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LC3, an autophagosome marker, is expressed on oligodendrocytes in Nasu-Hakola disease brains

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Abstract

Background: Nasu-Hakola disease (NHD) is a rare autosomal recessive disorder characterized by sclerosing leukoencephalopathy and multifocal bone cysts, caused by a loss-of-function mutation of either DAP12 or TREM2. TREM2 and DAP12 constitute a receptor/adaptor signaling complex expressed exclusively on osteoclasts, dendritic cells, macrophages, and microglia. Neuropathologically, NHD exhibits profound loss of myelin and accumulation of axonal spheroids, accompanied by intense gliosis accentuated in the white matter of the frontal and temporal lobes. At present, the molecular mechanism responsible for development of leukoencephalopathy in NHD brains remains totally unknown.

Methods: By immunohistochemistry, we studied the expression of microtubule-associated protein 1 light chain 3 (LC3), an autophagosome marker, in 5 NHD and 12 control brains.

Results: In all NHD brains, Nogo-A-positive, CNPase-positive oligodendrocytes surviving in the non-demyelinated white matter intensely expressed LC3. They also expressed ubiquitin, ubiquilin-1, and histone deacetylase 6 (HDAC6) but did not express Beclin 1 or sequestosome 1 (p62). Substantial numbers of axonal spheroids were also labeled with LC3 in NHD brains. In contrast, none of oligodendrocytes expressed LC3 in control brains. Furthermore, surviving oligodendrocytes located at the demyelinated lesion edge of multiple sclerosis (MS) did not express LC3, whereas infiltrating Iba1-positive macrophages and microglia intensely expressed LC3 in MS lesions.

Conclusions: These results propose a novel hypothesis that aberrant regulation of autophagy might induce oligodendrogliopathy causative of leukoencephalopathy in NHD brains.

Keywords: Autophagy, LC3, Leukoencephalopathy, Nasu-Hakola disease, Oligodendrocytes

Background

Nasu-Hakola disease (NHD), also designated polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL; OMIM 221770), is a rare autosomal recessive disorder, characterized by progressive presenile dementia and formation of multifocal bone cysts [1,2]. Although NHD patients are clustered in Japan and Finland, approximately 200 NHD cases are presently reported worldwide (http://www.orpha.net). Clinically, the patients show pathological bone fractures during the third decade of life, and a frontal lobe syndrome, such as loss of judgment and social inhibitions during the fourth decade of life, followed by progressive dementia and death until the fifth decade of life [3]. Pathologically, NHD brains exhibit extensive demyelination with sparing of subcortical U-fibers, accumulation of axonal spheroids, and intense astrogliosis predominantly in the white matter of frontal and temporal lobes and the basal ganglia [4]. Genetically, NHD is caused by the set of heterogeneous mutations located in one of the two genes, DNAX-activation protein 12 (*DAP12*), alternatively named TYRO protein tyrosine kinase-binding protein (*TYROBP*) on chromosome 19q13.1 or triggering receptor expressed on myeloid cells 2 (*TREM2*) on chromosome 6p21.1 [5-7]. Previous studies identified 7 different mutations in the *TYROBP* gene and 11 distinct mutations in the *TREM2* gene in NHD patients. The presence of multiple bone cysts, basal ganglia calcification, and genetic mutations of *TYROBP* or

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TREM2 in a pattern of autosomal recessive inheritance could differentiate NHD from hereditary diffuse leukoencephalopathy with spheroids (HDLS; OMIM 221820), a rare autosomal dominant disorder presenting with clinicopathological similarities to NHD, which is caused by genetic mutations in the colony-stimulating factor 1 receptor (CSF1R) gene [8].

TREM2, expressed exclusively on myeloid cells, such as osteoclasts, dendritic cells, macrophages, and microglia, acts as a receptor for as yet unidentified ligands. TREM2 constitutes a signaling complex with an adaptor molecule DAP12, leading to phosphorylation and activation of the downstream kinase named spleen tyrosine kinase (Syk), following the receptor engagement [9]. Syk transduces a wide range of downstream signals involved in activation of phosphatidylinositol-3 kinase (PI3K), phospholipase C (PLC), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) [10].

Increasing evidence indicated that a defect in microglial TREM2/DAP12 function plays a central role in the pathogenesis of NHD [11]. However, at present, the molecular mechanism responsible for development of leukoencephalopathy in NHD brains remains totally unknown. DAP12-knockout mice develop osteopetrosis, thalamic hypomyelination, and synaptic degeneration [12], being phenotypically different from osteolytic lesions and sudanophilic leukoencephalopathy found in NHD patients. Several studies showed that oligodendrocytes, along with microglia, express DAP12 [12,13]. However, follow-up studies could not verify oligodendroglial expression of DAP12 [14]. The synaptic function is also altered in DAP12 loss-of-function (KΔ75) mice, attributable to reduced expression of AMPA receptor GluR2 subunit and neurotrophin receptor TrkB [15]. Furthermore, the total number of microglia is greatly reduced in the brain of DAP12-deficient and loss-of-function mice [16,17]. These observations suggest that DAP12 signaling pathway plays a key role in development of microglia and maturation of synapses. Knockdown of TREM2 on cultured mouse microglia inhibits phagocytosis of apoptotic neurons, and stimulates production of proinflammatory cytokines, such as TNFα and IL-1β, suggesting that TREM2 plays a key role in the clearance of dying neural cells by microglia to resolve damage-induced inflammation [18]. In contrast to the suggested role of microglial TREM2 in the pathogenesis of NHD, we recently found that TREM2 is not expressed constitutively on human microglia, and Iba1-positive microglia are well preserved in the brains of NHD patients with DAP12 mutations [19].

Macroautophagy, hereafter called as autophagy, constitutes a lysosome-mediated degradation process that controls the quality of cytoplasmic components and organelles [20,21]. The process of autophagy involves the complex molecular machinery, composed of more than 30 autophagy-related (Atg) proteins and 50 lysosomal hydrolases. Autophagy is initiated by the formation of double-membrane-bound vesicles named autophagosomes that sequester cytoplasmic material in a non-degenerative compartment, followed by fusion with lysosomes, leading to degradation of the autophagic contents. They provide recycling pools of nutrients and membranes, being essential for maintenance of the cellular homeostasis and renovation. When the cells are exposed to protein-damaging insults, autophagy plays a key role in eliminating protein aggregates and damaged organelles, both of which are resistant to degradation by the ubiquitin-proteasome system (UPS) [20,21]. Mice defective in autophagy show severe neurodegeneration accompanied by an accumulation of ubiquitinated protein aggregates [22]. Furthermore, abnormal regulation of autophagy plays a central role in the pathogenesis of human neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), accompanied by neuronal accumulation of insoluble protein aggregates [23].

Because TREM2 serves as a phagocytic receptor of apoptotic neurons [18,24], and the efficient clearance of dead cells requires microtubule-associated protein 1 light chain 3A (LC3)-associated phagocytosis [25], we attempted to study the expression of LC3 in NHD brains by immunohistochemistry. Unexpectedly, we found that LC3 expression is enhanced on oligodendrocytes in NHD brains but not in control brains.

