Role of NLRP3 inflammasome activation in the pyroptotic cell death of glial cells

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ABSTRACT

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Inflammasome is a caspase-1-activating multi-protein complex composed of sensor molecules such as NOD-like receptor family, pyrin domain-containing 3 (NLRP3), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and procaspase-1. The inflammasome complex plays a crucial role in the induction of inflammatory responses mainly in myeloid cells such as macrophages. Assembly of the inflammasome complex induces the activation of caspase-1, which leads to the maturation and release of pro-inflammatory cytokines, such as interleukin (IL)-1β and IL-18. Also, active caspase-1 cleaves gasdermin D (GSDMD) which initiates generation of pores in plasma membrane and leads to 'pyroptosis', a form of programmed lytic cell death. Recent studies have proposed that NLRP3 inflammasome possibly contribute to neuroinflammation and neurodegenerative diseases. Also, NLRP3 inflammasome complexes are activated in glial cells and glial

inflammasome activation is critical to various neurologic disorders. However, the effects of glial pyroptosis in neuroinflammation are still poorly understood. Here, I examined the effect of mixed glial pyroptosis upon NLRP3 inflammasome activation. Active caspase-1 and IL-1β was secreted by lipopolysaccharide (LPS) and ATP stimulation in mixed glia. However, pyroptosis of mixed glia with LPS plus ATP treatment were reduced, not like bone marrow-derived macrophages (BMDMs). Furthermore, attenuated pyroptosis of mixed glia was not related to GSDMD, the effector of pyroptosis. Next, microglia were isolated from mixed glia and examined NLRP3 inflammasome-mediated pyroptosis. Interestingly, pyroptosis of microglia present in mixed glia showed a weaker response than pyroptosis of isolated microglia. Additionally, mixed glia showed less toxicity to SH-SY5Y, dopaminergic neuroblastoma cell line, than BMDMs upon LPS/ATP stimulation with coculture system. Collectively, mixed glia showed NLRP3 inflammasome-mediated attenuated pyroptosis than BMDMs and interaction between glial cells potentially reduced microglial pyroptosis.

Key words: mixed glia, microglia, macrophage, NLRP3 inflammasome, pyroptosis.

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I. INTRODUCTION

Inflammasomes are a group of cytoplasmic caspase-1-activating multiprotein complexes that are composed of a sensor protein such as NOD-like receptor (NLR) family, an adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and an effector protein procaspase-1[1,2]. The inflammasome complex is assembled and activated upon sensing of various cytoplasmic pathogen- or danger-associated molecular patterns mainly in myeloid cells[3]. Members of the NLR family, including NOD-like receptor family, pyrin domain-containing 1 (NLRP1), NLRP3 and NLRC4, and the cytosolic receptor absent in melanoma 2 (AIM2) are critical sensor proteins which senses each stimulus such as lipopolysaccharide (LPS) or viral nucleic acid[1]. Assembly of inflammasome triggers cleavage of procaspase-1 into active caspase-1, which subsequently cleaves

the pro-interleukin (IL)-1β and pro-IL-18 into their mature forms. At the sites of microbial infection or tissue injury, inflammasome signaling triggers the local inflammatory response by secreting these cytokines to manage host homeostasis. Also, active caspase-1 cleaves gasdermin D (GSDMD), separating its N-terminal pore-forming domain from the C-terminal repressor domain[4,5]. GSDMD N-terminals form GSDMD pores in the plasma membrane and trigger pyroptosis, a form of programmed lytic cell death.

NLRP3 is the most commonly studied inflammasome and stimuli which trigger NLRP3 inflammasome activation include pathogen-derived ligands such as microbial cell wall components, nucleic acids, and pore-forming toxins and endogenous danger signals like ATP, and uric acid crystals[1,6]. NLRP3 inflammasome is the major focus of interest as it is potentially involved in several autoinflammatory diseases, including arthritis, diabetes, obesity, and Alzheimer's disease[1,7,8]. Furthermore, the NLRP3 inflammasome is well studied within the central nerve system (CNS) and one of the vital contributors to neuroinflammation in an expansive spectrum of neurologic disorders[6,9,10]. Caspase-1 activation and consecutive pro-inflammatory cytokine release promote neuronal death that can lead to nerve system disorder. Recent studies demonstrated a notable contribution of NLRP3 inflammasome to the progression of Alzheimer's disease (AD)[11,12], Parkinson's disease[13] and multiple sclerosis[14]. Especially in AD, NLRP3 deficiency certainly attenuates AD phenotypes, including impaired spatial memory, in APP/PS1 mice[12]. Furthermore, amyloid β, a risk factor for AD pathogenesis, could activate the NLRP3 inflammasome in microglia[11]. Thus, glial NLRP3 inflammasome is possibly involved in pathogenesis of the neurodegenerative disease.

Microglia and astrocytes are the resident CNS glial cells and key regulators of neuroinflammation[15,16]. Microglia is involved in consistent sensing of changes in CNS environment and a housekeeping function that improves neuronal well-being and normal activity[17]. Furthermore, microglia are the major immune cells in CNS and act as phagocytes to clear the pathogen, accumulated proteins and debris[18,19]. However, upon CNS infection, injury or neurodegenerative disease, activated microglia might contribute to neuronal damage via the release of pro-inflammatory cytokines and neurotoxic products[20,21]. As myeloid lineage cells, NLRP3 inflammasome can be assembled in microglia upon stimulation with accumulated endogenous metabolites such as fibrillar amyloid β and alpha-synuclein aggregates[11,13]. Microglial NLRP3-mediated inflammasome signaling plays a significant role in neurodegenerative disease. Recent studies reported that ASC specks secreted by microglia bind rapidly to amyloid-β and promote the formation of amyloid-β oligomers and aggregates, acting as an inflammation-driven cross-seed for amyloid-β pathology in AD patients[11]. In addition, activation of the microglial NLRP3 inflammasome is a common pathway induced by both fibrillar α-synuclein and dopaminergic degeneration in the absence of α-synuclein aggregates[13].

Astrocytes are the most abundant glial cell type of the CNS and are crucial for brain homeostasis. They provide metabolites and growth factors to neurons, support synapse formation and plasticity, and regulate the extracellular balance of ions, fluid and neurotransmitters. Additionally, astrocytes are vital regulators of innate and adaptive immune responses in the damaged CNS[22]. Recent studies suggested that astrocyte deficiency during the chronic phase of experimental autoimmune encephalomyelitis (EAE) alleviates pathology and reduces leukocyte infiltration into the CNS[23]. Also, progressed pathology in mice with defective IFN-γ signaling in astrocytes is paralleled by emphasized demyelination during acute and chronic phases

of EAE with increased leukocyte infiltration during late stage of the disease[24]. Furthermore, recent studies suggested that astrocytes potentially secrete NLRP3 inflammasome-mediated pro-inflammatory cytokine IL-1β as well as microglia do[25,26].

Recent studies reported that microglia, unlike most other hematopoietic lineages, renew slowly at an average rate of 28% per year, and some microglia last for more than two decades[27,28]. These data provide that glial cells have a longer lifespan than other cells and show cell death resistances[29,30]. It has been reported that calcium/calmodulin-dependent protein kinase II (CaMKII) is constitutively activated in human astrocytes and protects the cells from apoptotic stimulation by Fas agonist[29]. Activated microglia may secrete LCN2 protein, which acts in an autocrine manner to sensitize microglia to the self-regulatory apoptosis[31]. However, glial pyroptosis, an inflammasome-mediated lytic cell death, in CNS inflammation are still poorly understood. Here, I examined about inflammasome-mediated pyroptosis of mixed glia and cross-talk between glial cells possibly ameliorate microglial pyroptosis.

