Implication of NLRP3 inflammasome in lipopolysaccharide-induced depressive like behaviors

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Implication of NLRP3 inflammasome in lipopolysaccharide-induced depressive like behaviors

Directed by Professor Je-Wook Yu

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ABSTRACT

Implication of NLRP3 inflammasome in lipopolysaccharide-induced depressive like behaviors

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For many years, central nervous system (CNS) was considered to be immune privileged, however, many reports now suggest that immune and inflammatory response can take place in the brain region. although mechanism of neuroinflammation remains to be unknown. Neuroinflammation is mediated by brain resident glial cells, mainly microglial cells and astrocytes, and infiltrated macrophages. These CNS-innate immune cells sense danger signals such as ATP and misfolded protein and cascade pro-inflammatory signaling. Inflammasome is cytosolic multi-protein complex that triggers caspase-1 activation and interleukin-1β (IL-1β) and interleukin-18 (IL-18) secretion in response to pathogen associated

molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs). NLRP3 inflammasome, which is best characterized, is known to be involved in pathogenesis of neurological disorders. Recently, several reports suggest that excessive activation of NLRP3 inflammasome upon amyloid-β plaque and α-synuclein aggregation leads to pathophysiology of Alzheimer's disease (AD) and Parkinson's disease (PD). Depressive disorder is one of the commonest psychiatric disorders, which is complex disorder with no established pathophysiological mechanism. Emerging papers are suggesting that inflammatory response can lead to comorbid depressive disorders. Recently, clinical data show that patients with depression have higher levels of inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factorα (TNFα) in peripheral serum. The kynurenine/tryptophan metabolism is known to be one of the important mechanisms, which mediates relevance of inflammation to depressive behaviors. Indoleamine 2,3-deoxygenase (IDO) – a key enzyme of kynurenine pathway in the CNS – is known to be driven by pro-inflammatory cytokines. Increase of IDO in glial cells leads to production of kynurenic acid (KA) and quinolinic acid (QA), which contribute to neuronal dysfunction, while the increase of IDO in neurons leads to 5-HT deficiency. Here, we demonstrated that NLRP3 inflammasome is closely implicated in lipopolysaccharide (LPS)-induced depressive like behaviors. Deficiency of Nlrp3 in mice demonstrated a significantly reduced depressive like behaviors after LPS-administration. Deficiency of Nlrp3 reduced recruitment of immune cells into brain hippocampus of LPS-induced depressive mouse. Moreover, we observed that active caspase-1 was significantly reduced in the hippocampus of $Nlrp3^{-/-}$ mice compared to wild type mice. Mechanistically, IDO was transcriptionally and functionally up-regulated in brains of LPS-injected WT mice, while it was not in $Nlrp3^{-/-}$ mice. In primary mixed glial cultures, we observed that NLRP3 inflammasome-activating stimulation increased the IDO gene expression and this effect was caspase-1 dependent. However, Recombinant IL-1β or IL-18 did not directly regulate IDO expression. Collectively, all our results indicate that NLRP3

inflammasome could contribute to progression of depressive like behaviors via regulating IDO activity in glial cells.

Key word : neuroinflammation, depressive disorder, NLRP3 inflammasome, Indoleamie 2,3-deoxygenase

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Ⅰ. INTRODUCTION

For decades central nervous system (CNS) has been considered to be immune privileged region, where inflammation can only occur when blood-brain barrier (BBB) break down.[1,2] However, it is recently known that inflammation is crucial component of major neurological disease.[3,4] Neuroinflammation can be initiated in response to various cues such as brain injury, infection, toxic metabolite and autoimmunity. Brain resident innate immune cell known as microglia and infiltrated immune cell can respond to various inflammatory signals by releasing pro-inflammatory cytokines and cytotoxic substances such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα) and neutrophil extracellular traps (NETs).[5,6] Excessive response of microglia to inflammation contributes to the pathogenesis of neurological disease by mediating neurotoxic events. Misfolded proteins in brain region including

amyloid-β plaque, tau tangle and α-synuclein have been reported to be able to activate microglia, leading to cause Alzheimer's disease (AD) and Parkinson's disease (PD).[7-9] Recently, inappropriate complement activation has been proposed to cause microglia to mediate excessive synaptic pruning, which leads to cognitive decline.[10,11]

Microglia is known to express pathogen recognition receptor (PRRs) that can trigger innate immune signaling pathway. PRRs have been identified with four different classes, which are Toll-like receptors (TLRs), C-type lectin receptors (CLRs), cytoplasmic proteins such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs).[12] TLRs and NLRs could sense DAMP (danger associated molecular patterns) as well as PAMP (pathogen associated molecular patterns), leading to transcriptional expression of inflammatory mediators. Particularly NLRs forms a multi-protein complex upon danger signals and controls activation of the proteolytic enzyme Caspase-1.[13]

Inflammasome is cytosolic protein complex composed of NLRs or absent in melanoma 2 (AIM2), ASC and caspase-1. The inflammasome is assembled upon pathogen infection or cell danger signals and promotes the maturation and release of pro-inflammatory cytokines including interleukin-1β (IL-1β) and interleukin-18 (IL-18).[13-15] NLRP3 is best characterized member of NLRP family which contains conserved NACHT domain, c-terminal leucin-rich repeat (LRR) domain and N-terminal pyrin domain (PYD). NLRP3 is critical responder of host defense system,[16] activated by microbes such as influenza A virus and Salmonella typhimurium (S.typhimurium) or by endogenous danger signals such as adenosine triphosphate (ATP) and misfolded proteins. Assembly of NLRP3 inflammasome induces auto-proteolysis of procaspase-1 and activation of caspase-1 leads to cleavage and subsequent release of IL-β and IL-18. The CNS is particularly sensitive to IL-1β and IL-18 signaling because many brain resident cells express receptors for these cytokines. Excessive and prolonged activation of NLRP3 inflammasome has been reported to contribute to pathological condition of CNS and lead to neurological

diseases.[14,17-19] It has been demonstrated that NLRP3 inflammasome is activated upon amyloid-β plaque, aggravating AD.[7,8] In case of PD, aggregation of α-synuclein could activate NLRP3 inflammasome.[9]

Depressive disorder is one of the commonest neuropsychiatric disorders, for approximately, 10 % being diagnosed with depression worldwide. The symptoms of major depressive disorder are depressed mood, diminished interest or pleasure in all activities, significant weight loss or weight gain and fatigue. Depression is complex disorder with no established physiological mechanism. However, there are several theories and hypothesis regarding the pathophysiology of depression. According to mainly dominated hypothesis, deficiency of monoamine neurotransmitter, particularly serotonin (5-HT) and norepinephrine (NE), can lead to procession of depression.[20] Some studies have shown that polymorphism of monoamine transporter cause reuptake of 5-HT and NE into presynaptic neuron, leading to monoamine deficiency in postsynaptic neurons. Reduced synthesis of 5-HT and NE from amino acid precursor or abnormally augmented catabolism by monoamine oxidase A (MAO-A) contributes to monoamine deficiency.[20] Neurotransmitter-receptor-second messenger signaling cascade such as cAMP pathway is known to be one of important factors in depressive disorder.[21] Excessive Stress has been considered to induce malfunction of hypothalamus-pituitary-adrenal (HPA) axis and increase corticortopn-releasing hormone (CRH), leading to depressive disorder.[20,22] Clinical treatment of antidepressant including tricyclic antidepressant (TCA), selective serotonin reuptake inhibitor (SSRI) results in increase 5-HT in synaptic cleft.

Recently, neuroinflammation has reported to contribute to depressive disorder.[23] Immune-to-brain communication pathway could lead to the production of pro-inflammatory cytokines and induce 5-HT deficiency in presynaptic neuron. Indoleamine 2,3-deoxygenase (IDO) is rate-limiting enzyme which shifts tryptophan from serotonin pathway to kynurenine pathway. It is known that IDO is induced by pro-inflammatory signals such as interferon-γ (IFNγ) during an immune response.[3,24] Kynurenine pathway is mainly mediated by microglia and astrocyte in CNS.[24] Its final

by-products, quinolinic acid (QA) and kynurenic acid (KA), are agonist and antagonist of neuronal N-methyl-D-aspartate receptor (NMDAr)[4,25,26] and known to be involved in pathophysiology of psychiatric disorder, regulating neuronal function.[24] It has been demonstrated that activation of kynurenine pathway induces toxicity on neuronal cells.[27,28]

The first study about correlation between inflammation and depression started from hepatitis C patients in 1987. Renault PF et al. showed that about 20 % of hepatitis C patients treated with IFNβ had been suffered from psychiatric side effect.[29] Since then, many clinical data have reported that depressive disorder patients show high level of pro-inflammatory cytokines including IL-1β, IL-6 and TNFα in peripheral serum.[30-33] Particularly increase of IL-1β and IL-18 indicates that inflammasome is implicated in pathophysiology of depression. Alcocer-Gomez E. et al. reported that gene expression of nlrp3 and caspase-1 and IL-1β level are increased in peripheral blood mononuclear cell (PBMC) of depression patients.[34] Although several animal studies suggest that immune response and NLRP3 inflammasome is involved in depressive disorder,[35-38] its molecular mechanism is still poorly understood.