Methods

Human brain tissues

Formalin-fixed paraffin-embedded brain tissues of the cerebral cortex, the hippocampus, and the basal ganglia derived from NHD and non-NHD cases were obtained from the Research Resource Network (RRN), Japan. Written informed consent was taken in all the cases at autopsy, following the regulation of the institutional ethics committees. The present study includes five NHD patients, composed of a 42-year-old man (NHD1), a 48-year-old woman (NHD2), a 44-year-old man (NHD3), a 32-year-old woman (NHD4), and a 38-year-old man (NHD5), four neuropsychiatric disease controls affected with myotonic dystrophy (MD), composed of a 68-year-old man (MD1), a 61-year-old man (MD2), a 60-year-old man (MD3), and a 53-year-old woman (MD4), four demyelinating disease controls affected with chronic progressive multiple sclerosis (MS), composed of a 29-year-old woman (MS1), a 40-year-old woman (MS2), a 43-year-old woman (MS3), and a 33-year-old man (MS4), and four subjects who died of non-neurological causes (NC), composed of a 63-year-old man who died of prostate cancer and acute myocardial infarction (NC1), a 67-year-old man who died of dissecting aortic aneurysm (NC2), a 57-year-old man who died of alcoholic liver

cirrhosis (NC3), and a 61-year-old man who died of rheumatoid arthritis with interstitial pneumonia (NC4). The homozygous mutation of a single base deletion of 141G (141delG) in exon 3 of DAP12 was identified in NHD1, NHD2, and NHD5 [19,26], while the genetic analysis was not performed in NHD3 [27] or NHD4 [28].

Immunohistochemistry

After deparaffination, tissue sections were heated in 10 mM citrate sodium buffer, pH 6.0 or 9.0 by autoclave at 110°C for 15 min in a temperature-controlled pressure chamber (Biocare Medical, Concord, CA, USA). They were treated at room temperature (RT) for 15 min with 3% hydrogen peroxide-containing methanol to block the endogenous peroxidase activity. They were then incubated with phosphate-buffered saline (PBS) containing 10% normal goat or rabbit serum at RT for 15 min to block non-specific staining, followed by incubation in a moist chamber at 4°C overnight with the primary antibodies listed in Table 1. We selected Nogo-A as the most reliable marker highly specific for oligodendrocytes in human brain tissue sections, as reported previously [29]. After washing with PBS, the tissue sections were incubated at RT for 30 min with horseradish peroxidase (HRP)-conjugated secondary antibodies (Nichirei, Tokyo, Japan), followed by incubation with diaminobenzidine tetrahydrochloride (DAB) substrate (Vector, Burlingame, CA, USA). They were processed for a counterstain with hematoxylin. Negative controls underwent all the steps except for exposure to primary antibody.

Western blot analysis

To prepare total protein extract, the cells were homogenized in the mammalian protein extraction reagent (M-PER; Thermo Scientific, Rockford, IL, USA) supplemented with a cocktail of protease inhibitors (Sigma, St. Louis, MO, USA). The protein extract was centrifuged at 12,000 rpm for 5 min at RT, separated on a 15% SDS-PAGE gel, and transferred onto nitrocellulose membranes. They were labeled at RT overnight with rabbit anti-LC3 antibody (PM036; MBL International, Woburn, MA, USA) that react with MAP1LC3A/B/C or goat anti-heat shock protein HSP60 antibody (sc-1052, N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) to standardize protein loading. Then, the membranes were incubated at RT for 60 min with HRP-conjugated anti-rabbit or anti-goat IgG (Santa Cruz Biotechnology). The specific reaction was visualized by exposing the membranes to a chemiluminescent substrate (Thermo Scientific).

Table 1 Primary antibodies utilized for immunohistochemistry in the present

Antibody	Supplier	Code (ID)	Origin	Antigen	Concentration
LC3	MBL	PM036	rabbit	recombinant human LC3B spanning amino acid residues 1-120 aa	diluted at 1: 5000
BECN1	AnaSpec	54229	rabbit	a peptide mapping near the N-terminus of human Beclin-1	0.2 μg/ml
NBR1	ProteinTech	16004-1-AP	rabbit	recombinant human NBR1-6xHis fusion protein	0.26 μg/ml
HDAC6	Santa Cruz Biotechnology	sc-11420	rabbit	a peptide spanning amino acid residues 916-1215 of human HDAC6	0.8 μg/ml
p62/SQSTM1	BD Bioscience	610832	mouse	a peptide spanning amino acid residues 257-437 of human p62	1 μg/ml
Ubiquitin	Dako	Z0458	rabbit	ubiquitin isolated from bovine erythrocytes	0.25 μg/ml
UBQLN1	Santa Cruz Biotechnology	sc-14652	goat	a peptide mapping within an internal region of human ubiquilin-1	1 μg/ml
Nogo-A	Santa Cruz Biotechnology	H-300	rabbit	a peptide mapping amino acids 700-1000 of human Nogo-A	0.1 μg/ml
MBP	Dako	N1564	rabbit	MBP purified from human brain	prediluted
CNPase	Sigma	11-5B	mouse	purified human CNPase	ascites fluid 1:500
Iba1	Wako	019-19741	rabbit	a synthetic peptide corresponding to the C-terminus of Iba1	0.5 μg/ml
GFAP	Dako	N1506	rabbit	GFAP purified from bovine spinal cord	prediluted
NF	Nichirei	412551 (2 F11)	mouse	NF purified from human brain	prediluted
Cleaved CASP3	Cell Signaling Technology	#9661 (Asp175)	rabbit	a peptide mapping amino-terminal residues adjacent to Asp175 of human caspase-3	1:100

Abbreviations: LC3, microtubule-associated protein 1 light chain 3; BECN1, Beclin 1; NBR1, neighbor of BRCA1 gene 1; HDAC6, histone deacetylase 6; SQSTM1, sequestosome 1; UBQLN1, ubiquilin-1; MBP, myelin basic protein; CNPase, 2',3'-cyclic nucleotide 3' phosphodiesterase; GFAP, glial fibrillary acidic protein; NF, neurofilament protein; and CASP3, caspase-3.