II. MATERIAL & MATHODS

1. Mice

C57BL/6 and *Nlrp3*$^{D301/NeoR}$ were from The Jackson Laboratory. All mouse strains were bred at Yonsei University College of medicine and under specific pathogen-free conditions. All experiments were in accordance with the guide for Care and Use of Laboratory Animals and approved by the Institutional Ethical Committee, Yonsei University College of Medicine.

2. Cell cultures

Primary mouse microglial cells were prepared from postnatal day P1-3 day mouse brain. Mouse brains were carefully dissected removing all the meninges and washed with Hanks' buffered salt solution. Brain tissues were dissociated by pipetting up and down with polished Pasteur pipettes (large/medium/small size hole) in DMEM/F12 (Gibco, Gran Island, NY, USA) medium, and then filter cell suspension through a cell strainer. Isolated mixed glial cells were grown in DMEM:F12 supplemented with 10% FBS (Gibco), L-glutamic acid (Sigma), Sodium pyruvate (Gibco), MEM NEAA (Gibco) and antibiotics at 37℃ with 5 % CO2. Medium was replaced completely 7 days after seeding, and then half of the medium volume was replaced every 3-4 days. Microglia were further enriched from the mixed glial cultures using mild trypsinization. SH-SY5Y was maintained with DMEM:F12 supplemented with 10% FBS and antibiotics. Mouse bone marrow-derived macrophages (BMDMs) were prepared from C57BL/6 or *Nlrp3*$^{D301/NeoR}$ (*Nlrp3*$^{-/-}$) mice as previously described. All BMDMs were prepared from the femurs and tibias of C57BL/6 mice. Harvested BMDMs were maintained in L929-conditioned DMEM (Corning, NY, USA),

supplemented with 10% FBS (Corning, NY, USA) and antibiotics. Culture medium was replaced with fresh medium 3 days after seeding, and then enriched cells were plated in appropriated plates.

3. Reagents and antibodies

LPS, ATP, nigericin, propidium iodide and MCC950 were purchased from Sigma-Aldrich. Y-VAD-CMK were purchased from BACHEM. Necrostatin-1 was purchased from MERCK. Anti-mouse caspase-1 and anti-NLRP3 antibodies were obtained from Adipogen (SanDiego, CA, USA). Anti-mouse IL-1β antibody was obtained from R&D Systems (Minneapolis, MN, USA). Anti-mouse GSDMD antibody was obtained from Abcam. Anti-mouse β-actin antibody was obtained from Santa cruz (Santa Cruz, CA, USA). Anti-CD45-BV421 antibody were obtained from BD Biosciences (SanJose, CA, USA). Anti-F4/80-APC antibody was purchased from eBIOSCIENCE. Anti-CD11b-APC antibody was purchased from Invitrogen.

4. Lactate Dehydrogenase (LDH) assay

Cell death was determined by the extracellular release of lactate dehydrogenase (LDH) using a CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega). Cell viability was assessed by quantification of LDH released in the supernatant, expressed as a percentage of total LDH released by the cells. LDH was measured from aliquots of supernatant with colorimetric quantification a using kit. Results were obtained by measuring light absorbance at 490 nm.

5. Immunoblot analysis

Cells were lysed in buffer containing 20 mM HEPES (pH 7.5), 0.5 % NP-40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and protease inhibitors. Soluble lysates were fractionated by SDS-PAGE and then transferred to PVDF membranes. In some experiments, cell culture supernatants were precipitated by methanol/chloroform extraction and then immunoblotted.

6. Assay of inflammasome/caspase-1 activation

To induce a conventional NLRP3 inflammasome activation, BMDMs or mixed glia, microglial cells were primed with LPS (0.25 ug/ml, 2.5 hr), followed with ATP treatment (2~2.5 mM, 40~50 min). Inflammasome activation was determined by the presence of active caspase-1 p20 and active IL-1β from culture supernatants in immunoblots, and by the extracellular IL-1β quantification using ELISA (BioLegend). For measure inflammation, IL-6 from culture supernatants were quantified by ELISA (BioLegend).

7. Flow cytometry to determine cell death

To measure cell death, mixed glia and BMDM isolated from *Nlrp3*$^{+/+}$ or *Nlrp3*-deficient mice were used. Following appropriate treatments, cell death was determined by flow cytometry. Cells were stained with propidium iodide (PI, Sigma-Aldrich) and analyzed by flow Jo. To measure pyroptosis-induced neuronal degeneration, mixed glia and BMDMs isolated from *Nlrp3*$^{+/+}$ mice were co-cultured with neuroblastoma SH-SY5Y cells. Following appropriate treatments, neuronal cell

death in the co-cultures was determined by flow cytometry. For the flow cytometry analyses, cells were stained with propidium iodide (PI, Sigma-Aldrich) and analyzed with the fluorescence of PI by flow cytometry.

8. Cell co-culture model in the transwell system

To observe pyroptosis-induced neuronal damage, SH-SY5Y cells were seeded in the lower chamber of a 6 well transwell system (0.4 μm pore; Corning, USA), while mixed glia and BMDMs were plated in upper chamber of transwell plate. The upper chamber was then placed above the lower chamber 24 hr after seeding and exposed to appropriate treatments.

9. Statistical analysis

All values were expressed as the mean SEM of individual samples. Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for comparison of all groups with control group, two-way ANOVA with Bonferroni post hoc test for comparison between WT and *Nlrp3*-deficient mouse groups, or Student's t-tests. The level of statistical significance was set at p values $\leq$ 0.05. Analyses were performed using GraphPad Prism.

III. RESULT

1. LPS plus ATP or nigericin treatment induces NLRP3 inflammasome activation in BMDMs

To examine NLRP3 inflammasome activation in macrophage, bone marrow-derived macrophages from wild type (WT) C57BL/6 mice were prepared. Harvested BMDMs were maintained in L929-conditioned media for differentiation to macrophages. Culture medium was replaced with fresh medium 3 days after seeding, and then enriched cells were plated in appropriated plates. BMDMs were stimulated with LPS plus ATP or nigericin for NLRP3 inflammasome activation (Fig 1). Cleaved caspase-1 p20 and IL-1β were secreted from BMDMs after appropriate treatment (Fig 1A, B). IL-6 were secreted from BMDMs upon LPS stimulation independent of NLRP3 inflammasome activation (Fig 1C).

Next, secretion of caspase-1 and IL-1β were examined from WT (*Nlrp3*$^{+/+}$) BMDM and *Nlrp3*-deficient BMDM upon LPS plus ATP or nigericin stimulation for verifying the role of NLRP3. LPS plus ATP or nigericin treatment triggered cleaved caspase-1 and mature IL-1β secretion from WT BMDMs but not from *Nlrp3*-deficient BMDMs (Fig 2A, B). Additionally, inhibitors of caspase-1 and NLRP3 were used for identifying whether the activity of caspase-1 and NLRP3 are crucial for IL-1β secretion from WT BMDMs upon LPS/ATP stimulation (Fig 2C, D). Caspase-1 inhibitor Y-VAD-CMK, NLRP3 inhibitor MCC950 and RIP1 kinase inhibitor necrostatin-1 (Nec 1) were treated before LPS/ATP treatment to WT BMDMs. Y-VAD-CMK and MCC950 inhibited cleaved caspase-1 and IL-1β secretion from BMDMs as determined by immunoblots and ELISA.