The aim of this present study is to examine whether NLRP3 inflammasome is implicated in inflammation comorbid depressive disorder. Here, we provide evidences that NLRP3 inflammasome activation could contribute to depressive like behaviors in lipopolysaccharide-administrated mouse depression model via regulation of IDO enzyme in brain glial cells.

Ⅱ. MATERIALS AND METHODS

1. Mice and treatment

All mice care and use procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals and approved by the Animal Care Committee of Yonsei University College of Medicine. All mice were maintained in specific pathogen-free conditions. Experiments were performed on 8- to 10-week-old male and female C57BL/6 mice or Nlrp3-deficient mice. Lipopolysaccharide was purchased from Sigma. Mice were treated with LPS (intraperitoneal, 1.8 mg/kg) and then underwent behavior testing 24 hr later.

2. Behavioral experiments

A. *Forced swim test*

FST was conducted after TST. Each mouse was placed individually in a cylinder (diameter : 15 cm, height : 22 cm) containing 15 cm of tap water maintained 24±1℃. The water was changed every test. Mice were placed into the water for 6 min and video recorded. First 2 min were adaptation time for new environment and the last 4 min were scored for duration of immobility.

B. *Tail suspension test*

At 24 hr after LPS injection, TST was performed. Each mouse was suspended by the tail with adhesive tape and head was about 10 cm above the floor. Mice were hung for 6 min and video recorded. First 2 min were for adaptation time for new environment and only last 4 min were scored. The time of immobility was determined after establishing a threshold level that would exclude all movements.

C. *Sucrose preference test*

At the start of the experiment, mice were habituated to drink a 1.5% sucrose solution for 2 day. After adaptation, mice were deprived of water for 24 hr. At the end of the 24 hr, LPS was injected and 2 mice were co-housed and given access to two bottles (containing water or 1.5 % sucrose). Sucrose and water consumption was measured every 24 hr for 3 day and the sucrose preference was calculated as a percentage of the consumed 1.5 % sucrose solution relative to the total liquid intake. The position of the two bottles was switched every 24 hr for 3 day to ensure that the mice did not develop a side preference.

3. Primary mixed glia cell culture and treatment

Primary mixed glial cell culture was established from C57BL/6 mice or Nlrp3-deficient mice. Mixed glia was prepared from whole brain (without cerebellum) of neonatal (up to 3-day-old) mice pups. After removing meninges and washing with HBSS, brain tissue was dissociated with Pasteur pipette. Isolated glial cells were grown in DMEM:F12 1:1 (GIBCO, USA), which was supplemented with 10%FBS and 100 units/ml penicillin-streptomycin and incubated at 37℃ with 5 % CO_2.

4. Reagents and antibody

LPS and ATP were purchased from Sigma-Aldrich. Z-VAD-fmk was obtained from Bchem. Recombinant IL-1β (#211-11B) was purchased from Peprotech and IL-18 (50073-MNCE) was from Sino Biological Inc.. Glibenclamide (G0639) and NAC (A7250) were purchased from Sigma-Aldrich. Anti-IL-1β antibody (AF-401-NA) was obtained from R&D systems, anti-IL-18 antibody (sc-6179) was from Santa Cruz Biotechnology, Inc.. Anti-caspase-1 (AG-20B-0042) was purchased

from Adipogen and anti-NLRP3 antibody (ALX-804-881-C100) was from Enzo Life Sciences.

5. Caspase-1 assay

Caspase-1 activity of Brain hippocampus was assayed with FAM-FLICA™ in vitro caspase detection kit (ImmunChemistry Technologies, LLC., USA). After collecting whole intact brain, we dissect medial section of the brain with brain slicer matrix and isolated hippocampus. After dissociating hippocampal tissue with Pasteur pipette, cells were stained with CD45 antibody, CD11b antibody and FAM-YVAD-FMK up to 3 hr. After washing with FACS buffer 2 times, samples were analyzed with FACSVerse (BD).

6. KYN and TRP measurements

Brain Kynurenine (Kyn) / tryptophan (Trp) ratio was analyzed to assess IDO enzymatic activity. Brian hemisphere was homogenized in the presence of lysis buffer containing 25 mM Tris-Cl (pH7.5), 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS and protease inhibitor. Homogenates were then centrifuged at 12,500 rpm for 2 times at 4℃. Collected supernatants was normalized with lysis buffer and then analyzed for HPLC.

7. Cytokine measurements

After the behavioral test, we collected blood samples from the mice to quantify the secretion of pro-inflammatory cytokines in response to LPS injection. Cardiac blood was collected and clotted overnight at 4 ℃. Collected blood was centrifuged for 30 min at 6000 rpm after clotting. The samples were immediately assayed or stored at -80℃. The concentration of IL-1β, IL-6 and TNFα was measured by

ELISA kit (R&D System, Minneapolis, MN, USA) according to manufacturer's instructions.

Whole brain tissues were homogenized in the presence of lysis buffer containing of 25 mM Tris-Cl (pH7.5), 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS and protease inhibitor. The lysates were centrifuged at 12500 rpm for 2 times at 4 ℃. The supernatant was collected and assayed for IL-1β, IL-6 and TNFα according to manufacturer's instructions.

8. Immunoblot assay

Cell culture supernatants were precipitated by addition of an equal volume of methanol and 0.25 volumes of chloroform. The mixtures were vortexed and then centrifuged at 12,000 rpm for 10 min. The upper phase was discarded and 500 uL methanol was added to the interphase. This mixture was centrifuged at 12,000 rpm for 10 min and the protein pellet was dried at room temperature, resuspended in SDS sample buffer and boiled for 8 min. Samples was separated by SDS-PAGE, transferred to PVDF membrane, and then immunoblotted using appropriated antibodies as indicated.

9. RNA isolation and quantification of mRNA expression

To quantify mouse brain tissue mRNA expression, total RNA from brain hemisphere was prepared using Trizol reagent (Invitrogen, Carsbad, CA, USA). To quantify mRNA expression of cultured cell, total RNA was isolated with same reagent. 1 ug of RNA was reverse transcribed with PrimeScript™RT Master Mix (Takara Clontech, Mountain View, CA, USA). Template DNA was amplified by quantitative real-time PCR (RT-PCR) using SYBR Premix Ex Taq™ Ⅱ (Takara Clontech, Mountain View, CA, USA) and StepOne plus real-time PCR instrument

(Applied Biosystems, Foster, CA, USA). It was initiated with one cycle of 95℃ for 30 sec, followed by 40 cycles of 95℃ for 5 sec and 60℃ for 34 sec. Final dissociation state was performed with 95℃ for 15 sec, 60℃ for 1 min and 95℃ for 15 sec. Following amplification, product purity was assessed by melting curve analysis. Duplicate cycle thresholds (Ct values) were obtained for each sample and averaged. The values for Rn18s were then used to normalize the expression of target gene with the delta Ct method. The specific primer pairs were purchased from Genotech (Daejeon, Korea).

Table 1. Primers used for RT-PCR

Gene	Forward primer	Reverse primer
Mouse *Ido*	GGCTAGAAATCTGCCTGTGC	AGAGCTCGCAGTAGGGAACA
Mouse *Il1β*	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG
Mouse *Il6*	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
Mouse *Nlrp3*	ATGCTGCTTCGACATCTCCT	AACCAATGCGAGATCCTGAC
Mouse *Rn18s*	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC

10. Statistical analysis

All values were shown as the mean $\pm$ standard error of the mean (SEM). The data were analyzed with student's t-test. The p values ≤ 0.05 were considered significant.

Ⅲ. RESULT

1. Deficiency of Nlrp3 in mice reduces LPS-induced depressive like behaviors

To study the implication of NLRP3 inflammasome in depressive like behaviors induced by immune response, we administered PBS or 1.8 mg/kg dose of LPS to WT and $Nlrp3^{-/-}$ mice. We first examined whether LPS induces sickness response by assessing changes of body weight loss 24 hr post injection (Figure 1). As expected, there were significant changes of body weight both in WT and $Nlrp3^{-/-}$ mice. We further investigated whether LPS-administration induces depressive like behaviors by performing Tail Suspension Test (TST), Forced Swimming Test (FST) and Sucrose Preference Test (SPT) after 24 hr injection. TST and FST are known to be normally used to measure behavioral despair, while SPT is used to measure anhedonia which is one of the major symptoms of depressive disorder.[39] Indeed, LPS-administration induced an evident increase of duration of immobility in TST and FST in WT mice. Interestingly, this behavioral alteration was not significant in $Nlrp3^{-/-}$ mice (Figure 2A and B). To assess whether LPS induces anhedonia, we carried out SPT. Figure 1C shows that LPS-administration resulted in anhedonic symptoms by decreasing sucrose consumption compared to normal tap water in WT mice. $Nlrp3^{-/-}$ mice tended to be slightly protective from anhedonia compared to WT mice (Figure 2C).