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

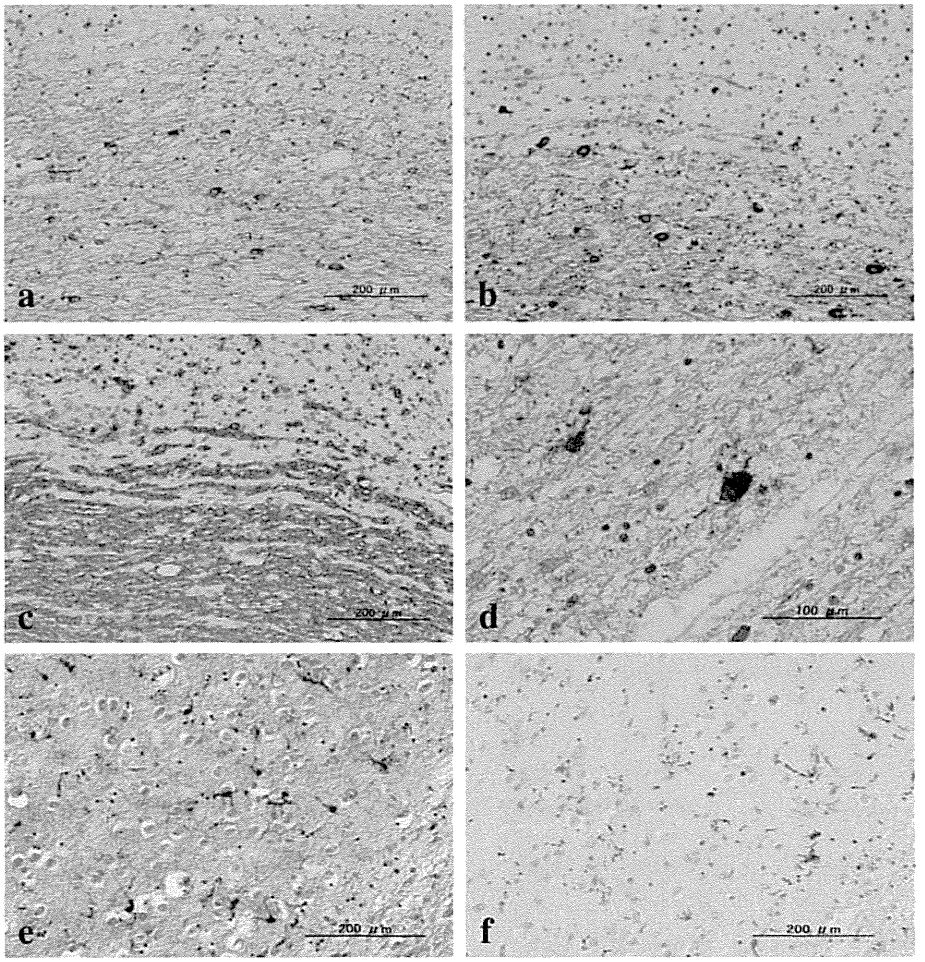

Figure 1 Surviving oligodendrocytes express LC3 in NHD brains. The serial brain sections of NHD cases were processed for immunohistochemistry. The panels **(a-f)** represent **(a)** the periventricular white matter, LC3, **(b)** the same field as **(a)**, Nogo-A, **(c)** the same field as **(a)**, MBP, 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.

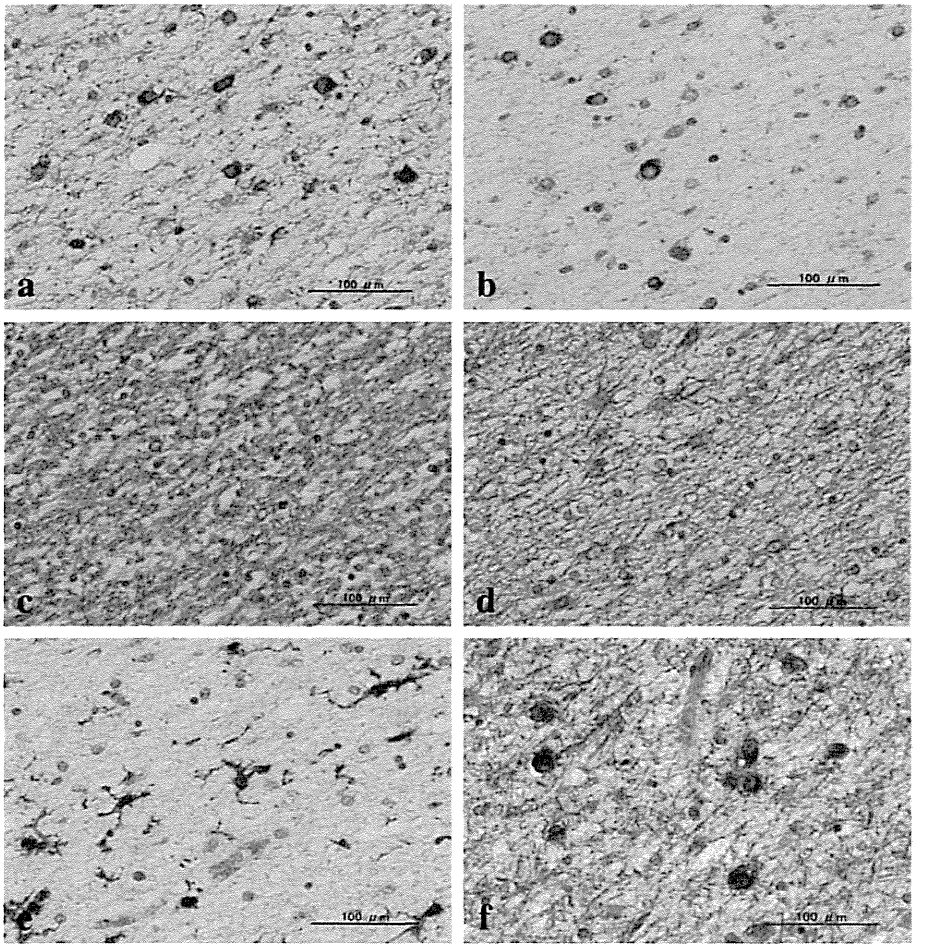

Figure 2 Surviving oligodendrocytes express LC3 in NHD brains. The serial brain sections of NHD cases were processed for immunohistochemistry. The panels **(a-f)** represent the identical field of the frontal white matter labeled with **(a)** LC3, **(b)** Nogo-A, **(c)** MBP, **(d)** GFAP, **(e)** Iba1,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

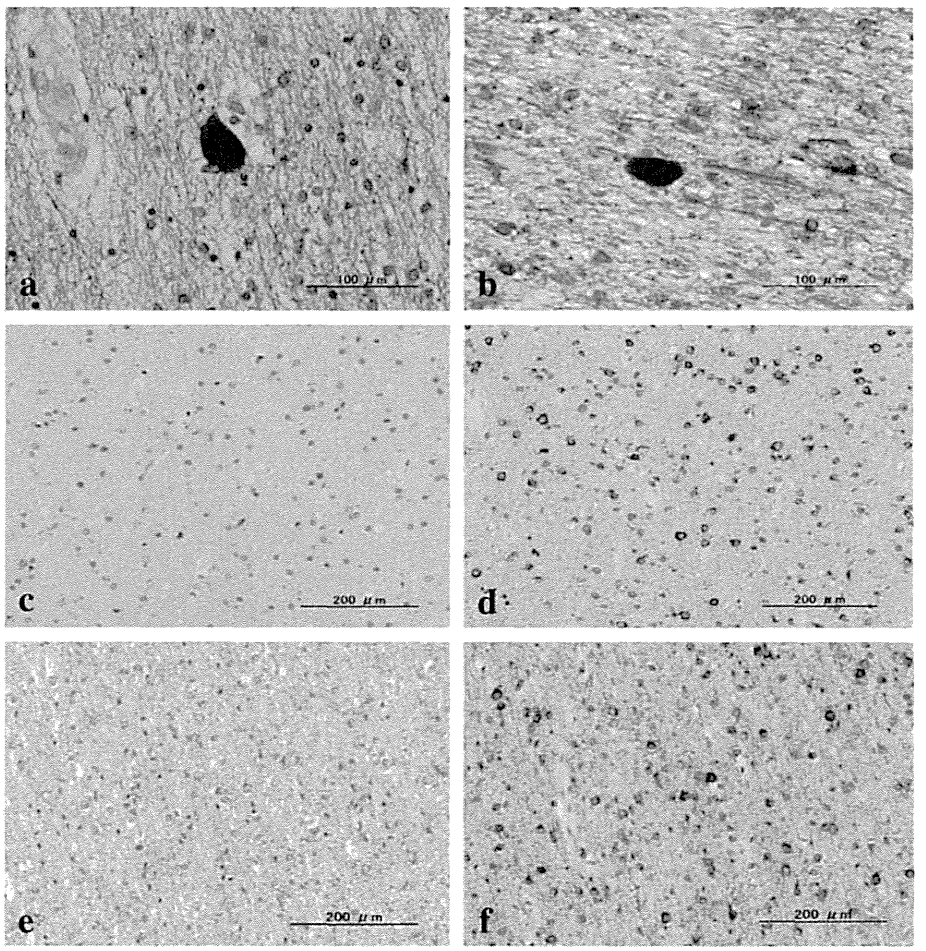

Figure 3 Oligodendrocytes do not express LC3 in control brains. The serial brain sections of NHD, myotonic dystrophy (MD), and non-neurological control (NC) cases were processed for immunohistochemistry. The panels **(a-f)** represent **(a)** NHD, the basal ganglia, LC3, **(b)** NHD, the frontal white 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,