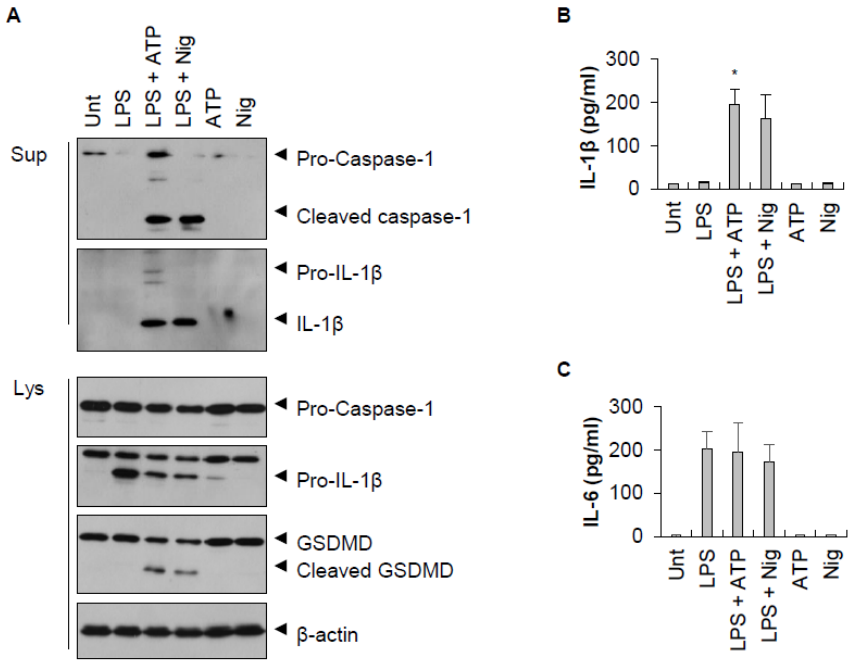


Figure 1. Secretion of cleaved caspase-1 and IL-1β were induced by LPS plus ATP or nigericin treatment in BMDMs. (A) Mouse BMDMs were treated with LPS (0.25 ug/ml) for 2.5 hr, followed by treatment with ATP (2.5 mM) or nigericin (Nig, 5 uM) for 50 min. Culture supernatants (Sup) and cellular lysates (Lys) were immunoblotted with the indicated antibodies. (B, C) Culture supernatants were assayed for extracellular levels of IL-1β and IL-6 by ELISA. (n=2). The values were expressed as the mean ±SEM. *P<0.05

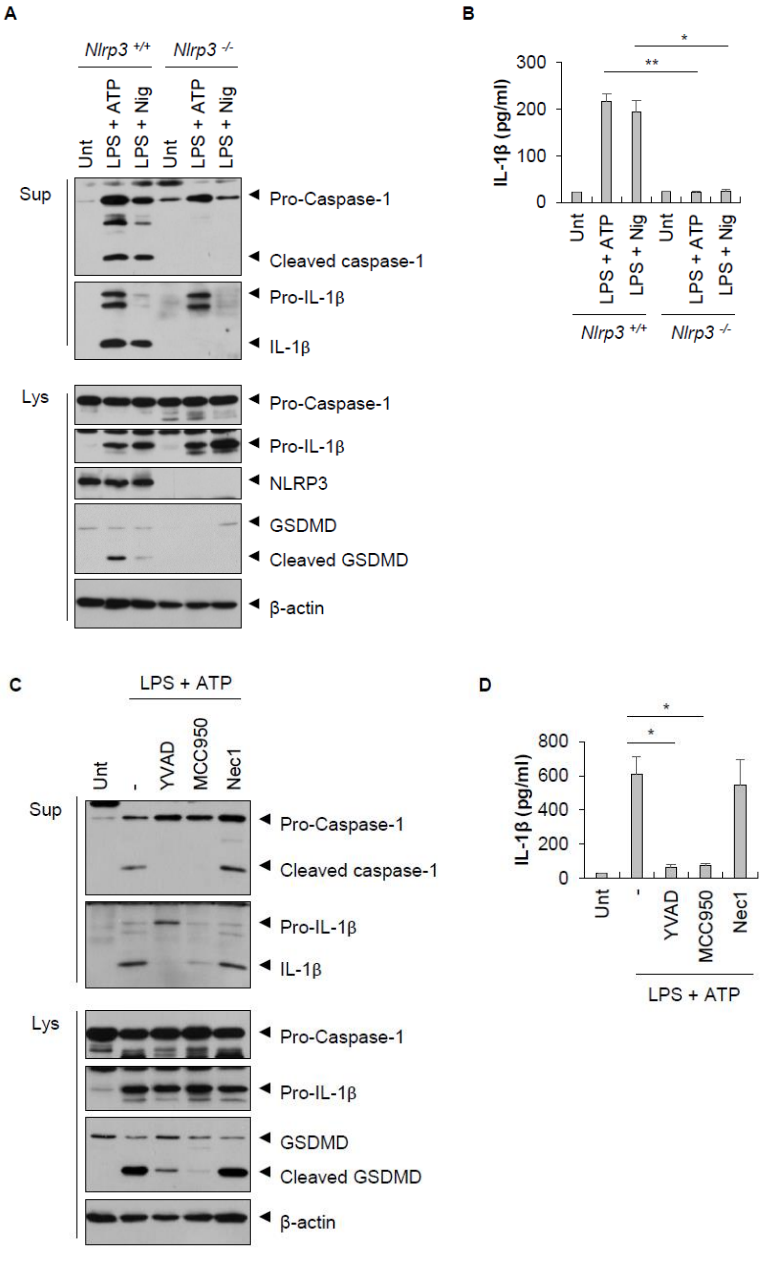


Figure 2. LPS plus ATP or nigericin treatment induces inflammasome activation in NLRP3 and caspase-1-dependent manner. (A, B) *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ BMDMs were treated with LPS (0.25 ug/ml) for 2.5 hr, followed by treatment with ATP (2.5 mM) or nigericin (Nig, 5 uM) for 50 min. (n=2). (C, D) WT BMDMs were pretreated with caspase-1 inhibitor Y-VAD-CMK, NLRP3 inhibitor MCC950 and RIP1 kinase inhibitor nec1 for 30 min and treated with LPS (0.25 ug/ml, 2.5 hr) followed by treatment with ATP (2.5 mM, 1 hr). (n=2). The values were expressed as the mean ±SEM. *P<0.05, **p<0.01

2. BMDMs undergo pyroptosis upon LPS plus ATP or nigericin treatment.

Next, pyroptosis, a programmed lytic cell death induced by inflammasome activation were examined[32,33]. Cell death from BMDMs upon LPS plus ATP or nigericin stimulation were measured (Fig 3A, B). Lactate dehydrogenase (LDH) assay and propidium iodide (PI) staining were used for measuring cell death. As expected, WT BMDMs undergo pyroptosis in response to LPS plus ATP or nigericin treatment. *Nlrp3*-deficient BMDMs with LPS plus ATP or nigericin stimulation decreased release of LDH as well as WT BMDMs treated inhibitors of caspase-1 or NLRP3 (Fig 3C, D).

