendrocyte-type 2 astrocyte (O2A) progenitor cells named OS3 was processed for western blot with (a) anti-LC3 antibody PM036 and relabeled with (b) anti-HSP60 antibody for standardization of protein loading. The lanes (1, 2) indicate a 48 hour-treatment of OS3 cells with (1) the equal v/v% concentration of dimethyl sulfoxide (DMSO) or (2) 1 μM rapamycin.

**Additional file 2: Figure S2.** Surviving oligodendrocytes express HDAC6 in NHD brains. The serial brain sections of NHD cases were processed for immunohistochemistry. The panels (a-d) represent (a) the perivascular white matter, LC3, (b) the same field as (a), cleaved CASP3, (c) the same filed as (a), NBR1, and (d) the same field as (a), HDAC6 with a close-up view in inset.

**Additional file 3: Figure S3.** Oligodendrocytes do not express LC3 in early lesions of MS brains. The serial brain sections of MS cases were processed for immunohistochemistry. The panels (a, b) represent (a) an early lesion in the frontal white matter, LC3, some macrophages are positive, and (b) the same field as (a), Nogo-A.

### Abbreviations

AD: Alzheimer's disease; CNPase: 2',3'-cyclic nucleotide 3' phosphodiesterase; DAP12: DNAX-activation protein 12; HDAC6: Histone deacetylase 6; LC3: microtubule-associated protein 1 light chain 3; MD: Myotonic dystrophy; MS: Multiple sclerosis; NBR1: Neighbor of BRCA1 gene 1; NHD: Nasu-Hakola disease; PD: Parkinson's disease; TREM2: Triggering receptor expressed on myeloid cells 2; UPS: Ubiquitin-proteasome system.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

JS and YK performed immunohistochemical analysis. JS drafted the manuscript. NM, SY, KJ, NA, KN, and AT provided NHD brain tissues. KA, YS, and TI validated the pathological diagnosis of all autopsied brains. All authors read and approved the final manuscript.

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### Author details

[1]Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University, Tokyo, Japan. [2]Department of Psychiatry, University of Yamanashi, Faculty of Medicine, Yamanashi, Japan. [3]Department of Pathology and Laboratory Medicine, Kohnodai Hospital, National Center for Global Health and Medicine, Chiba, Japan. [4]Department of Pathology, Kanagawa Rehabilitation Center, Kanagawa, Japan. [5]Department of Neurology, NHO Hyogo-Chuo Hospital, Hyogo, Japan. [6]Brain Pathology Research Center, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan. [7]Department of Neurology, Institute of Clinical Medicine, University of Tsukuba, Ibaraki, Japan. [8]Department of Laboratory Medicine, National Center Hospital, NCNP, Tokyo, Japan. [9]Department of Psychiatry, National Center Hospital, NCNP, Tokyo, Japan.

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# Human Astrocytes: Secretome Profiles of Cytokines and Chemokines

**Sung S. Choi[1☯], Hong J. Lee[1☯], Inja Lim[2], Jun-ichi Satoh[3], Seung U. Kim[1,4*]**

**1** Medical Research Institute, Chung-Ang University College of Medicine, Seoul, Korea, **2** Department of Physiology, Chung-Ang University College of Medicine, Seoul, Korea, **3** Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University, Kiyose, Japan, **4** Division of Neurology, Department of Medicine, UBC Hospital, University of British Columbia, Vancouver, Canada

## Abstract

Astrocytes play a key role in maintenance of neuronal functions in the central nervous system by producing various cytokines, chemokines, and growth factors, which act as a molecular coordinator of neuron-glia communication. At the site of neuroinflammation, astrocyte-derived cytokines and chemokines play both neuroprotective and neurotoxic roles in brain lesions of human neurological diseases. At present, the comprehensive profile of human astrocyte-derived cytokines and chemokines during inflammation remains to be fully characterized. We investigated the cytokine secretome profile of highly purified human astrocytes by using a protein microarray. Non-stimulated human astrocytes in culture expressed eight cytokines, including G-CSF, GM-CSF, GROα (CXCL1), IL-6, IL-8 (CXCL8), MCP-1 (CCL2), MIF and Serpin E1. Following stimulation with IL-1β and TNF-α, activated astrocytes newly produced IL-1β, IL-1ra, TNF-α, IP-10 (CXCL10), MIP-1α (CCL3) and RANTES (CCL5), in addition to the induction of sICAM-1 and complement component 5. Database search indicated that most of cytokines and chemokines produced by non-stimulated and activated astrocytes are direct targets of the transcription factor NF-kB. These results indicated that cultured human astrocytes express a distinct set of NF-kB-target cytokines and chemokines in resting and activated conditions, suggesting that the NF-kB signaling pathway differentially regulates gene expression of cytokines and chemokines in human astrocytes under physiological and inflammatory conditions.