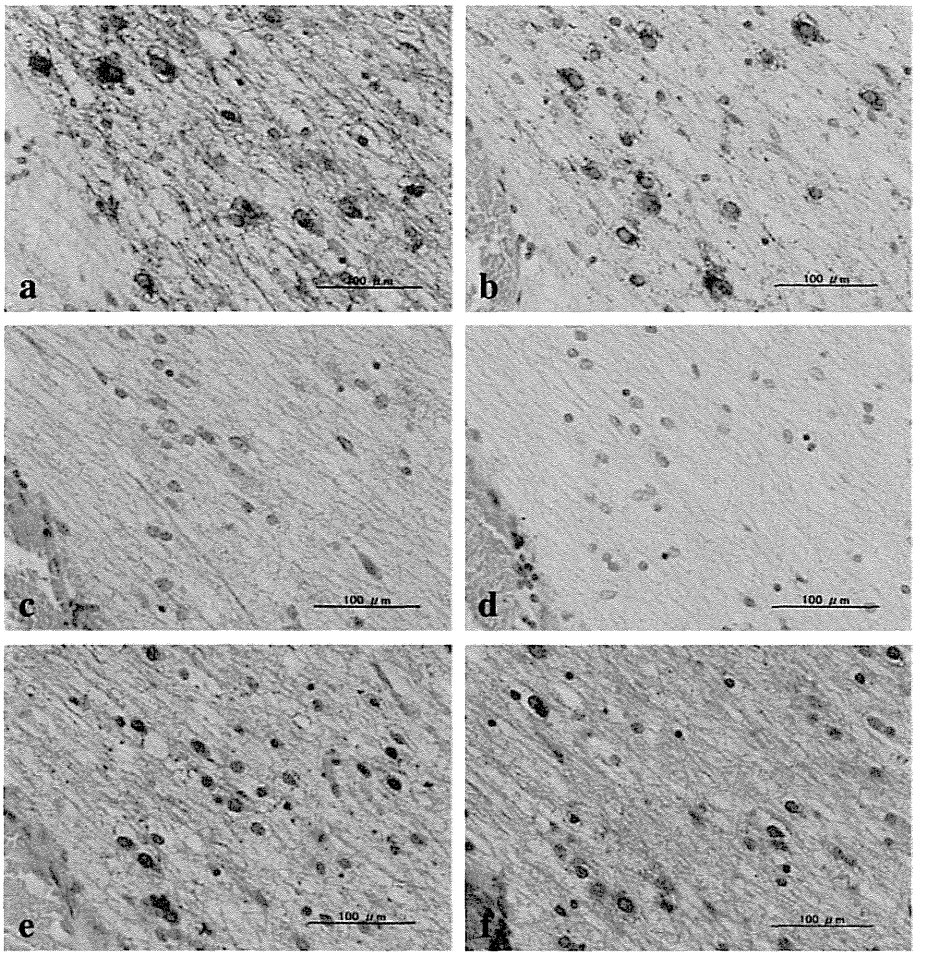

Figure 4 Surviving oligodendrocytes do not express Beclin 1 or p62 in NHD brains. The serial brain sections of NHD cases were processed for immunohistochemistry. The panels **(a-f)** represent the identical field of the frontal white matter labeled with **(a)** LC3, **(b)** Nogo-A, **(c)** Beclin 1,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

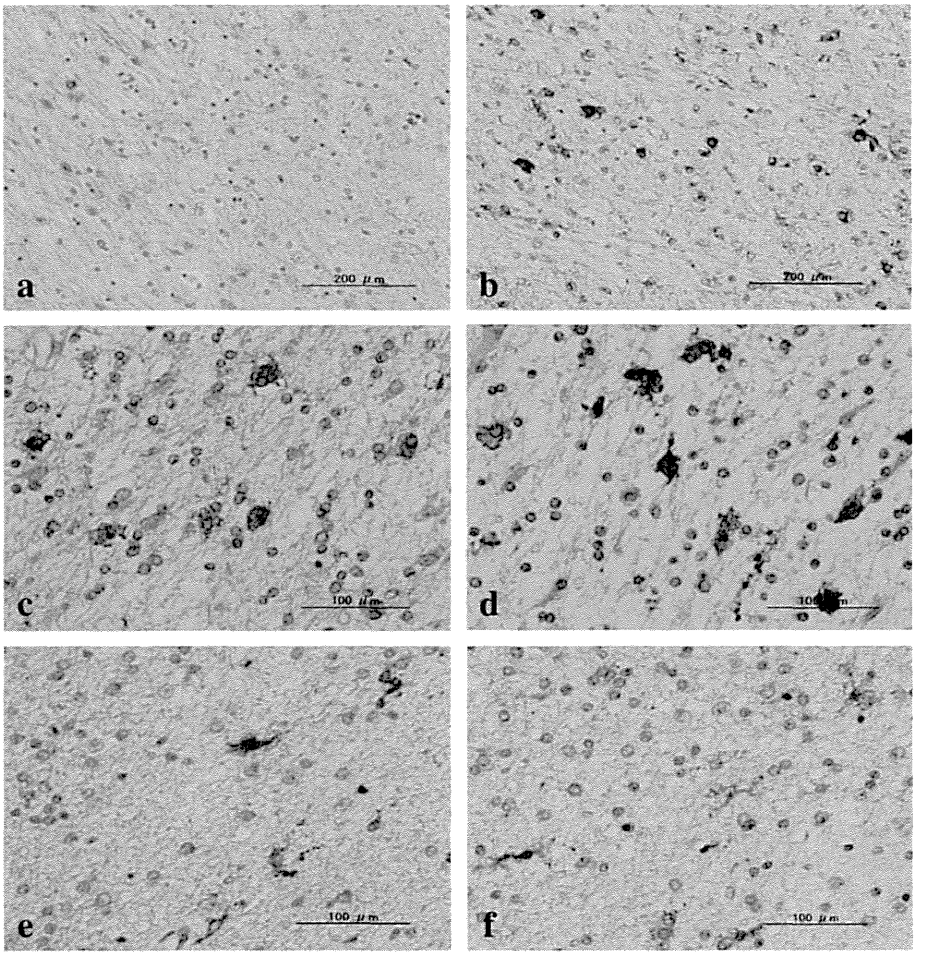

Figure 5 Macrophages and microglia but not oligodendrocytes express LC3 in MS brains. The serial brain sections of multiple sclerosis (MS) cases were processed for immunohistochemistry. The panels **(a-f)** represent **(a)** the edge of demyelinated lesions in the parietal white matter, LC3, **(b)** 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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Human Astrocytes: Secretome Profiles of Cytokines and Chemokines

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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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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 5×10^7 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