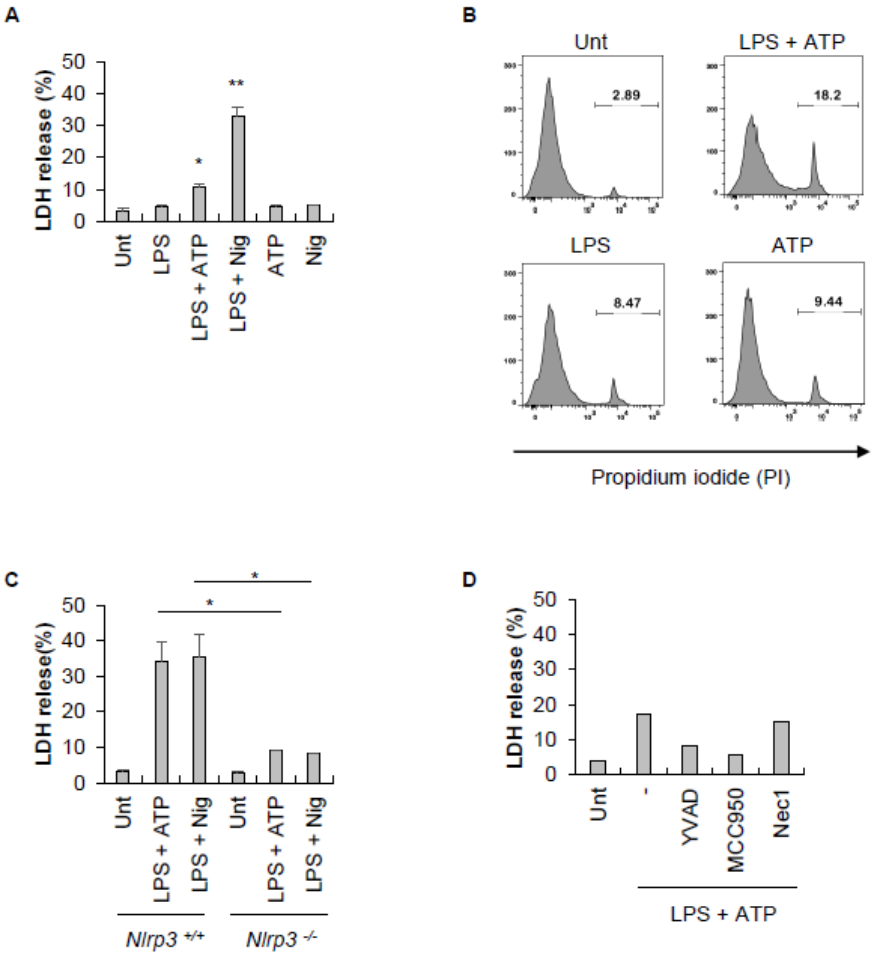


Figure 3. LPS plus ATP or nigericin stimulation induces pyroptosis in BMDMs. (A, B) WT BMDMs were treated LPS (0.25 ug/ml) for 2.5 hr followed by treatment with ATP (2.5 mM) or nigericin (Nig, 5 uM) for 50 min. Culture supernatants and lysis were assayed for measurement of cell death by LDH assay and PI staining. (C) *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ BMDMs were treated with LPS (0.25 ug/ml) for 2.5 hr followed by ATP (2.5 mM, 50 min) or nigericin (5 uM, 50 min) treatment. Cell death were measured by LDH assay using cell supernatant and lysate. (D) WT BMDMs were treated caspase-1 inhibitor Y-VAD-CMK, NLRP3 inhibitor MCC950 and RIP1 kinase inhibitor necrostatin-1 (nec1) for 30 min. After then, LPS (0.25 ug/ml) was treated for 2.5 hr, followed by treatment with ATP (2.5 mM) for 40 min. Cell death of BMDMs were measured by LDH assay. (n=2). The values were expressed as the mean ±SEM. *$p<0.05$, **$p<0.01$

3. Primary mixed glia upon LPS plus ATP or nigericin stimulation undergo attenuated pyroptosis.

To confirm NLRP3 inflammasome activation in primary mixed glia, primary mixed glia from pups of WT C57BL/6 mice were obtained. Primary mixed glia were treated with LPS followed by treatment with ATP or nigericin. Cleaved caspase-1 and mature IL-1β secretion from primary mixed glia were verified after appropriate treatment. (Fig 4A, B). Then pyroptosis in mixed glia induced by LPS plus ATP or nigericin stimulation were examined (Fig 4C). After appropriate treatment, pyroptosis of mixed glia were reduced, not like pyroptosis of BMDMs.

To verify reduced pyroptosis of mixed glia upon NLRP3 inflammasome activation, mixed glia and BMDMs were treated with LPS followed by sustained ATP or nigericin treatment (Fig 5). Cleaved caspase-1 and IL-1β were secreted from mixed glia and BMDMs after appropriate treatment (Fig 5A). However, mixed glia were less vulnerable to pyroptosis than BMDMs (Fig 5B, C).

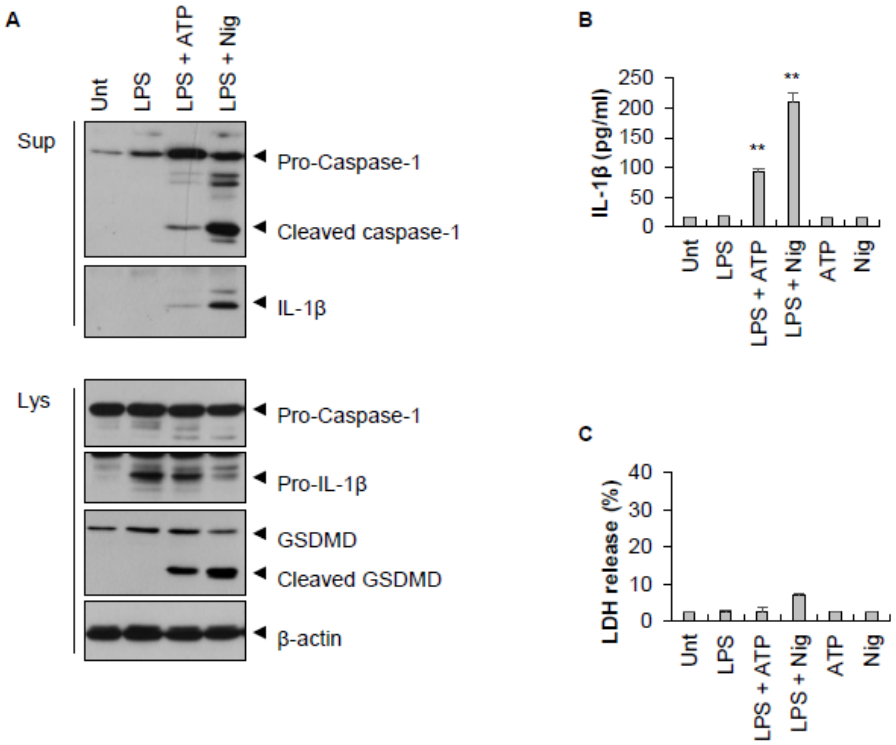


Figure 4. LPS plus ATP or nigericin stimulation induces reduced pyroptosis in primary mixed glia. (A, B) Primary mixed glia were treated with LPS (0.25 ug/ml) for 2.5 hr followed by ATP (2.5 mM) or nigericin (Nig, 5 uM) treatment for 40 min. Culture supernatants were assayed for extracellular IL-1β secretion by ELISAs. (C) Cell death was measured by LDH assay using cell supernatant and lysate of primary mixed glia. (n=2). The values were expressed as the mean ±SEM. **p<0.01