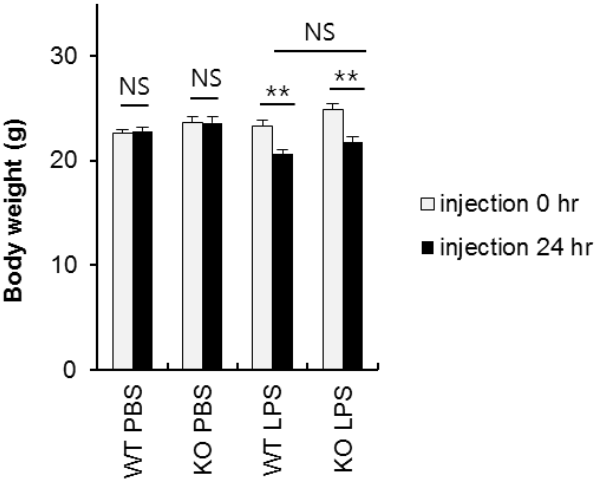

Figure 1. LPS-administration induces body weight loss both in WT and Nlrp3$^{-/-}$ mice. Mice were injected i.p with either PBS or LPS (1.8 mg/kg). Body weight was measured 24 hr after injection. **$p < 0.005$ compared to injection 0 hr. Values are means $\pm$ SEM (n= 11-13 mice / group).

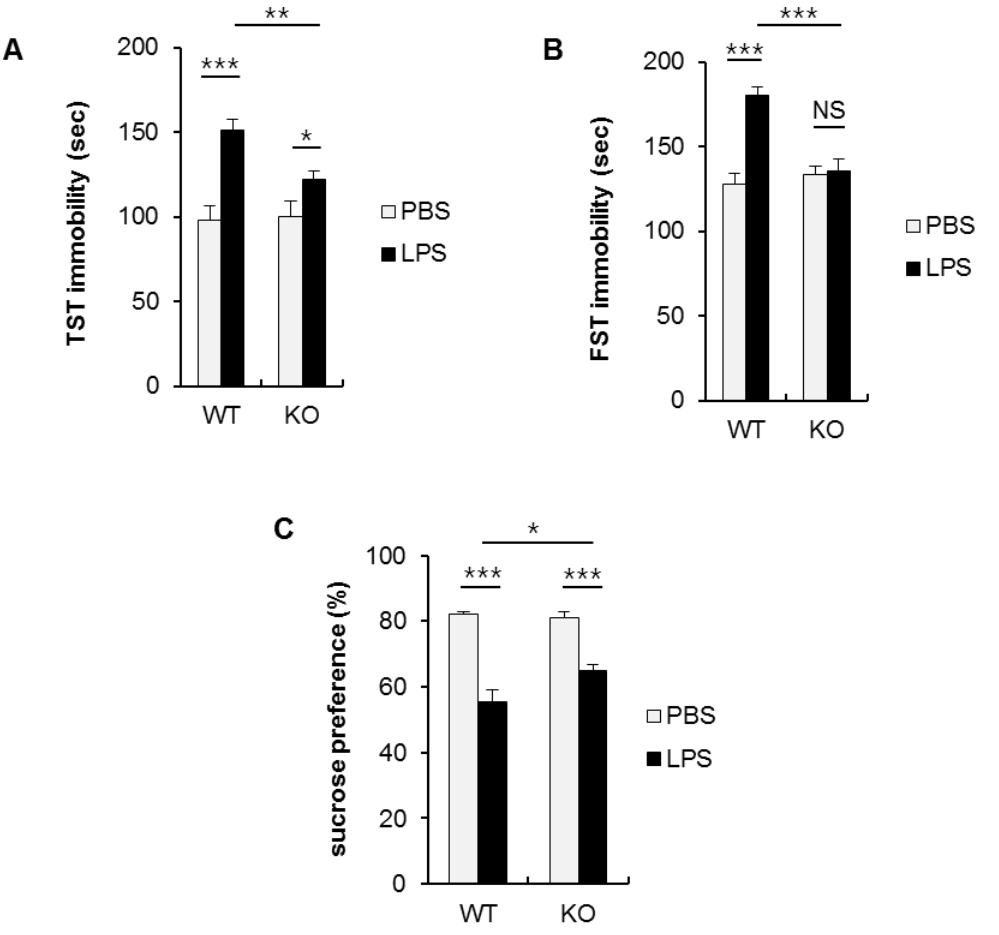

Figure 2. Nlrp3$^{-/-}$mice show reduced depressive like behaviors after LPS-administration. Mice were injected i.p with either PBS or LPS (1.8 mg/kg). (A) TST and (B) FST were performed during 6 min, 24 hr after injection. *p< 0.05, **p< 0.005, ***p< 0.0005 compared to PBS. Values are means ± SEM (n= 11-13 mice / group) (C) SPT was performed during 3 days. *p< 0.05, ***p< 0.0005 compared to PBS. Values are means ± SEM (n= 5-6 mice / group).

2. Pro-inflammatory cytokines in peripheral serum and brain are elevated in LPS-induced depressive mouse

To determine whether LPS-induced depressive like behaviors were associated with differential expression of peripheral pro-inflammatory cytokines, we collected cardiac serum 6 hr and 24 hr after injection and analyzed with ELISA assay. By 6 hr, there was a significant increase of IL-1β, IL-6 and TNFα (Figure 3A-C). Interestingly, $Nlrp3^{-/-}$ mice showed a slight decrease of IL-1β level in serum after LPS-administration (Figure 3D). However, there were no significant changes of IL-6 and TNFα levels (Figure 3E-F). To determine the effect of pro-inflammatory cytokines in brain region, we collected fresh whole brain and assayed ELISA. As expected, the levels of IL-1β, IL-6 and TNFα increased after LPS-administration. This result is in a good agreement with another LPS-induced depression mouse model study.[40] IL-1β and IL-6 levels were increased by 6 hr though TNFα was slightly elevated with no statistical significance (Figure 4A-C). Interestingly, there was no significant changes of central pro-inflammatory cytokine levels between two strains (Figure 4D-F).

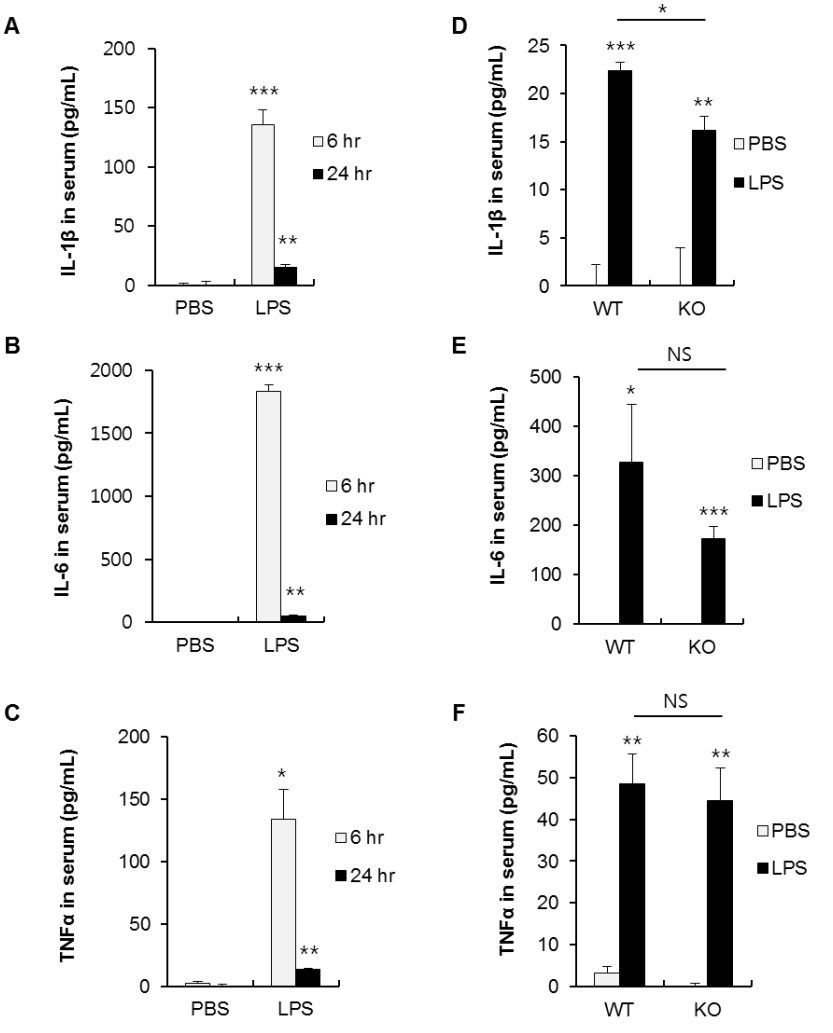

Figure 3. Peripheral pro-inflammatory cytokines are increased in LPS-administered WT and Nlrp3$^{-/-}$ mice. Mice were injected i.p with either PBS or LPS (1.8 mg/kg). (A-C) Fresh cardiac serum was collected 6 hr or 24 hr after injection and (D-F) 24 hr after injection. Collected serum was assayed for detection (A and D) IL-1β, (B and E) IL-6 and (C and D) TNFα. *p< 0.05, **p< 0.005, ***p< 0.0005 compared to PBS. Values are means ± SEM (n= 4 mice / group).