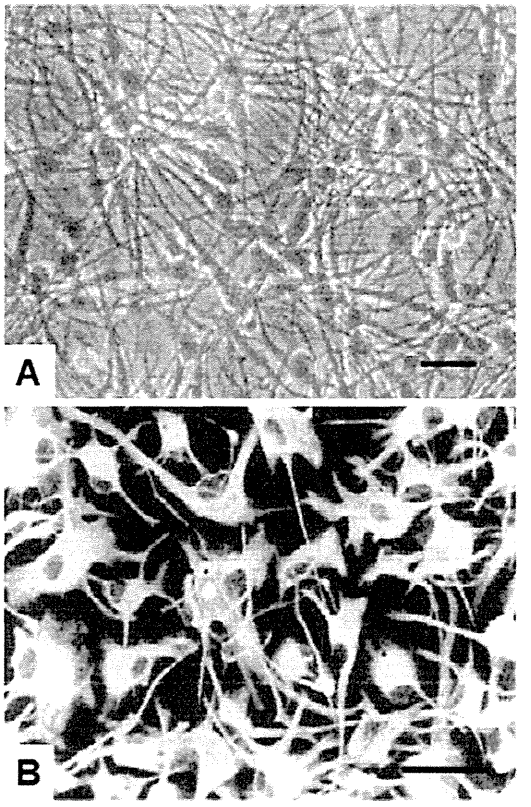

Figure 1. The purity of human astrocytes in culture exceeded 99% glial fibrillary acidic protein (GFAP) immunoreactivity-positive. Astocytes shown are at the normal non-stimulated resting state. A: Phase contrast microscopy. B: Immunostaning with anti-GFAP antibody.
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Table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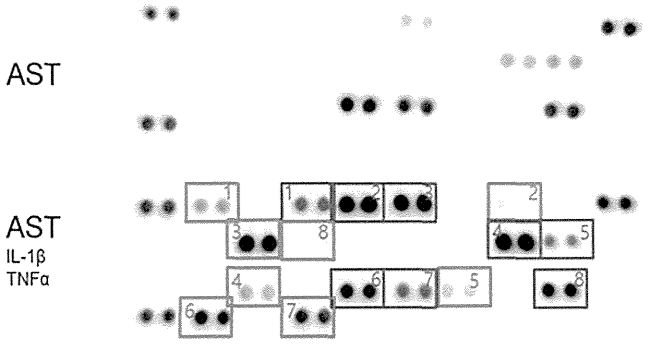

	Newly Expressed cytokines/chemokines		Changed expression
1	C5/C5a (Complement Component 5/5a)	1	G-CSF (CSFβ, CSF-3)
2	sICAM-1 (CD54)	2	GM-CSF (CSFα, CSF-2)
3	IL-1β (IL-1F2)	3	GROα (CXCL1)
4	IP-10 (CXCL10)	4	IL-6
5	MIP-1α (CCL3)	5	IL-8 (CXCL8)
6	RANTES (CCL5)	6	MCP-1 (CCL2)
7	TNF-α (TNFSF1A)	7	MIF (GIF, DER6)
8	IL-1ra (IL-1F3)	8	Serpin E1 (PAI-1)

Figure 2. Proteome profiler array of human cytokines/chmokines. The array is capable of detecting a panel of 36 cytokines, chemokines, and soluble mediators. Top panel shows expression of cytokines/chemokines in resting unstimulated human astrocyrtes, and middle panel shows expression of cytkines/chemokines in human astrocytes stimulated with IL-1β and TNF-α. Items circled in red are newly expressed cytokines in activated astrocytes and ones circled in blue are cytokines changed expression in activated aqstrocytes. In bottom panel, newly expressed cytokines and cytokines changed expression are listed separately.
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antibody (1:4, mouse; Kim Lab), a oligodendrocyte cell type-specific marker, or anti-human CD68 antibody (1:200, mouse, Millipore), a microglial marker, for 48 hrs at 4°C, followed by Alexa Fluor 594-conjugated anti-mouse IgG for 1 hr at RT. Cultures processed for immunocytochemistry were examined under an Olympus laser confocal fluorescence microscope.

Cytokine and chemokine profiling

Human astrocytes were incubated in culture medium with or without inclusion of a mixture of 10 ng/mL recombinant human IL-1β (Peprotech, Rocky Hill, NJ) and 10 ng/mL recombinant human TNF-α (Peprotech). At 24 hours after treatment, the conditioned media were harvested and processed for profiling of cytokines and chemokines on the human cytokine array panel A (R&D system, Minneapolis, MN), which is capable of detecting a panel of 36 cytokines, chemokines, and soluble mediators, such as complement 5/5a, CD40 ligand, G-CSF, GM-CSF, GROα, I-309, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, IP-10, I-TAC, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, SDF-1, TNFα, and sTREM-1 (Table 1). The array membranes were reacted with the mixture of conditioned media and the antibody cocktail for 18 hrs at 4°C. After several washing, they were briefly incubated with secondary antibodies conjugated with horseradish peroxidase (HRP). Then, the membranes were exposed to HRP substrate. The intensity of the reaction was quantified on the Da vinci imaging system (Seoul, Korea).

Molecular network analysis

We imported Entrez Gene IDs corresponding to cytokine and chemokine genes into the Core Analysis tool of Ingenuity Pathways Analysis (IPA) (Ingenuity Systems; www.ingenuity.com). IPA is a commercial knowledgebase that contains information on approximately 3,000,000 biological and chemical interactions with definite scientific evidence. By uploading the list of Gene IDs, the network-generation algorithm identifies focused genes integrated in global molecular networks. IPA calculates the score p-value that reflects the statistical significance of association between the genes and the networks by Fisher's exact test. We considered p-value$<$0.05 as a significant association. The information on known NF-kB target genes was collected from web accessible databases constructed by Dr. Thomas Gilmore, Boston University (www.bu.edu/nf-kb/gene-resources/target-genes) and by Bonsai Bioinformatics, Laboratoire d'Informatique Fondamentale de Lille (LIFL), Université Lille 1 (bioinfo.lifl.fr/NF-KB), as described previously [6].

Results

Cytokine and chemokine profiles of human astrocytes in culture

The purity of human astrocytes in culture exceeded 99% by GFAP labeling without any contamination of the cells expressing CD68 (microglia), galactocerebroside (oligodendrocytes) or tubulin βIII (neurons) (Fig. 1). Non-stimulated resting astrocytes incubated

Table 3. Cytokines absent in normal resting human astrocytes.

Gene	Genebank	Gene name
CD40 Ligand	NM_000074	CD40 ligand
I-309 (CCL1)	NM_002981	chemokine (C-C motif) ligand 1
IFN-γ (Type II IFN)	NM_000619	interferon, gamma
IL-1α (IL-1F1)	NM_000575	interleukin 1, alpha
IL-2	NM_000586	interleukin 2
IL-4	NM_000589	interleukin 4
IL-5	NM_000879	interleukin 5 (colony-stimulating factor, eosinophil)
IL-10	NM_000572	interleukin 10
IL-12 p70	NM_000882/	interleukin 12A (natural killer cell stimulatory factor 1)
IL-13	NM_002188	interleukin 13
IL-16	NM_172217	interleukin 16
IL-17	NM_002190	interleukin 17A
IL-17E	NM_022789	interleukin 25
IL-23	NM_016584	interleukin 23, alpha subunit p19
IL-27	NM_145659	interleukin 27
IL-32	NM_001012633	interleukin 32
I-TAC (CXCL11)	NM_005409	chemokine (C-X-C motif) ligand 11
MIP-1β (CCL4)	NM_002984	chemokine (C-C motif) ligand 4
SDF-1 (CXCL12)	NM_199168	chemokine (C-X-C motif) ligand 12
sTREM-1	NM_018643	triggering receptor expressed on myeloid cells 1

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in the culture medium without addition of cytokines expressed 8 out of 36 cytokines and chemokines tested, such as G-CSF, GM-CSF, IL-6, GROα (CXCL1), IL-8 (CXCL8), MCP-1 (CCL2), MIF and serpin E1 (Table 2, Fig. 2). Other cytokines and chemokines were undetectable in the conditioned media of non-stimulated astrocytes (Table 3). Following a 24 hr-exposure to a mixture of IL-1β and TNF-α, the expression levels of 6 cytokines, including G-CSF, GM-CSF, IL-6, GROα, IL-8 and Serpin E1, were elevated substantially (Table 4, Figs. 2 and 3), whereas the levels of both MCP-1 and MIF showed a minor reduction (Table 5, Figs. 2 and 3). In astrocytes activated with IL-1β/TNF-α for 24 hrs, there was new indcution of cytokines and chemokines including IL-1β, IL-1ra, TNF-α, IP-10 (CXCL10), MIP-1α (CCL3), RANTES (CCL5), sICAM-1 (CD54) and complement component 5 (C5a) (Table 6, Figs. 2 and 3), suggesting an existence of the positive autoregulatory feedback loop for expression of IL-1β and TNF-α. Among them, upregulated expression of RANTES was the most prominent (Fig. 3B). By database search on known NF-kB target genes, nearly all cytokines and chemokines produced by non-stimulated and activated astrocytes are direct targets of the transcription factor NF-kB, except for C5a, IL-1ra, and MIF, although the genes encoding C5a and MIF have NF-kB binding sites in the promoter regions by literature search on PubMed [7,8].