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* E-mail: sukim@mail.ubc.ca

☯ These authors contributed equally to the work.

## Introduction

Astrocytes belong to one of three major types of neuroglia in the central nervous system (CNS) and play active roles in many neuronal functions: maintaining ion and pH homeostasis, promoting the synthesis and removal of neurotransmitters, providing glucose supply and antioxidant defense, and regulating synaptic activity by producing various cytokines, chemokines, growth factors, and metabolites, all of which act as a molecular coordinator of neuron-glia communication [1]. At the site of neuroinflammation, astrocyte-derived cytokines and chemokines play both neurotoxic (inflammatory) and neuroprotective (immunoregulatory) roles in the brains of human neurological diseases, such as multiple sclerosis (MS), Alzheimer's disease (AD), Parkinson's disease (PD), and HIV-1 associated dementia (HAD) [2]. However, at present, the exact profile of human astrocyte-derived cytokines and chemokines during inflammation remain mostly unclear, possibly attributable to the limited availability of non-malignant human astrocyte cell lines that serve as an *in vitro* model of normal human astrocytes. In the present study, we characterized the comprehensive profiles named "secretome" [3] of cytokines and chemokines derived from cultured normal human astrocytes, compared under resting and activated conditions by using a protein microarray.

## Materials and Methods

### Ethics statement

Fetal brain tissue was obtained from a 15 weeks human fetus and dissociated cells prepared as described previously [4,5]. Human tissue collected for research purpose was approved by the Chung-Ang University Ethics Committee on Human Subject (Certificate #09-0041). Pregnant women gave written informed consents for clinical procedure and research use of the embryonic tissue in accordance with the declaration of Helsinki.

### Human astrocytes in culture

The brain tissue isolated from a fetus of 15 weeks gestation, was dissociated into single cells by incubation with 0.25% trypsin in phosphate-buffered saline (PBS) for 30 min, as described previously [4,5]. Dissociated cells were suspended in the culture medium, composed of the Dulbecco's modified Eagle medium (DMEM) with high glucose (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 20 μg/mL gentamicin (Sigma, St Louis, MO). Dissociated cells were plated at $5\times10^7$ cells/T75 flask and were grown in an incubator with 5% $CO_2$ atmosphere. In primary brain cell cultures, all microglia freely floating in the medium were removed.

**Table 1.** Proteome profiler array of human cytokines/chmokines used in the present study.

| Location | Control | Expression ratio in stimulated astrocytes |
|---|---|---|
| A3, A4 | Complement Component 5/5a (C5/C5a) | Up |
| A5, A6 | CD40 Ligand (CD154) | - |
| A7, A8 | G-CSF (CSFβ, CSF-3) | Up |
| A9, A10 | GM-CSF (CSFα, CSF-2) | Up |
| A11, A12 | GROα (CXCL1) | Up |
| A13, A14 | I-309 (CCL1) | - |
| A15, A16 | sICAM-1 (CD54) | Up |
| A17, A18 | IFN-γ (Type II IFN) | - |
| B3, B4 | IL-1α (IL-1F1) | - |
| B5, B6 | IL-1β (IL-1F2) | Up |
| B7, B8 | IL-1ra (IL-1F3) | Up |
| B9, B10 | IL-2 | - |
| B11, B12 | IL-4 | - |
| B13, B14 | IL-5 | - |
| B15, B16 | IL-6 | Up |
| B17, B18 | IL-8 (CXCL8) | Up |
| C3, C4 | IL-10 | - |
| C5, C6 | IL-12 p70 | - |
| C7, C8 | IL-13 | - |
| C9, C10 | IL-16 (LCF) | - |
| C11, C12 | IL-17 | - |
| C13, C14 | IL-17E | - |
| C15, C16 | IL-23 | - |
| C17, C18 | IL-27 | - |
| D3, D4 | IL-32 | - |
| D5, D6 | IP-10 (CXCL10) | Up |
| D7, D8 | I-TAC (CXCL11) | - |
| D9, D10 | MCP-1 (CCL2) | Down |
| D11, D12 | MIF (GIF, DER6) | Down |
| D13, D14 | MIP-1α (CCL3) | Up |
| D15, D16 | MIP-1β (CCL4) | - |
| D17, D18 | Serpin E1 (PAI-1) | Up |
| E3, E4 | RANTES (CCL5) | Up |
| E5, E6 | SDF-1 (CXCL12) | - |
| E7, E8 | TNF-α (TNFSF1A) | Up |
| E9, E10 | sTREM-1 | - |