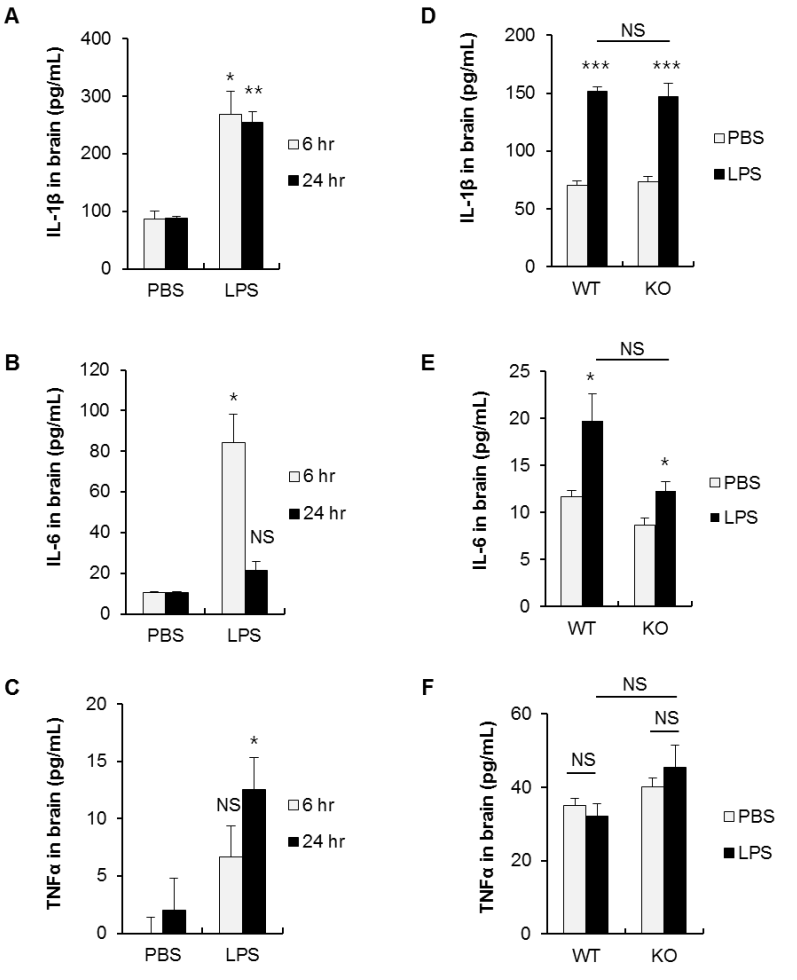

Figure 4. Central pro-inflammatory cytokines are increased in LPS-administered WT and Nlrp3$^{-/-}$ mice. Mice were injected i.p with either PBS or LPS (1.8 mg/kg). (A-C) Fresh hippocampus tissue was collected after 6 hr or 24 hr injection and (D-F) 24 hr after injection. Tissue homogenates were immediately assayed for detection (A and D) IL-1β, (B and E) IL-6 and (C and D) TNFα. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ compared to PBS. Values are means ± SEM (n= 4 mice / group).

3. Deficiency of Nlrp3 in mice impairs LPS-induced recruitment of $CD45^+CD11b^+$ immune cells into hippocampus

It is recently known that hippocampus may have a role in the pathophysiology of depressive disorder.[41] Recently, functional magnetic resonance imaging (fMRI) study suggested that abnormal hippocampal activation is detected in patients with major depressive disorder (MDD).[42] As pro-inflammatory cytokines are known to be mainly produced from immune cells, we assessed whether deficiency of Nlrp3 results in differential ratio of $CD45^+CD11b^+$ immune cell population in hippocampus. After collecting fresh hippocampal tissue 24 hr after injection, isolated single cells were stained with CD45 and CD11b antibody, which is marker dfor lymphocyte and monocyte. Figure 5 shows how to gate CD45 / CD11b double positive cells out of total hippocampal singlet. It indicates that $CD45^+CD11b^+$ immune cells were elevated in LPS-injected mice hippocampus of WT but interestingly not in $Nlrp3^{-/-}$ (Figure 5A and B).

4. Deficiency of Nlrp3 reduces caspase-1 activity of hippocampal immune cells in LPS-induced depressive mice

To further test our hypothesis that brain caspase-1 activity is critical for developing LPS-induced depressive like behaviors, brain hippocampal tissues were assayed FLICA. Following NLRP3 inflammasome activation and assembly, procaspase-1 is known to be cleaved through auto-proteolytic maturation. FLICA, Fluorescent Labeled Inhibitors of CAspases, is known to covalently bind with active site of caspase enzymes. After collecting fresh hippocampal tissue 24 hr after injection, isolated cells were stained with CD45, CD11b and FAM-YVAD-FMK and counted $FLICA^+$ cells out of $CD45^+CD11b^+$ immune cells. We found that caspase-1 activity was abnormally increased in WT mice after LPS-administration, showing that FLICA percentage of PBS-injected mouse was 27.8 % while LPS-

injected mouse was 61.3 %. In case of Nlrp3$^{-/-}$ mice, there was no significant change of FLICA percentage, showing that PBS-injected mouse was 30.4 % while LPS-injected mouse was 38.6 % (Figure 6A and B). We could detect noticeable shift of peaks from PBS group to LPS group in WT mice, while there was no shift of peaks between groups in Nlrp3$^{-/-}$ mice (right side of Figure 6A).

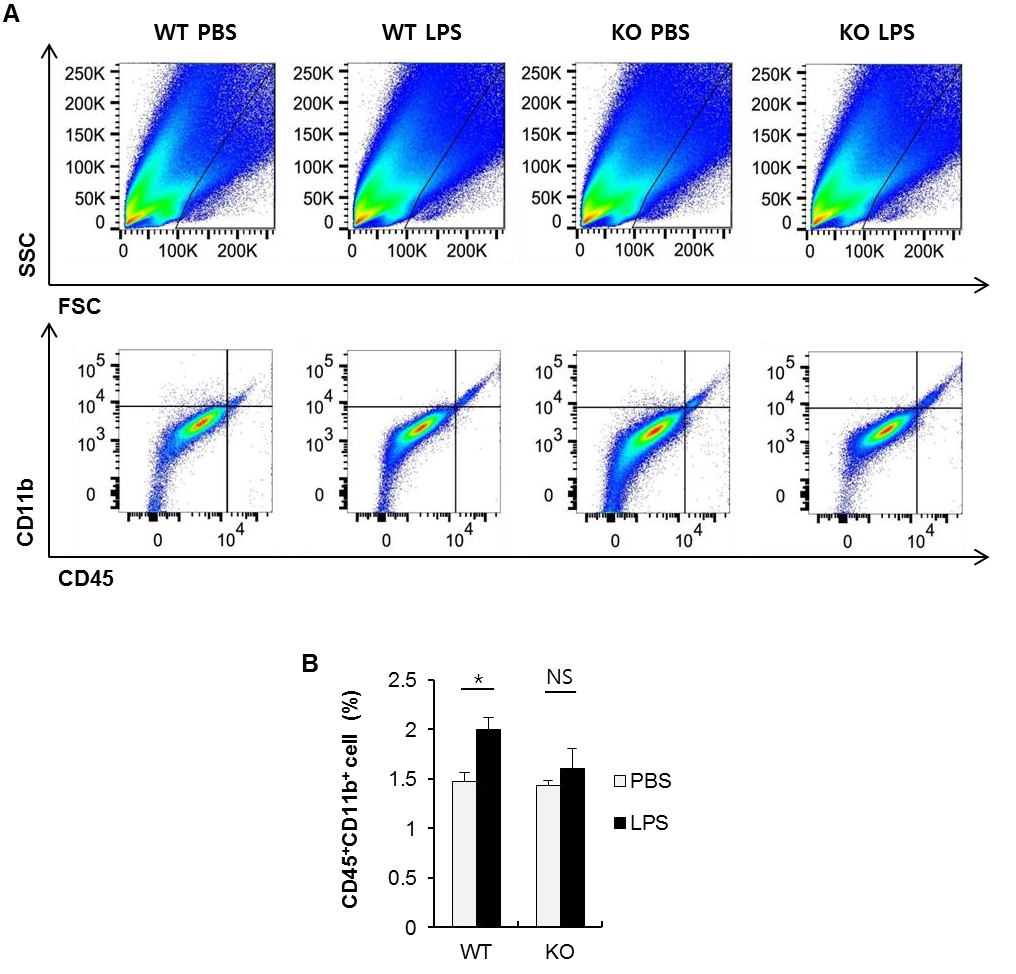

Figure 5. $CD45^{+}CD11b^{+}$ immune cells are abnormally recruited into hippocampus in LPS-administered WT mice but not in $Nlrp3^{-/-}$. Mice were injected i.p with either PBS or LPS (1.8 mg/kg). (A and B) Fresh hippocampus tissue was collected 24 hr after injection and isolated into single cells for staining with CD45 (2 μM) and CD11b (2 μM) for 2 hr, and analyzed by flow cytometry. *p< 0.05 compared to PBS. Values are means ± SEM (n= 4 mice / group).