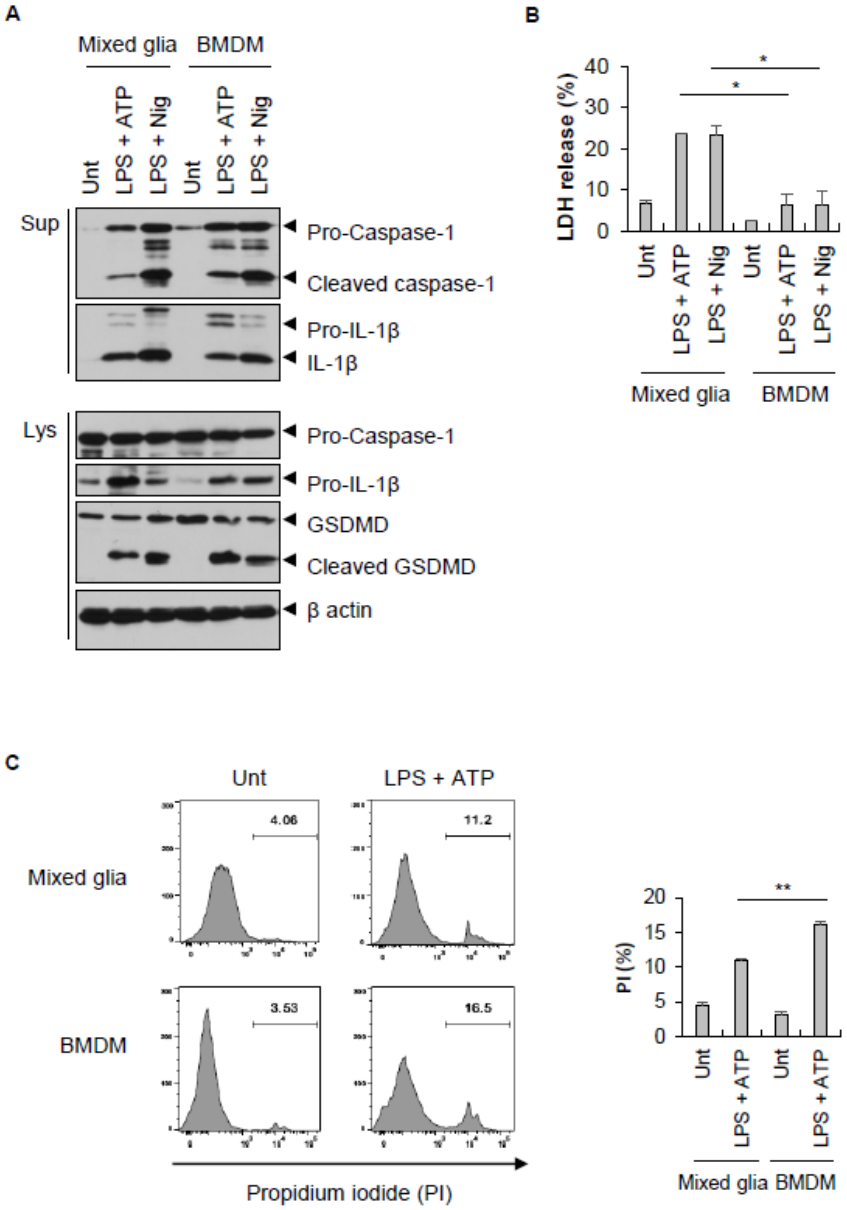


Figure 5. Primary mixed glia are less vulnerable to pyroptosis than BMDMs. (A) WT BMDMs and primary mixed glia were treated with LPS (0.25 ug/ml) for 2.5 hr followed by treatment with ATP (2 mM) for 2 hr. (B) Culture supernatants and lysis were assayed for measurement of cell death by LDH assay. (C) Flow cytometric analysis of BMDMs and mixed glia treated with LPS (0.25 ug/ml) for 2.5 hr followed by ATP (2 mM) for 2 hr. After appropriate treatment, BMDMs and mixed glia were stained with PI. (n=2). The values were expressed as the mean ±SEM. *p<0.05, **p<0.01

4. Mixed glia is insensitive to NLRP3-dependent pyroptosis than BMDMs.

It has been reported that GSDMD are cleaved by activate caspase-1 and cleaved GSDMD-N terminals induce pyroptosis by forming pores in plasma membrane[4]. To elucidate pyroptosis resistance of mixed glia, NLRP3 inflammasome activation was induced by LPS/ATP treatment (Fig 6). Cleaved GSDMD were observed in BMDMs and mixed glia in response to prolonged LPS/ATP treatment (Fig 6A, B). However, mixed glia showed attenuated pyroptosis in a GSDMD-independent manner upon prolonged LPS/ATP stimulation, not like BMDMs (Fig 6C, D).

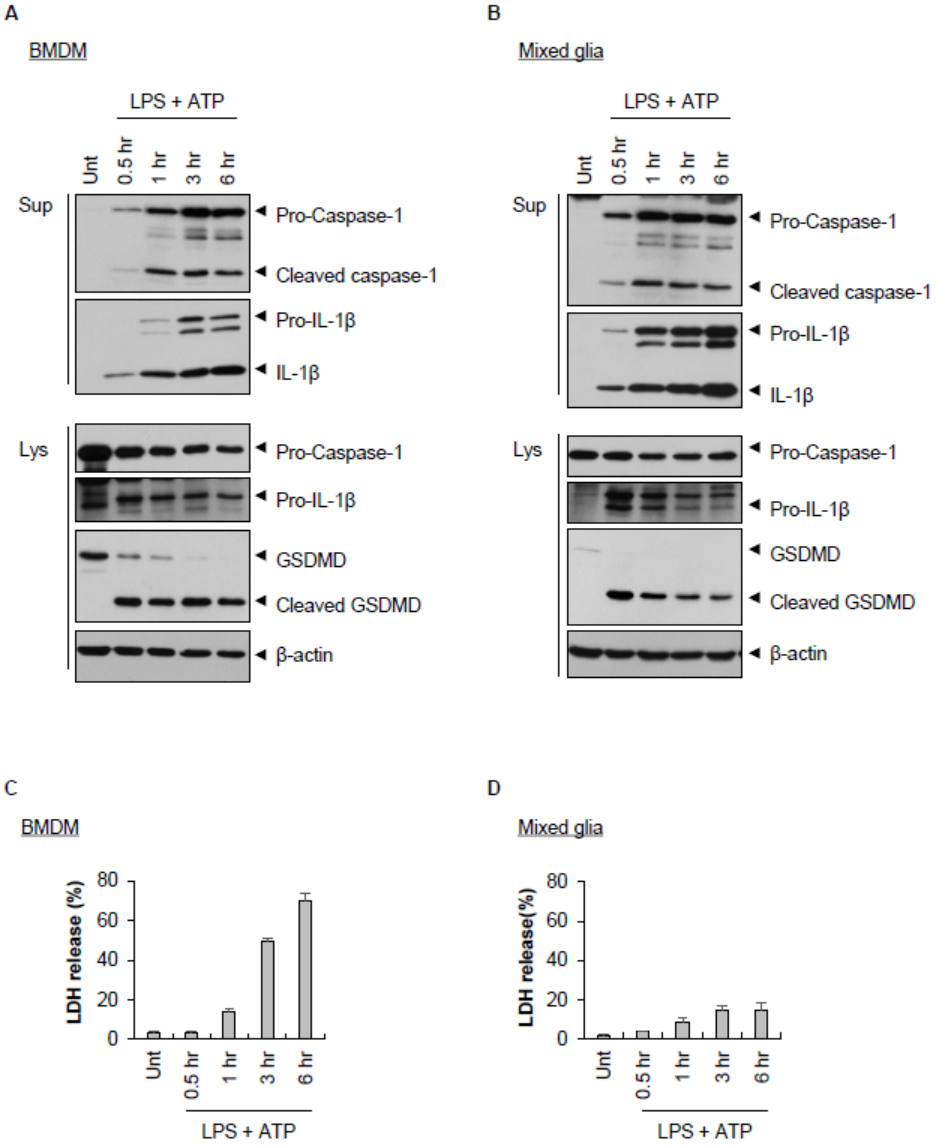


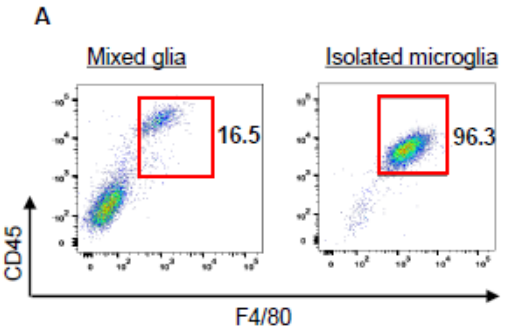
Figure 6. Reduced pyroptosis of mixed glia is not related to GSDMD. WT BMDMs and primary mixed glia were treated with LPS (0.25 ug/ml) for 2.5 hr followed by ATP treatment (2 mM) for 0.5, 1, 3, 6 hr. (A, B) Immunoblots from WT BMDMs and primary mixed glia cultures. (C, D) Culture supernatants and lysis of BMDMs and mixed glia were assayed for measurement of cell death by LDH assay after appropriate treatment. (n=4). The values were expressed as the mean ±SEM. **$p<0.01$