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After replating the cultures for three to five times by treatment with trypsin, most of neurons and oligodendrocytes underwent cell death and detached off, while astrocytes were firmly attached onto the flask surface, resulting in enrichment of highly purified astrocytes.

## Immunocytochemistry

Human astrocytes cultured on poly-L-lysine-coated Aclar plastic coverslips (9 mm in diameter) were fixed in methanol for 10 min at −20°C. The cells were incubated with primary antibodies specific for GFAP (1:1,000, rabbit; Millipore, Billerica, MA), a cell type specific marker for astrocytes, or tubulin βIII antibody (1:200, mouse, Millipore), a. neuron specific marker, for 48 hrs at 4°C followed by Alexa Fluor594-conjugated anti-rabbit IgG or anti-mouse IgG for 1 hr at room temperature (RT). For immunostaining for cell type markers for oligodendrocytes and microglia, astrocytes on coverslips were fixed in 4% paraformaldehyde for 2 min, washed in PBS, incubated in anti-galactocerebroside

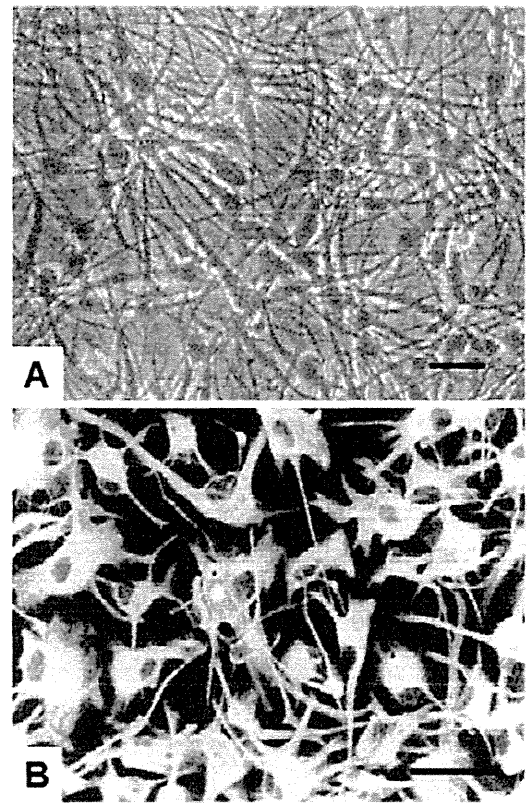


**Figure 1. The purity of human astrocytes in culture exceeded 99% glial fibrillary acidic protein (GFAP) immunoreactivity-positive.** Astocytes shown are at the normal non-stimulated resting state. A: Phase contrast microscopy. B: Immunostaning with anti-GFAP antibody.
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**Table 2.** Cytokines expressed in normal resting human astrocytes.

| Gene | Genbank | Gene Name |
|---|---|---|
| G-CSF | NM_000759 | colony stimulating factor 3 (granulocyte) |
| GM-CSF | NM_000758 | colony stimulating factor 2 |
| MCP-1 (CCL2) | NM_002982 | chemokine (C-C motif) ligand 2 |
| GROα (CXCL1) | NM_001511 | chemokine (C-X-C motif) ligand 1 |
| MIF | NM_002415 | macrophage migration inhibitory factor |
| IL-6 | NM_000600 | interleukin 6 |
| IL-8 (CXCL8) | NM_000584 | interleukin 8 |
| Serpin E1 | NM_000602 | plasminogen activator inhibitor type 1 |

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