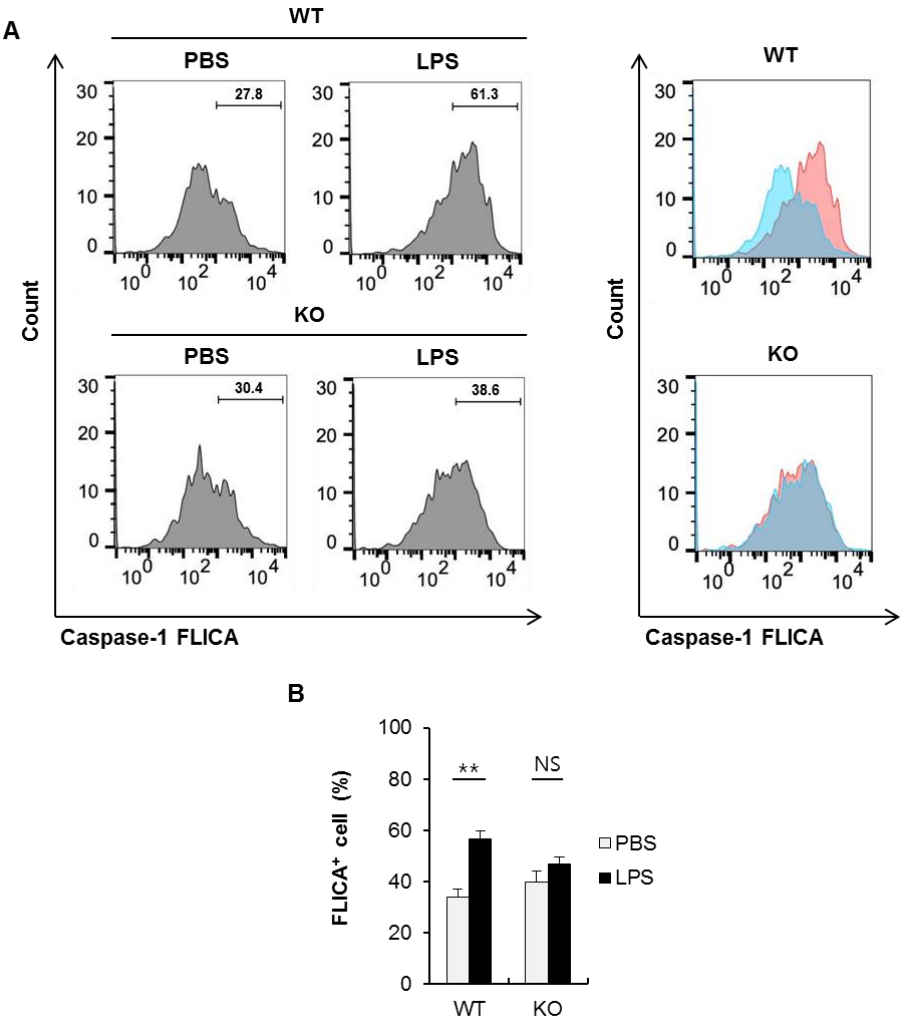

Figure 6. Caspase-1 activity is significantly increased in WT hippocampal immune cells but not in Nlrp3$^{-/-}$. Mice were injected i.p with either PBS or LPS (1.8 mg/kg). (A and B) Fresh hippocampus tissue was collected 24 hr after injection and isolated into single cells for staining with CD45 (2 μM), CD11b (2 μM) and FLICA (30X) for 2 hr, and analyzed with flow cytometry. **$p < 0.005$ compared to PBS. Values are means ± SEM (n= 4 mice / group).

5. Deficiency of Nlrp3 in mice reduces IDO mRNA transcription and enzymatic activity of brain in response to LPS

Indoleamine 2,3-deoxygenase (IDO) is known to be involved in depressive disorder, regulating Kynurenine / Tryptophan (Kyn/Trp) ratio. It has been previously reported that IDO is induced by pro-inflammatory signals such as IL-1β, IL-6 and IFNγ.[3] To assess whether IDO is associated with inflammatory signal cascade, we analyzed pro-inflammatory cytokines and IDO mRNA expression in brain hemisphere (Figure 7). LPS-administration significantly increased expression of IL-1β and IL-6 (Figure 7A and B). As we expected, NLRP3 mRNA level was elevated in LPS-administered mice (Figure 7C). Surprisingly, IDO mRNA expression, which was undetectable in PBS group, was markedly increased upon LPS-administration (Figure 7D). Expression of these cytokines and IDO was specifically increased in medial and occipital region (Figure 7A-D). This result may show a good agreement with previous report that provided evidence of microstructural changes in bipolar disorder patients.[43]

To study whether IDO induction contributes to NLRP3-dependent alteration in depressive like behaviors, we compared mRNA transcripts in brain (medial and occipital regions) of WT and $Nlrp3^{-/-}$ mice. Increase of IDO brain mRNA level was blocked in $Nlrp3^{-/-}$ mice after LPS-administration, while expression of IL-1β and IL-6 was still elevated (Figure 8A). To further investigate whether IDO enzymatic activity is NLRP3-dependent, we compared brain homogenates of two strains (Figure 8B). It is known that IDO is a key factor which initiates kynurenine pathway upon its enzymatic activation, leading to degradation of tryptophan. Corresponding with existing literatures, we found that Kyn/Trp ratio was increased in response to LPS. However, we observed that deficiency of Nlrp3 in mice resulted in reduction of IDO enzymatic activity upon LPS-administration, although P-value could not reach 0.05 (Figure 8B).

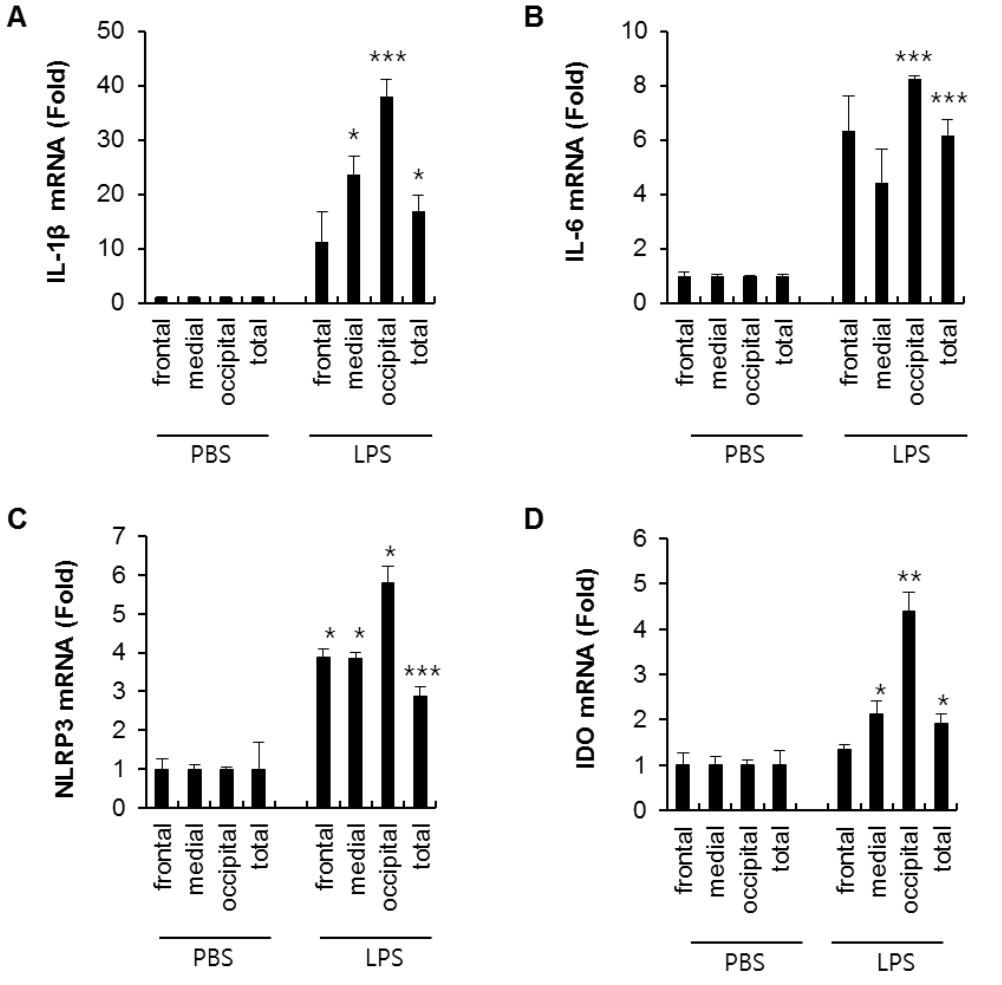

Figure 7. mRNA transcription of pro-inflammatory cytokines and IDO is increased in brains of LPS-induced depressive mice. Mice were injected i.p with either PBS or LPS (1.8 mg/kg). (A-D) Expression of mRNA transcripts in brain hemisphere was determined by quantitative RT-PCR for detection of IL-1β, IL-6, NLRP3 and IDO. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ compared to PBS. Values are means ± SEM (n= 3 mice / group).