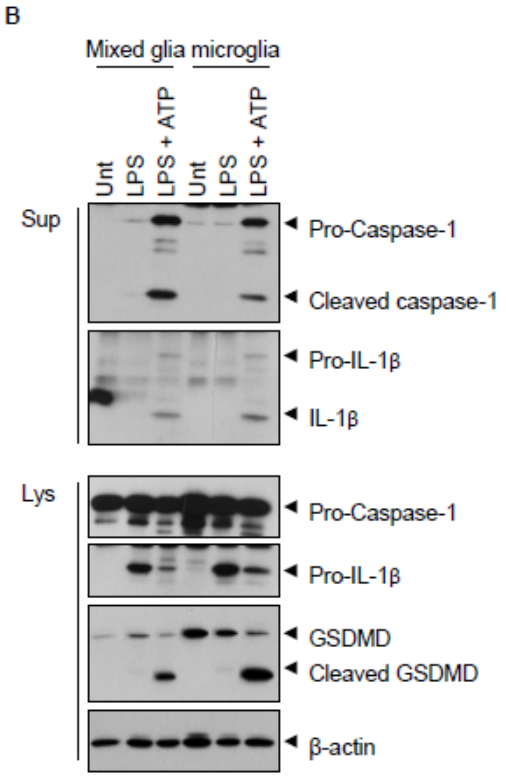
5. Cross-talk between glial cells possibly attenuates pyroptosis.

Since mixed glia contain microglia and studies have been reported that microglia can activate NLRP3 inflammasome[6,17], microglial inflammasome activation and pyroptosis were examined. To examine that, microglia were isolated from primary mixed glia cultures. Mixed glia cultures after about 10~15 days, microglia were isolated by mild trypsinization. To confirm microglia population, isolated cells were stained by microglia marker F4/80 and CD45 and analyzed by flow cytometry (Fig 7A). Next, microglial NLRP3 inflammasome activation was compared with mixed glia (Fig 7B, C). Cleaved caspase-1 and IL-1β were secreted from isolated microglia and mixed glia upon LPS/ATP stimulation.

Since glial cell death resistances have been reported[31], microglial pyroptosis upon LPS/ATP treatment were examined. When mixed glia and microglia were treated with LPS followed by treatment with ATP, pyroptosis of microglia was observed much more than that observed from mixed glia (Fig 8).

Previously, interactions between astrocytes and microglia have been reported in many studies[31,34,35]. Therefore, pyroptosis between microglia in mixed glia and isolated microglia were compared (Fig 9). After LPS/ATP stimulation to mixed glia and microglia, both glial cells were gated with microglial marker CD11b. Interestingly, LPS/ATP treatment caused much more population of PI-positive isolated microglia than PI-positive population of microglia in mixed glia (Fig 9B).





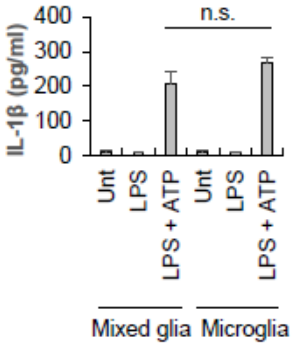
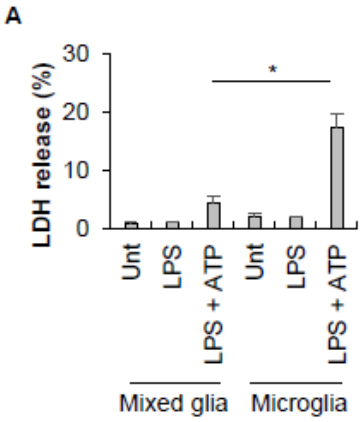


Figure 7. Microglia are isolated from mixed glia and secrete IL-1β induced by LPS/ATP stimulation. (A) Primary mixed glia cultures after about 10~15 days, microglia were isolated by mild trypsinization. Microglia were stained with microglia marker F4/80 and CD45-specific antibodies and analyzed via flow cytometry. (B, C) Microglia and mixed glia were treated with LPS (0.25 ug/ml for 2.5 hr) followed by the treatment with ATP (2.5 mM for 50 min). Levels of IL-1β from mixed glia and microglia treated with LPS/ATP treatment were measured by ELISA. (n=2). The values were expressed as the mean ±SEM. n.s. not significant



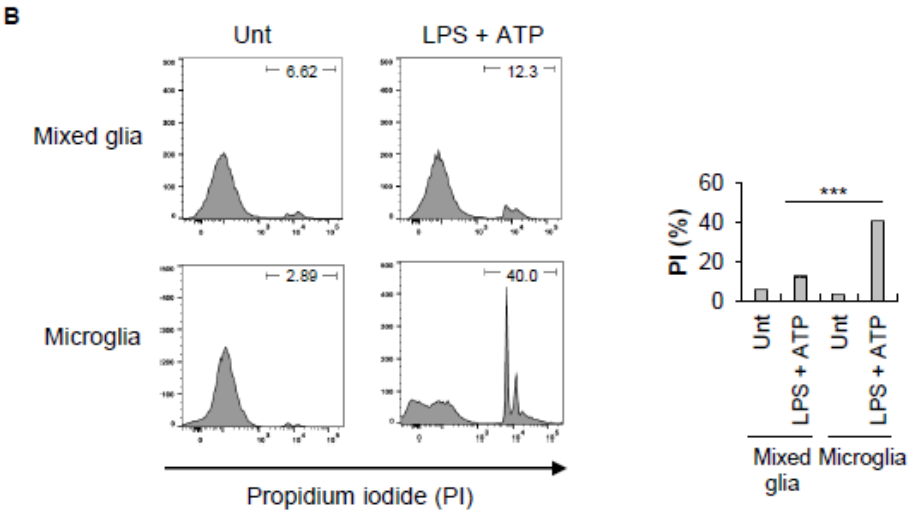


Figure 8. Microglia are more susceptible to pyroptosis than mixed glia. (A) Primary mixed glia and microglia were treated with LPS (0.25 ug/ml for 2.5 hr) followed by ATP (2.5 mM for 50 min). Cell death of glial cells were measured by LDH assay. (n=2). (B) Mouse mixed glia and microglia were treated with LPS (0.25 ug/ml, 2.5 hr) followed by treatment with ATP (2 mM, 2 hr). Cell death of primary mixed glia and microglia was measured by PI staining and analyzed by flow cytometry. (n=2). The values were expressed as the mean ±SEM. *$p<0.05$, ***$p<0.001$