Molecular network of cytokine and chemokine secretome of human astrocytes

When the list of Entrez Gene IDs corresponding to 14 up-regulated and 2 down-regulated cytokines in IL-1β/TNF-α-activated human astrocytes (Tables 4 and 5, Figs. 2 and 3) were imported into IPA, we identified the molecular network defined by "Cell-to-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking" as the most significant functional network relevant to the set of imported genes (p = 1.00E-13) (Table 7, Fig. 4). The network defined by "Cellular Movement, Hematological System Development and

Table 4. Cytokines upregulated in human astrocytes following IL-1β/TNFα treatment.

Gene	Fold	Genbank	Gene Name
G-CSF	19.74	NM_000759	colony stimulating factor 3 (granulocyte)
GM-CSF	43.25	NM_000758	colony stimulating factor 2 (granulocyte-macrophage)
GROα (CXCL1)	10.27	NM_001511	chemokine (C-X-C motif) ligand 1
IL-6	6.09	NM_000600	interleukin 6
IL-8 (CXCL8)	1.47	NM_000584	interleukin 8
Serpin E1(PAI-1)	1.30	NM_000602	plasminogen activator inhibitor type 1

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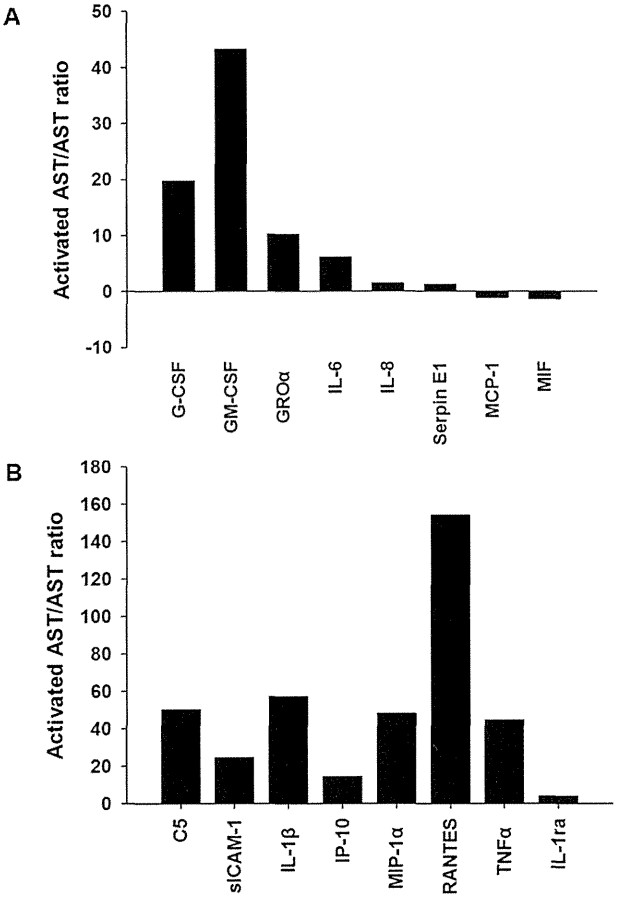

Figure 3. Cytokines/chemokines changed their secretion levels in human astrocytes stimulated with IL-1β and TNF-α. A: Forty five-fold increase in secertion of GM-CSF and twenty-fold increase in secretion of G-CSF are shown here. A minor reduction in expression of MCP-1 and MIF is also shown. B. Among the elevated levels of cytokines/chemokines in human astrocytes stimulated with IL-1β and TNF-α, upregulated expression of RANTES was the most prominent with more than 150-fold increase.
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Function, Immune Cell Trafficking" (p = 1.00E-6) was the second rank significant functional network (Table 7). IPA also indicated that nuclear factor NF-kB/RelA serves as an upstream regulator of the imported genes (p = 7.36E-24), validating the results of database search on NF-kB target genes described above. In IPA analysis, up-regulated molecules, such as MIP-1α (CCL3), RANTES (CCL5), GM-CSF, sICAM1, IL-1β, IL-6, IL-8 (CXCL8), and TNF-α, and a down-regulated molecule MCP-1 (CCL2), were categorized into NF-kB target genes located in the NF-kB signaling pathway (Fig. 4).

Discussion

Due to the limited availability of human brain tissues, only a small number of studies have previously reported the cytokine production profiles of normal human astrocytes [9–11]. In the present study, we characterized the more comprehensive profile of cytokine and chemokine named "secretome" derived from non-stimulated resting and activated human astrocytes by using a protein microarray. We found that both non-stimulated and IL-1β/TNFα-activated astrocytes produce distinct sets of cytokines and chemokines, nearly all of which represent direct targets of transcription factor NF-kB. One exception is MCP-1 (CCL2), a direct target of NF-kB [12], was down-regulated in activated astrocytes following exposure to IL-1β and TNF-α. In contrast, a previous study has indicated that the activated adult human astrocytes in culture produced increased amounts of MCP-1 [11]. The discrepancy between our results and previous findings is attributable to a difference in maturation of cultured cells, i.e. fetal versus adult astrocytes employed. These results suggest that the NF-kB signaling pathway differentially regulates gene expression of cytokines and chemokines in human astrocytes under physiological and inflammatory environments.

NF-kB acts as a central regulator of innate and adaptive immune response, stress response, cell proliferation, and apoptosis [13]. The NF-kB family proteins consist of five members, such as RelA (p65), RelB, c-Rel, NF-kB1 (p105), and NF-kB2 (p100). The latter two are processed proteolytically into p50 and p52, respectively. The NF-kB family proteins constitute either homodimers or heterodimers, except for RelB that exclusively forms heterodimers. The NF-kB dimers interact with consensus DNA sequences termed the kB site located on promoters to activate or repress transcription of target genes. Only p65 and c-Rel act as a potent transcriptional activator, whereas p50 and p52 homodimers generally repress transcription, leading to differential regulation of gene expression of NF-kB targets [14]. We found that MCP-1 (CCL2), a target of NF-kB, is down-regulated in NF-kB-activated human astrocytes. Importantly, NF-kB target genes often activate NF-kB itself, providing a positive regulatory loop that amplifies and perpetuates inflammatory responses [15]. IL-1 β and TNF-α are the prototypes of NF-kB activators for the canonical NF-kB signaling pathway. We found that non-stimulated human astrocytes do not constitutively produce IL-1β or TNF-α, while activated human astrocytes could produce both, as described previously [9,11]. In contrast, a previous study has shown that IL-1β is undetectable at both mRNA and protein levels in non-stimulated or cytokine-stimulated cultured human astrocytes [10]. A different study from the same group showed that IL-8 (CXCL8) is undetectable in non-stimulated human fetal astrocytes in culture [16], being inconsistent with our results. In our study, non-stimulated astrocytes expressed a panel of NF-kB targets, such as G-CSF, GM-CSF, IL-6, GROα (CXCL1), IL-8 (CXCL8), MCP-1 (CCL2), MIF and serpin E1, suggesting that the NF-kB signaling pathway is constitutively active to a certain extent in normal human astrocytes in culture. Notably, GM-CSF serves as an anti-apoptotic and neurotrophic factor [17].