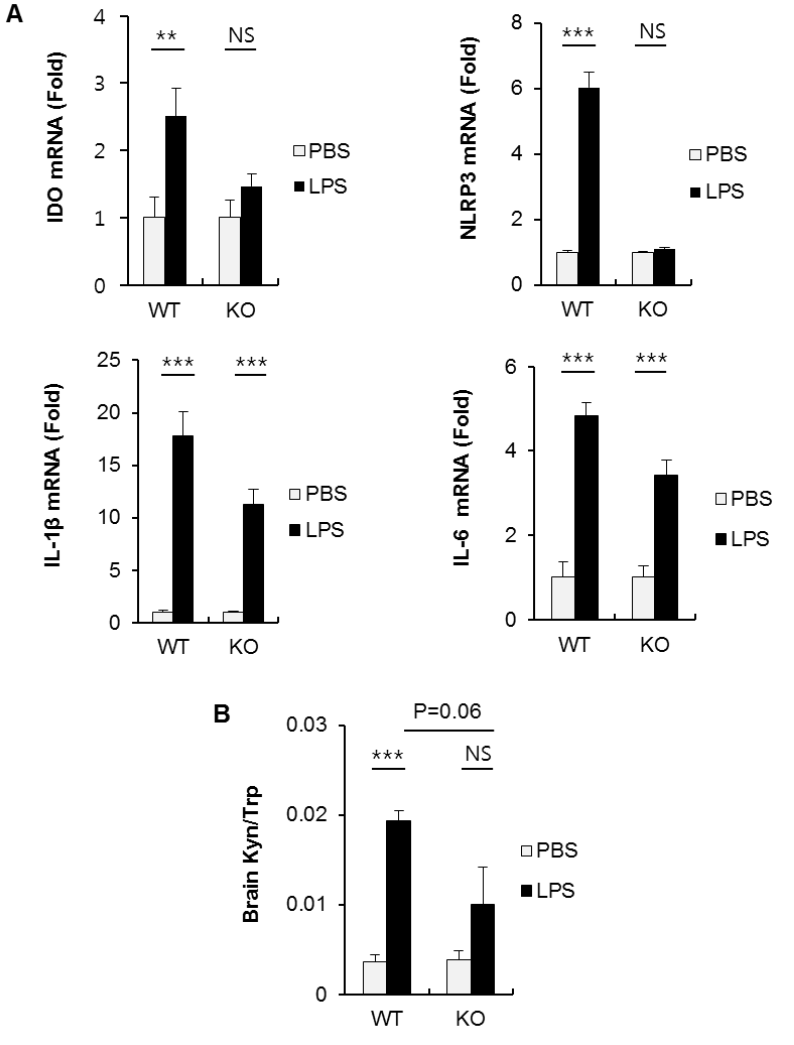

Figure 8. IDO mRNA expression and enzymatic activity are reduced in brain homogenates of Nlrp3$^{-/-}$ mice. Mice were injected i.p with either PBS or LPS (1.8 mg/kg). (A) Expression of mRNA transcripts in brain hemisphere was determined by quantitative RT-PCR for detection of IL-1β, IL-6, NLRP3 and IDO. (B) Brain lysates were analyzed for HPLC. **p< 0.005, ***p< 0.0005 compared to PBS. Values are means ± SEM (n= 4 mice / group).

6. IDO mRNA expression is abnormally up-regulated upon NLRP3 stimulation in primary mixed glial culture

To study whether IDO is directly associated with NLRP3 activation signal, we cultured primary mixed glia (mainly microglia and astrocyte). It is known that triggering LPS-primed cells with NLRP3 activator could lead to assembly of NLRP3 inflammasome complex, maturation of caspase-1, and secretion of IL-1β and IL-18. We primed primary mixed glial cells with LPS and then treated with ATP for activation of caspase-1 (Figure 9A and 9B). We observed that IDO mRNA expression was significantly increased upon LPS plus ATP stimulation (Figure 9A). Immunoblot data of cultured supernatant showed that release of active caspase-1 p20, IL-1β and IL-18 was associated with IDO induction (Figure 9B).

7. Inhibition of caspase-1 activation significantly reduces IDO mRNA expression

To confirm our hypothesis that active caspase-1 p20 increase IDO mRNA expression, we assessed whether IDO mRNA expression restores its basal level upon caspase-1 inhibition. In primary mixed glial culture, inhibition of caspase-1activity with Zvad reduced abnormally-increased IDO level upon LPS plus ATP stimulation (Figure 10A), blocking IL-1β and IL-18 secretion, but not IL-6 (Figure 10B and C). Further, we observed that inhibition of caspase-1 activity with treatment of glibenclamide and NAC, which are known to block potassium (K^+) efflux and inhibit reactive oxygen species (ROS) respectively, reduced IDO mRNA expression (Figure 10D and E).

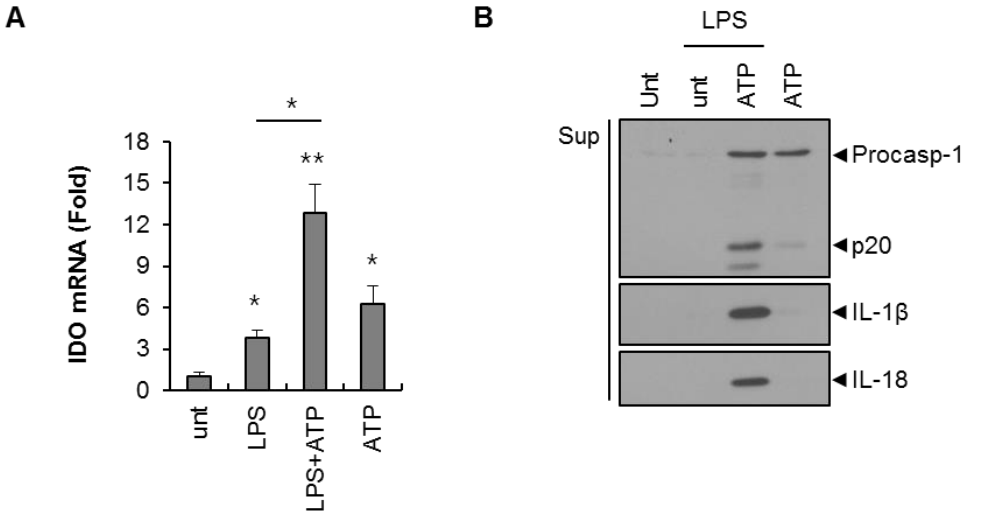

Figure 9. IDO mRNA is increased upon NLRP3 inflammasome stimulation in primary mixed glial culture. Primary glial cells were treated with LPS (0.5 μg/ml) and ATP (2 mM). For induction of IDO mRNA, glial cells were determined by quantitative RT-PCR. *$p < 0.05$, **$p < 0.005$ compare to untreated sample. (B) Culture supernatant was analyzed for immunoblotting. Data are representative of at least three independent experiments.