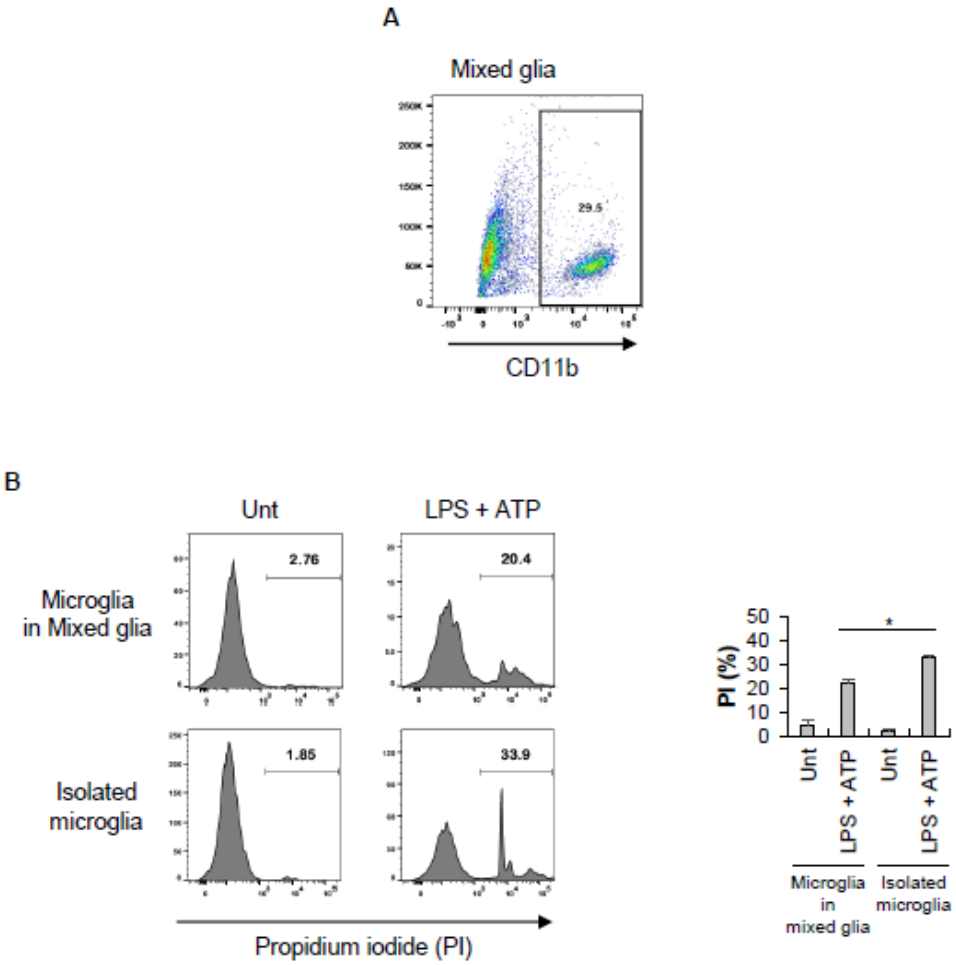


Figure 9. Glial interaction possibly attenuates microglial pyroptosis. (A, B) Primary mixed glia and microglia were treated with LPS (0.25 ug/ml, 2.5 hr) followed by ATP treatment (2 mM, 2 hr). After appropriate treatment, Primary mixed glia cultures were stained with microglia marker CD11b-specific antibody and cell death marker PI and analyzed by flow cytometry. (n=2). The values were expressed as the mean ±SEM. *$p<0.05$

6. NLRP3-dependent pyroptosis in BMDMs and mixed glia leads to neuronal cell death.

Recent studies reported that pyroptosis is possibly mediated with neurologic disease[36]. To examine effects of pyroptosis mediated NLRP3 inflammasome activation in BMDMs and mixed glia to neurotoxicity, dopaminergic neuroblastoma cell line SH-SY5Y were used for quantification of neurotoxicity. SH-SY5Y were plated at multiwell plate and BMDMs or mixed glia were plated at upper chamber (Fig 10). BMDMs and mixed glia were treated with LPS plus ATP and the damage of co-cultured SH-SY5Y cells were quantified by PI staining (Fig 10B). Interestingly, SH-SY5Y co-cultured with BMDMs upon LPS/ATP stimulation showed increased PI population than that co-cultured with mixed glia.

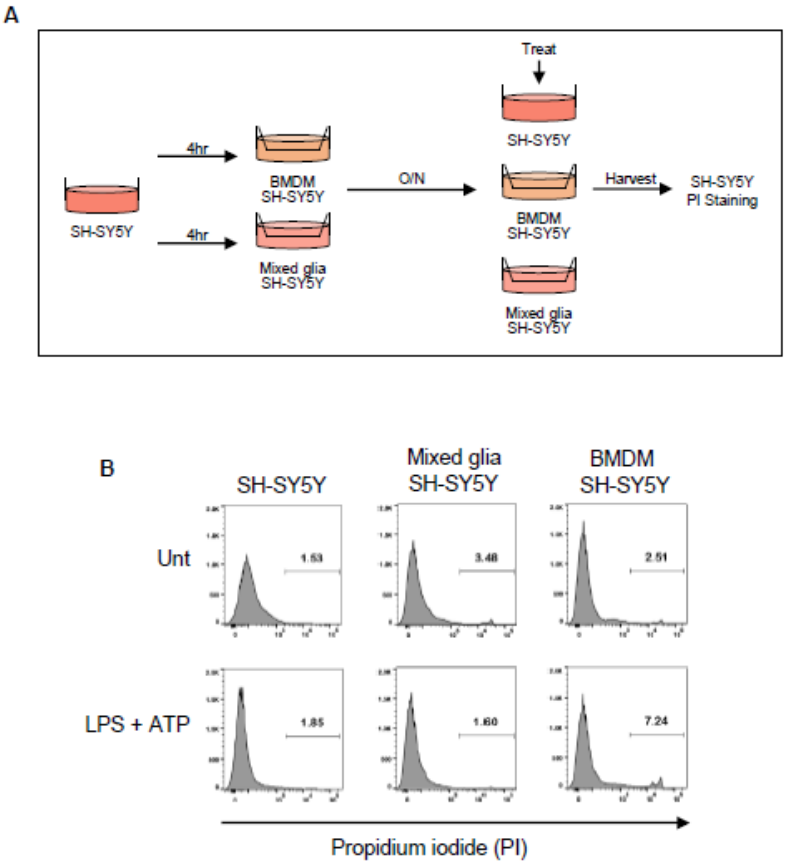


Figure 10. Inflammasome-mediated pyroptosis triggers neuronal cell death. (A, B) Transwell system pattern diagram of BMDMs, mixed glia and SH-SY5Y cells. First, SH-SY5Y cells were plated in 6 well plate. After 4 hr, BMDMs or mixed glia were plated in transwell upper chamber. Next day, LPS (0.25 ug/ml for 2.5 hr) and ATP (2 mM for 2 hr) were treated to upper chamber. After appropriate treatment, SH-SY5Y cells were stained with PI and analyzed by flow cytometry.

IV. DISCUSSION

Recent studies reported inflammatory caspases (caspase-1, -4,-5 and -11) lead to pyroptotic cell death due to gasdermin D (GSDMD)[32]. Pyroptosis is morphologically and mechanistically different from other forms of cell death. Pyroptosis initiates in caspase-1-dependent manner and induces rapid plasma-membrane rupture which leads to release of pro-inflammatory intracellular contents, not like apoptosis, a non-inflammatory cell death[37,38]. Recent studies have proposed potential connection between pyroptosis and pathogenesis such as sepsis[39], autoimmune disease[5] and neurologic disease[36]. In this regard, inflammasome activation and consequent pyroptosis may support host defense against pathogens and various pathogenesis. However, the mechanisms underlying pyroptosis induced by NLRP3 inflammasome activation in glial cells remains unclear. In this study, I tried to demonstrate attenuated pyroptosis of mixed glia upon NLRP3 inflammasome activation and effects of glial pyroptosis resistances.

At first, I demonstrated NLRP3 inflammasome activation in BMDMs upon LPS plus ATP or nigericin simulation. Cleaved caspase-1 and mature IL-1β were secreted from WT BMDMs upon LPS plus ATP or nigericin stimulation. By using *Nlrp3*-defient BMDMs and inhibitors, I examined that LPS plus ATP or nigericin stimulation induces secretion of cleaved caspase-1 and IL-1β from BMDMs in NLRP3 and caspase-1-dependent manner. Furthermore, cleaved GSDMD, the effector of pyroptosis, were observed in BMDMs upon appropriate treatment.