Table 5. Cytokines downregulated in human astrocytes following IL-1β/TNFα treatment.

Gene	Fold	Genbank	Gene Name
MCP-1 (CCL2)	−1.04	NM_002982	chemokine (C-C motif) ligand 2
MIF	−1.28	NM_002415	macrophage migration inhibitory factor

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Table 6. Cytokines newly induced in human astrocytes following IL-1β/TNFα treatment.

Gene	Fold	Genbank	Gene Name
IL-1β	57.17	NM_000576	interleukin 1, beta
IL-1rα	3.72	NM_002182	interleukin 1 receptor accessory protein
TNFα	44.75	NM_000594	tumor necrosis factor (TNF superfamily, member 2)
sICAM-1	24.38	NM_000201	intercellular adhesion molecule 1 (CD54)
C5	50.25	NM_001735	complement component 5
IP-10 (CXCL10)	14.27	NM_001565	chemokine (C-X-C motif) ligand 10
MIP-1α (CCL3)	48.05	NM_002983	chemokine (C-C motif) ligand 3
RANTES (CCL5)	154.13	NM_002985	chemokine (C-C motif) ligand 5

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Chemokines constitutes a group of structurally related proteins that play a pivotal role in regulation of immune cell trafficking involved in inflammatory and immunoregulatory processes in the CNS [18]. In the present study, molecular network of cytokine and chemokine secretome of activated human astrocytes strongly supported the view that these soluble factors are critically involved in regulation of the cellular interaction and trafficking of immune cells. Various chemokine receptors, such as CCR2 with the ligand MCP-1 and CXCL2 with multiple ligands, such as Groα, Groβ and IL-8, are up-regulated in brain lesions of trauma, ischemia, and multiple sclerosis (MS) [19]. MIP-1 belongs to a family of C-C chemokines with a potent chemotactic activity for neutrophils and other leukocytes, composed of two members MIP-1α (CCL3) and MIP-1β (CCL4), both of which exhibit very similar but not identical proinflammatory properties through binding differentially to the receptors CCR1, CCR4 or CCR5. The upregulated expression of MIP-1α and MIP-1β was identified in acute MS lesions [20]. However, we identified MIP-1α (CCL3) but did not detect the expression of MIP-1 β (CCL4) in non-stimulated or activated human astrocytes in culture, suggesting that the gene regulatory mechanism is different between MIP-1α and MIP-1 β, although both are directly regulated by NF-kB.

RANTES is a member of C-C chemokines involved in the pathogenesis of MS and HIV-1 encephalitis by binding to the receptors CCR1, CCR3 or CCR5 [21,22]. A previous study has shown that fetal human astrocytes upon activation by IL-1β secretes a large amount of RANTES (CCL5) protein [23], supporting our observation that RANTES is the most prominently up-regulated chemokine in activated human astrocytes in culture. Importantly, RANTES plays a neuroprotective role in ischemic brain injury [24]. The expression of IP-10 (CXCL10), secreted by monocytes, endothelial cells, and fibroblasts in response to IFN-γ, shows a chemotactic activity for T cells, NK cells, dendritic cells and monocytes/macrophages through binding to the receptor CXCR3. We found that IP-10 production is greatly enhanced in activated human astrocytes in culture. Notably, the expression of IP-10 and CXCR3 is up-regulated in the brains of AD, where CXCR3 is expressed constitutively on neurons, while IP-10 expression is enhanced in a subset of reactive astrocytes surrounding senile plaques [25].

In conclusion, the comprehensive cytokine and chemokine secretome of activated human astrocytes, closely linked to NF-kB activation, suggested that astrocyte-derived cytokines and chemokines play a central role in proinflammatory (neurotoxic) and immunoregulatory (neuroprotective) responses in the CNS.

Table 7. Top 3 molecular networks of cytokine and chemokine secretome in human astrocytes.

Rank	Functional Networks	Focused Molecules	p-Value
1	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking	**CCL3**, **CCL5**, **CSF2**, **CSF3**, **CXCL1**, chitinase, Collagen Alpha1, Collagen type II, Cpla2, elastase, Eotaxin, ERK1/2, ETS, Fcer1, Fcgr2, Ferritin, Fibrin, HLA-DR, IL-17f dimer, **IL-1R**, IL17a dimer, IL17R, IL1RAP, Il8r, IRAK, Lfa-1, Lymphotoxin, lymphotoxin-alpha1-beta2, NfkB-RelA, NfkB1-RelA, Nr1h, PI3K (family), Rxr, Scavenger receptor class A, VitaminD3-VDR-RXR	1.00E-13
2	Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	26 s Proteasome, Akt, AMPK, BCR (complex), calpain, **CCL2**, Cdk, Collagen type IV, Cyclin A, Cyclin E, cyclooxygenase, Fibrinogen, gelatinase, **GM-CSF**, Growth hormone, HDL, Ige, Ikb, JINK1/2, Laminin, LDL, **MIF**, N-cor, NADPH oxidase, Nos, PDGF (complex), PDGF BB, PRKAA, Ptk, Rb, Rock, **Serpine1**, Smad, Sphk, TGF-beta	1.00E-06
3	Infectious Disease, Cell-To-Cell Signaling and Interaction, Cellular Growth and Proliferation	Calcineurin protein(s), CD3, collagen, CYP, estrogen receptor, Hdac, hemoglobin, Histone h4, Hsp27, Hsp70, Hsp90, Icam, Iga, IgG1, Igm, **IL-6**, **IL-8**, Immunoglobulin, Interferon alpha, Ldh, Mek, Nfat (family), Notch, P38 MAPK, p70 S6k, Pro-inflammatory Cytokine, Rap1, Rsk, Serine Protease, Sod, SRC (family), STAT5a/b, **TNF**, TSH, U1 snRNP	1.00E-05

Functional networks were studied by importing Entrez Gene IDs of 14 up-regulated and 2 down-regulated cytokines in IL-1β/TNF-α-activated human astrocytes into the core analysis tool of IPA. They are listed with functional networks, focused molecules, and p-value of the Fisher's exact test. The first rank network is illustrated in Fig. 4.