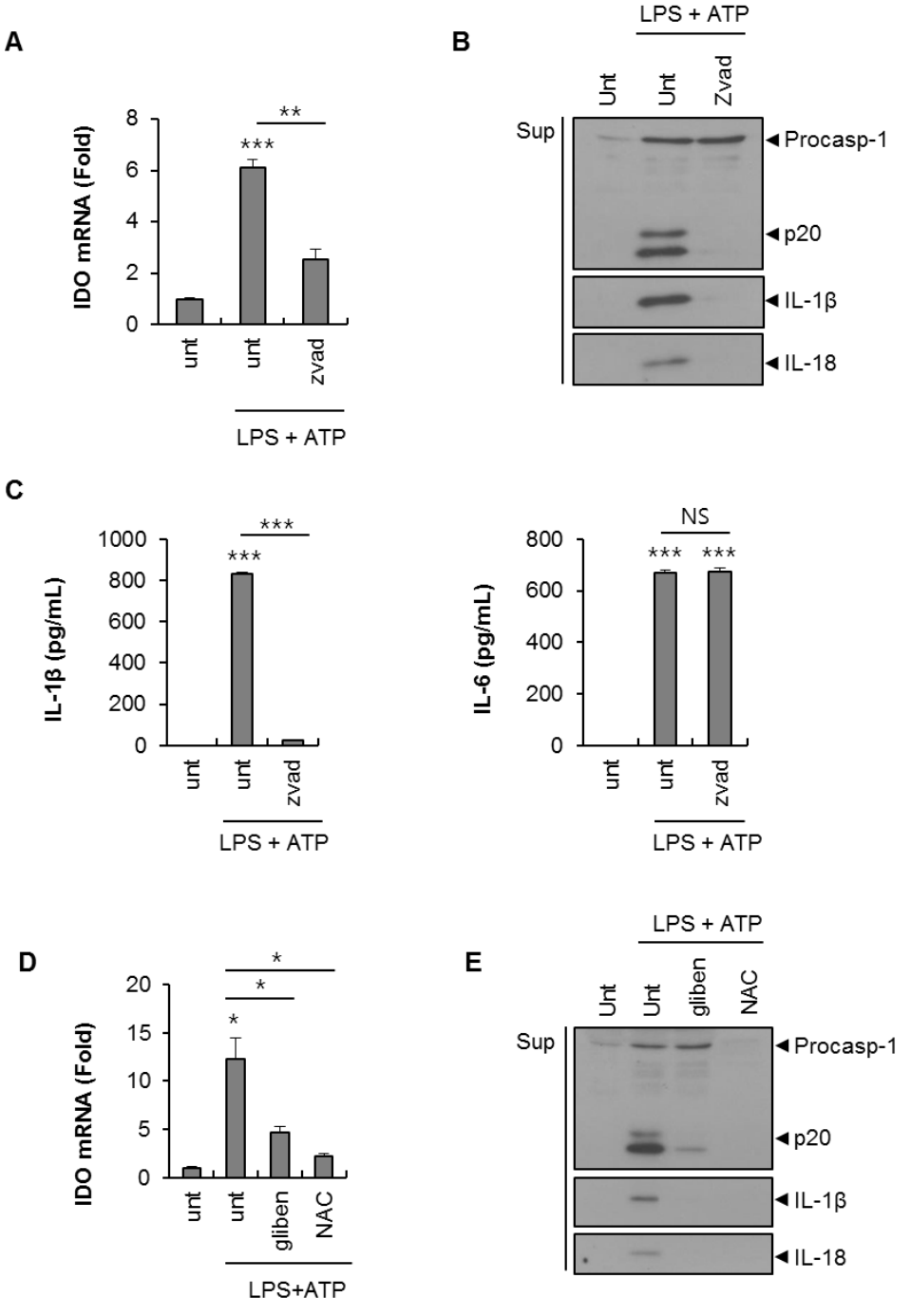
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Figure 10. Caspase-1 inhibition restores IDO mRNA level in primary mixed glial culture. (A) Primary glial cells were primed with LPS (0.5 μg/ml) or LPS plus zvad (40 μM) and then treated with ATP (2 mM). For induction of IDO mRNA, glial cells were determined by quantitative RT-PCR. **p< 0.005, ***p< 0.0005 compared to untreated sample (B) Culture supernatant was analyzed for immunoblotting. (C) Culture supernatant was analyzed for ELISA for detection of IL-1β and IL-6. ***p< 0.0005 compared to untreated sample. (D and E) Primary glial cells were primed with LPS (0.5 μg/ml) or LPS plus glibenclamide (100 μM) or LPS plus NAC (40 mM), followed by ATP treatment (2mM). Data are representative of at least three independent experiments.

8. Recombinant IL-1β and IL-18 have no effect on IDO mRNA expression

IL-1β and IL-18 are known to be maturated by active caspase-1 p20 and then secreted. To assess the direct effect of IL-1β and IL-18 on IDO mRNA expression, primary mixed glial cells were treated with recombinant IL-1β and IL-18. In contrary to our expectation, IL-1β and IL-18 could not induce IDO mRNA expression, although IDO was significantly induced by its positive control IFNγ (Figure 11).

9. Anti-depressant does not inhibit caspase-1 activation but reduces IL-1β secretion via blocking pro-IL-1β production

Previously anti-depressant medication is known to have anti-inflammatory effects. We examined whether anti-depressant could inhibit caspase-1 activation and IL-1β secretion. We used Amitryptyline (AT), which is one of the major tricyclic anti-depressant (TCA). Pretreatment of WTBMM with AT had no effect on caspase-1 cleavage (Figure 12A). However, AT blocked LPS-induced pro-IL-1β production and this led to decrease of IL-1β secretion (Figure 12A and C). This observed inhibitory effect of AT on IL-1β secretion was confirmed in primary mixed glia. In an agreement with results of WTBMM, AT could not inhibit caspase-1 activation but reduced IL-1β secretion via blocking pro-IL-1β production in primary mixed glial culture (Figure 12B and D). Further, we observed AT had no effect on IDO mRNA expression (Figure 12E).

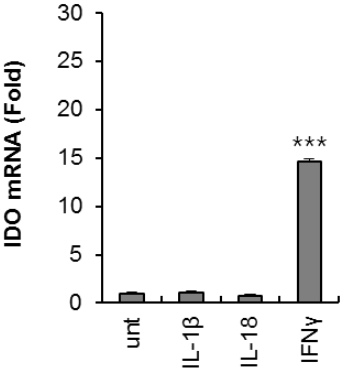

Figure 11. Recombinant IL-1β and IL-18 have no significant effect on IDO mRNA expression. Primary glial cells were treated with IL-1β (20 ng/ml), IL-18 (20 ng/ml) and IFNγ (20 ng/ml). Cells were determined by quantitative RT-PCR for detection of IDO mRNA. ***$p < 0.0005$ compared to untreated sample. Data are representative of at least three independent experiments.

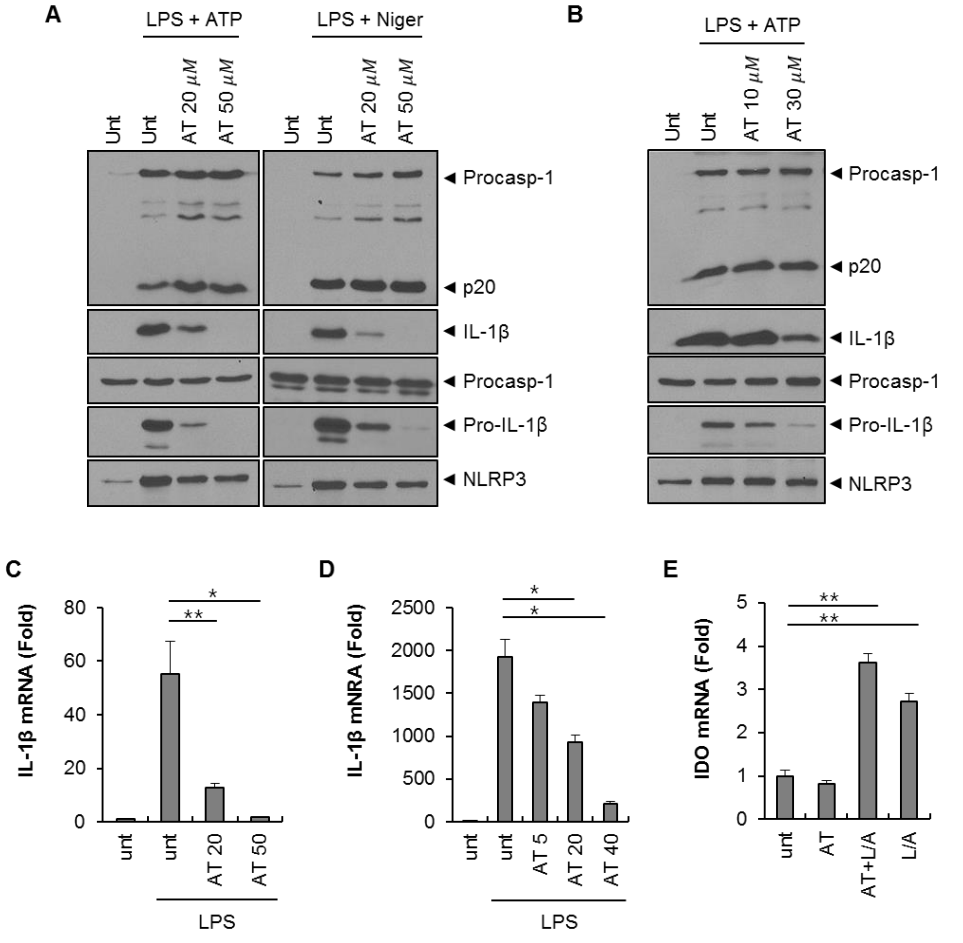

Figure 12. Anti-depressant treatment induces reduction of IL-1β production. (A) WTBMM and (B) Primary mixed glia were primed with *Amitriptyline* (as indicated) and LPS (0.25 μg/ml), followed with ATP (2.5 mM). Medium supernatants and cell lysates were analyzed for immunoblotting with indicated antibody. (C) WTBMM and (D) primary mixed glia were analyzed for induction of IL-1β by quantitative RT-PCR, after treatment of *Amitriptyline* (as indicated) and LPS (0.25 μg/ml). (E) Primary mixed glial cells were primed with AT (30 μM) and LPS (0.5 μg/ml), followed with ATP (2 mM), for analyzing IDO mRNA. *$p < 0.05$, **$p < 0.005$ compared to untreated sample. Data are representative of at least three independent experiments.