Glial cells have longer lifespan than other immune cells[27] and recent studies have reported to establish the differences between BMDMs and glial cells in the inflammasome activation and the effects[40,41]. However, pyroptosis of glial cells is still poorly understood. These studies indicated that cleaved caspase-1 and IL-1β were

secreted from primary mixed glia upon NLRP3 inflammasome activation as same as BMDMs. Interestingly, pyroptosis of mixed glia was much less susceptible than pyroptosis of BMDMs. It suggests that unlike BMDMs, mixed glial cells were less susceptible to pyroptosis upon NLRP3 inflammasome activation.

It has been reported that cleaved GSDMDs oligomerize to form pyroptotic pores and induce pyroptosis[4]. However, GSDMD cleavage in glial cells have been studied yet. In this study, I demonstrated whether attenuated pyroptosis of mixed glia is related to GSDMD cleavage. Pyroptosis of mixed glia were still attenuated upon prolonged LPS/ATP stimulation and GSDMD cleavage were observed as same as that of BMDMs. It suggests that mixed glial reduced pyroptosis is not related to GSDMD cleavage. Recent studies reported that endosomal sorting complexes required for transport (ESCRT)-III system which contribute membrane repairment could promote cellular survival mechanisms during pyroptosis[42]. Therefore, identifying the characteristics of ESCRT system in glial cells potentially contributes to explain reduced glial pyroptosis.

As a population of mixed glia, microglia are major immune cell and microglial inflammasome activation has been well studied. Therefore, microglia were isolated from mixed glia and compared NLRP3 inflammasome activation and pyroptosis between mixed glia and microglia. It demonstrated that mixed glial NLRP3 inflammasome activation processed secretion of caspase-1 and IL-1β as same as microglia. However, pyroptosis of microglia was more susceptible than mixed glia upon NLRP3 inflammasome activation. Multiple glial cells are present in mixed glia and it has been reported that glial cells interaction could affects immune response of other glial cells[35,43,44]. So, I examined whether other glial cells in mixed glia could affect pyroptosis of microglial cells. Interestingly, microglia in mixed glia were less

susceptible in pyroptosis than isolated microglia upon NLRP3 inflammasome activation. It suggested that cross-talk between glial cells possibly attenuates microglial pyroptosis. Therefore, identifying what factors of glial interaction could affect pyroptosis resistance of microglia is required.

Since pyroptosis is a proinflammatory lytic cell death[37], I expected that reduced pyroptosis of mixed glia could affects to neurotoxicity. In this study, I found that pyroptosis of BMDMs showed more toxicity to SH-SY5Y than pyroptosis of mixed glia. It suggests that pyroptosis is more toxic to neuronal cells than inflammasome activation of immune cells.

Collectively, this study indicates that mixed glial cells showed NLRP3 inflammasome-mediated weaker pyroptosis than BMDMs and glial interaction potentially attenuates microglial pyroptosis.

V. CONCLUSION

Here, I demonstrated that mixed glial cells are less susceptible to NLRP3 inflammasome-mediated pyroptosis than macrophages. BMDMs were treated with LPS plus ATP or nigericin, which activates NLRP3 inflammasome. Activation of NLRP3 inflammasome in macrophage induces cleaved caspase-1, mature IL-1β secretion and pyroptosis, a programmed lytic cell death. In primary mixed glia, NLRP3 inflammasome activation induces activation of caspase-1 and IL-1β release as same as macrophages but not pyroptosis. Pyroptosis of primary mixed glia was attenuated upon LPS/ATP stimulation. Furthermore, cleavage of GSDMDs, effectors of pyroptosis, in mixed glia upon LPS/ATP treatment were observed but pyroptosis of mixed glia was still reduced. Moreover, microglia were isolated from mixed glia, and microglial NLRP3 inflammasome activation was examined upon LPS/ATP stimulation. Cleaved caspase-1, mature IL-1β release and pyroptosis were observed in microglia after LPS plus ATP or nigericin stimulation. In addition, isolated microglia from mixed glia were more susceptible to pyroptosis than microglia in mixed glia. Furthermore, neurotoxicity mediated pyroptosis was observed in the transwell system. SH-SY5Y co-cultured with BMDM upon LPS/ATP treatment were more damaged than SH-SY5Y co-cultured with mixed glia. Collectively, all these data provide evidence that pyroptosis of mixed glia is less susceptible than pyroptosis of macrophage and glial interaction possibly attenuates microglial pyroptosis.

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ABSTRACT (IN KOREAN)

아교세포의 pyroptosis 에 대한 NLRP3 인플라마좀 활성의 역할

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황 보 름

인플라마좀(inflammasomes)은 감염이나 스트레스 등 선천성 면역 방어체계와 관련하여 염증성 사이토카인의 성숙을 유도하는 분자적 기작이 되는 다중단백질 복합체이다. 주로 대식세포와 같은 골수세포에서 일어나는 인플라마좀 복합체는 NLR 단백질과 같은 sensor 단백질과 연결 단백질 ASC 그리고 procaspase-1 의 결합으로 이루어져 있다. 인플라마좀 복합체의 활성은 pro-caspase-1 을 cleaved caspase-1 으로 활성화시키고 이는 pro-IL-1β, pro-IL-18 와 같은 염증성 사이토카인을 활성형태로 바꾸어 면역반응에 기여한다. 인플라마좀의 활성은 pyroptosis 라는 세포용해사멸을 유도하는데, 최근 연구를 통해 cleaved caspase-1 이 세포내 존재하는 gasdermin D (GSDMD)의 활성을 유도하고

이를 통해 잘린 GSDMD 의 N 말단이 막공을 형성함으로써 pyroptosis 에 기여한다는 것이 알려졌다. 미세아교세포와 성상아교세포는 중추신경계에 상주하는 아교세포이자 병원균에 대항하는 신경염증의 주된 조절자로서 인플라마좀 역시 활성화시킬 수 있다. 최근 교세포의 NLRP3 인플라마좀 활성이 신경염증과 신경퇴행성 질환에 기여한다는 보고가 이어지고 있다. 아교세포들은 다른 면역세포들에 비해 긴 수명을 갖고 있고 apoptosis 와 같은 세포사에 저항성을 갖고 있다는 가능성이 제기되어왔다. 그러나 중추신경계 내 아교세포들의 pyroptosis 와 그 영향에 대한 연구 결과는 아직 부족하다. 본 논문을 통해 mixed glia 의 NLRP3 인플라마좀의 활성을 통한 pyroptosis 에 대해 확인하고자 하였다. Lipopolysaccharide (LPS) 와 ATP 의 처리에 의해 mixed glia 는 cleaved caspase-1 과 IL-1β 를 분비하였지만 골수유래대식세포 (Bone marrow-derived macrophages, BMDMs) 보다 약화된 pyroptosis 를 보였다. Pyroptosis 에 주된 단백질인 GSDMD 의 분열은 mixed glia 의 약화된 pyroptosis 와는 무관하였음을 확인 후 mixed glia 에서 미세아교세포를 분리하여 pyroptosis 를 확인하였다. Mixed glia 내에 존재하는 미세아교세포와 분리된 미세아교세포 간에 NLRP3 inflammasome 의 활성을 통한 pyroptosis 를 비교한 결과 mixed

glia 내의 미세아교세포에게서 pyroptosis 가 약화되어있음을 확인하였다. 결론적으로, 본 논문을 통해 아교세포의 약화된 pyroptosis 와 아교세포간의 상호작용에 의해 미세아교세포의 pyroptosis 가 약화될 수 있을 것이라는 가능성을 확인할 수 있었다 .

핵심되는 말: mixed glia, 미세아교세포, 대식세포, NLRP3 인플라마좀, pyroptosis.

PUBLICATION LIST

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