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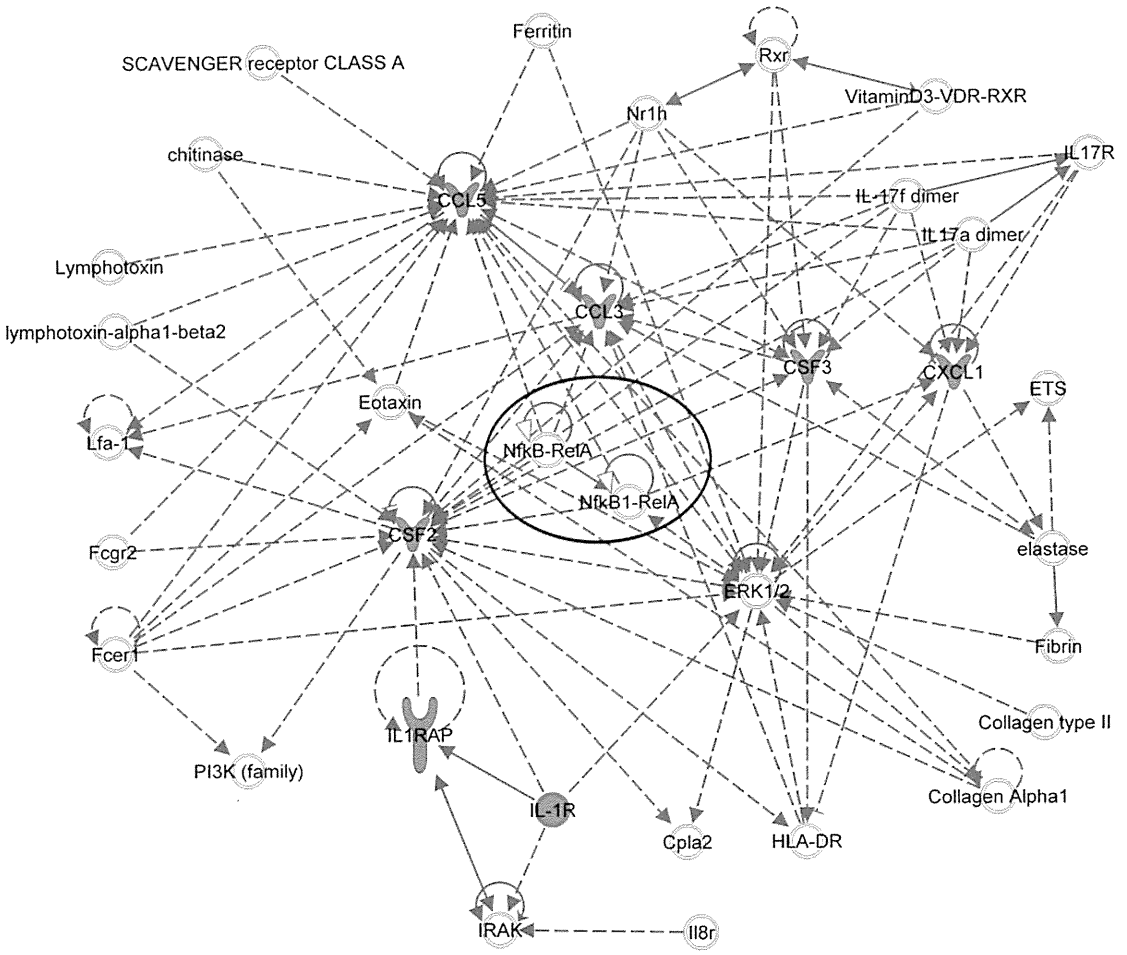

Figure 4. Molecular network showing cytokine and chemokine secretomes of human astrocytes. Entrez Gene IDs corresponding to 14 upregulated and 2 downregulated cytokines in IL-1β/TNF-α-activated human astrocytes were imported into the Ingenuity Pathways Analysis (IPA). The most significant molecular network relevant to the imported genes (red arrows) is shown. NF-kB is highlighted by blue circle. CCL5 (RANTES), CCL3 (MIP-1α), CXCL1 (GROα), CSF2 (GM-CSF), CSF3 (G-CSF), IL1R and IL1RAP are indicated by red.
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Author Contributions

Conceived and designed the experiments: HJL SUK. Performed the experiments: SSC HJL. Analyzed the data: SSC HJL JIS SUK. Contributed reagents/materials/analysis tools: IL JIS. Wrote the paper: SSC HJL JIS SUK.

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Research Article

Molecular Network of NLRP3 Inflammasome Activation-Responsive Genes in a Human Monocyte Cell Line

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Abstract

Background: Inflammasome, activated by pathogen-derived and host-derived danger signals, constitutes a multimolecular signaling complex that serves as a platform for caspase-1 (CASP1) activation and interleukin-1β (IL-1β) maturation. The activation of NLRP3 inflammasome requires two-step signals. The first "priming" signal enhances gene expression of inflammasome components. The second "activation" signal promotes the assembly of inflammasome components. Deregulated activation of NLRP3 inflammasome contributes to the pathological processes of Alzheimer's disease (AD) and multiple sclerosis (MS). However, at present, the precise mechanism regulating NLRP3 inflammasome activation and deactivation remains largely unknown.

Methods: By genome-wide gene expression profiling, we studied the molecular network of NLRP3 inflammasome activation-responsive genes in a human monocyte cell line THP-1 sequentially given two-step signals.

Results: We identified the set of 83 NLRP3 inflammasome activation-responsive genes. Among them, we found the NR4A nuclear receptor family NR4A1, NR4A2, and NR4A3, the EGR family EGR1, EGR2, and EGR3, the IκB family NFKBIZ, NFKBID, and NFKBIA as a key group of the genes that possibly constitute a negative feedback loop for shutting down inflammation following NLRP3 inflammasome activation. By molecular network analysis, we identified a complex network of NLRP3 inflammasome activation-responsive genes involved in cellular development and death, and immune and inflammatory responses, where transcription factors AP-1, NR4A, and EGR serve as a hub.

Conclusion: NLRP3 inflammasome activation-responsive genes constitute the molecular network composed of a set of negative feedback regulators for prompt resolution of inflammation.

Keywords: Inflammasome; NLRP3; NR4A1; NR4A2; NR4A3

Introduction

Inflammasome serves as a multi molecular signaling complex involved in activation of caspase-1 (CASP1) and maturation of interleukin-1β (IL-1β) and IL-18 [1,2]. A wide variety of exogenous and endogenous stimuli, characterized by microbe-derived pathogen-associated molecular patterns (PAMPs) and host- or environment-derived danger-associated molecular patterns (DAMPs), are recognized by an intracellular sensor called the NOD-like receptors (NLRs), resulting in rapid induction of inflammasome formation by ordered assembly of self-oligomerizing components.

Among various classes of inflammasome, the nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 3 (NLRP3) inflammasome has been most intensively studied. It is composed of NLRP3, the adaptor molecule named apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and the precursor form of the cysteine protease pro-CASP1 [1,2]. NLRP3 contains a central nucleotide-binding and oligomerization (NACHT) domain essential for activation of the signaling complex via ATP-dependent oligomerization, flanked by a C-terminal leucine-rich repeat (LRR) pivotal for ligand sensing and autoregulation and a N-terminal pyrin (PYD) domain involved in a homotypic protein-protein interaction between NLRP3 and ASC. The molecular interaction of NLRP3 with ASC recruits pro-CASP1 by a homotypic interaction of caspase activation and recruitment (CARD) domains between ASC and pro-CASP1. Subsequently, the proximity-induced pro-CASP1 oligomerization causes autocatalytic activation of CASP1, resulting in processing of pro-IL-1β or pro-IL-18 into biologically active IL-1β and IL-18. Both of them act as a central regulator for induction of cytokines and chemokines that amplify inflammation by recruiting immune effector cells.

The activation of NLRP3 inflammasome requires two-step signals (Figure 1) [3,4]. The first "priming" signal termed Signal 1, such as microbe-derived lipopolysaccharide (LPS), enhances gene expression of inflammasome components and target proteins via activation of transcription factor nuclear factor-kappa B (NF-κB). The second "activation" signal termed Signal 2 promotes the organized assembly of inflammasome components. The second signal involves three major mechanisms, such as generation of reactive oxygen species (ROS), lysosomal protease leakage, and the potassium efflux [1,2]. Mitochondria often serve as the principal source of ROS. Blockade of mitophagy induces accumulation of ROS-generating mitochondria that activates NLRP3 inflammasome [5]. Furthermore, oxidized

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