Ⅳ. DISCUSSION

This study was designed to analyze whether NLRP3 inflammasome contributes to inflammation comorbid depressive disorder. Further objective of this study was to investigate through what molecular mechanism NLRP3 inflammasome is involved in depressive disorder which is the commonest psychiatric disorder. Further, the results obtained in our animal behavioral study indicate that deficiency of Nlrp3 have protective effect on LPS-induced depressive like behaviors. These behavioral differences of $Nlrp3^{-/-}$ mice could be explained by inhibited recruitment of immune cells in hippocampal region and by reduced caspase-1 activity. Further, IDO expression in brain region is increased NLRP3-dependently in LPS-induced depressive mouse model. In our Primary mixed glial cell study, we observed that caspase-1 activation upon NLRP3 stimuli directly increase IDO expression, which corresponds with our animal study.

As mentioned, inflammation may play a key role in the development of many neurological diseases. Emerging data suggest that NLRP3 inflammasome is involved in pathophysiology of AD and PD.[7-9] With these previous studies, we can postulate that immune activation through NLRP3 activation has a role for many psychiatric diseases including depressive disorder. Although its possibility of implication in various CNS disease, its precise mechanism is still elusive.[14] So understanding the mechanism that NLRP3 inflammasome contribute to neurological disorders and identifying its potential inhibitor would offer a new way to develop a treatment.

IDO is key enzymne key enzyme that mediates kynurenine pathway. Recently, although IDO activation has been proposed to bridge the relationship between inflammation and depression, its precise molecular mechanism remains elusive. So identifying what inflammatory molecules can regulate IDO activity would allow us to find new antidepressant.

Regulation of caspase-1 activity is known to be mainly mediated in microglial cells and infiltrated macrophages in CNS. The present study demonstrated that recruitment of immune cells and caspase-1 activity were increased in hippocampus in LPS-induced depressive mouse. At the same time, our data show that IDO expression was up-regulated in brain with elevation of IL-1β, IL-6 and NLRP3 gene expression. It can be inferred that IDO is induced by NLRP3 inflammasome signals. These in vivo findings were confirmed with our primary glial cell culture, showing that NLRP3 stimulating condition increases IDO gene expression caspase-1 dependently. How active caspase-1 p20 could directly up-regulate IDO gene expression has to be further studied in depth.

It has been repeatedly reported that serum concentrations of IL-1β and IL-18 were increased in depressive disorder patients. We could not detect significant difference of IL-1β in brain homogenates between WT and Nlrp3$^{-/-}$ mice, because using ELISA assay does not allow us to distinct from pro-IL-1β and cleaved IL-1β. Since IL-1β and IL-18 are maturated by active caspase-1 and released, we presumed that these cytokines may regulate IDO expression. However, we observed that recombinant IL-1β or IL-18 could not directly regulate IDO gene level in primary glial cell culture. Moreover, we could not detect IDO induction upon IL-1β and IL-18 treatment in HT22-mouse hippocampal cell line. (data now shown) These findings are contrast to the results of Zunszain et al., who studied IDO induction in human hippocampal progenitor cells. Their results show that IL-1β treatment increased functional IDO enzyme.[44] It has to be still studied whether IL-1β or IL-18 has an effect on IDO level both in glial cells and neurons.

This study has novel findings in that we showed relevance of NLRP3 inflammasome to depressive disorder with comparative study using Nlrp3$^{-/-}$ mouse strain and further pointed out NLRP3 activation directly regulate IDO which is a key enzyme of depression.

Ⅴ. CONCLUSION

Here, we demonstrated that deficiency of Nlrp3 in mice had protective effects on LPS-induced depressive like behaviors. Although there was no difference in pro-inflammatory cytokine levels in brain tissue, $Nlrp3^{-/-}$ mice had lower IL-1β level in peripheral serum. Moreover, we observed that deficiency of Nlrp3 reduced immune cells recruitment and caspase-1 activity in brain hippocampus of LPS-induced depressive mouse. Moreover, we detected that IDO transcription and enzymatic activity were reduced in $Nlrp3^{-/-}$ mice brain after LPS-administration. Mechanistically, in primary mixed glial cell culture, NLRP3 activation up-regulated IDO gene expression caspase-1 dependently, although precise mechanism remains to be further investigated. Collectively all our data provide evidence that NLRP3 inflammasome is closely implicated in inflammation-comorbid depressive disorder via regulating IDO activity in glial cells (Figure 13).

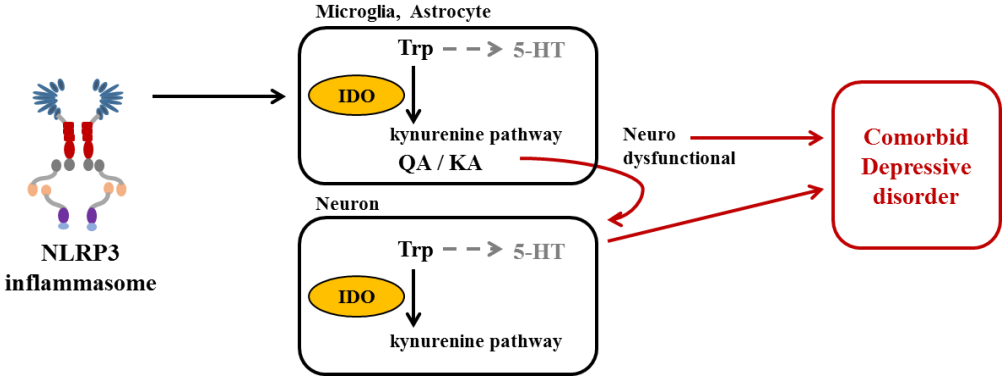

Figure 13. NLRP3 inflammasome is closely implicated in pathophysiological mechanism of depressive disorder.

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ABSTRACT (In Korean)

Lipopolysaccharide 유발 우울증 모델에서 NLRP3 인플라마좀의 연계성 연구

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전 선 아

다양한 요인들에 의해서 체내의 염증반응이 진행되면, 뇌 영역을 포함한 중추신경계도 염증반응에 손상을 받아 뇌 질환의 한 원인이 되는 것으로 알려져 있다. 아직 뇌 염증반응을 유발하는 자극과 기전은 명확하지 않지만, 병원균 유래(PAMP) 또는 손상된 조직에서 유래된(DAMP) 분자적 패턴 및 스트레스 조건은 중추신경계의 염증을 유발하는 것으로 생각되고 있다. 특히 최근 패턴인식수용체 신호경로 중 하나인 인플라마좀(Inflammasome)이 퇴행성 뇌 질환인 알츠하이머 발병에 중요한 역할을 한다고 보고되었다. 이를 통해, 뇌 염증 및 뇌 염증이 관여하는 뇌 질환에서 인플라마좀이 기여할 가능성이 제안되었지만, 아직 많은 연구가 이루어지지 않은 실정이다. 또한 인플라마좀과 우울증 (Depressive disorder)의 발병에 대한 연관성은 아직 보고된 것이 거의 없지만, 말초계 및 중추신경계의 염증반응이 유도되면 신경세포 내의 신경전달물질의 합성이 저해되거나 분해되는 것으로 알려져 있다. 또한 뇌 영역에서

일어나는 아교세포 (Glial cell) 및 대식세포 (Macrophage)의 염증반응 결과로 분비되는 염증성 사이토카인이 신경세포의 비정상적인 기능을 유도하여 뇌 질환을 유발한다고 알려져 있다. 본 연구에서는 NLRP3 인플라마좀의 활성이 우울증세를 유발할 수 있음을 확인하였다. 이는 마우스 행동실험을 통해서, NLRP3 결핍 마우스가 염증반응에 의해 동반되는 우울증세를 유도하였을 때, 정상 마우스보다 경미한 증상을 보임을 확인하였다. 또한, 기존에 우울증 발병에 중요한 역할을 한다고 알려진 뇌 해마(Hippocampus)상에서, 정상 마우스는 우울증세를 유도하였을 때, 더 많은 면역세포들이 모이는 것을 확인하였지만, NLRP3 결핍 마우스는 그렇지 않음을 확인하였다. 또한, 이러한 면역세포들이 발현하는 caspase-1 프로테아제의 활성이 NLRP3 결핍 마우스에서 감소되어 있는 것을 확인하였다. 기존연구에 따르면, Indoleamine 2,3-deoxygenase (IDO) 효소는 세로토닌의 합성을 저해함으로써 우울증을 촉진한다고 알려져 있었다. 본 연구에서는 염증반응에 의해 우울증상이 유도된 마우스에서 IDO 의 유전자발현과 효소활성이 NLRP3 의존적으로 증가되어 있음을 확인하였다. 마우스 뇌에서 분리한 아교세포배양을 통해서, NLRP3 인플라마좀의 활성화가 caspase-1 프로테아제의 활성을 유도함으로써 IDO 의 발현를 증가시킴을 확인하였다. 따라서 본 연구에서는 NLRP3 의 과도한 활성화가 IDO 의 전사를 직접적으로 증가시킴으로써, 우울증세를 유도할 수 있음을 밝혀냈다.

핵심되는 말 : 뇌 염증, 우울증, NLRP3 인플라마좀, 인돌아민 2,3-이산소화효소

PUBLICATION LIST

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