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NLRP3 Inflammasome Contributes to Lipopolysaccharide-induced Depressive-Like Behaviors via Indoleamine 2,3-dioxygenase Induction

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Abstract

Background: Inflammation may play a significant role in the pathogenesis of depression, although the molecular target for the treatment of inflammation-mediated depressive symptoms remains to be elucidated. Recent studies have implicated the NLRP3 inflammasome in various psychiatric disorders, including depression. However, the underlying mechanism by which NLRP3 inflammasome activation mediates the progression of depressive-like behaviors remains poorly understood.
Methods: We examined whether NLRP3 deficiency influenced depressive-like behaviors and cerebral inflammation following systemic administration of lipopolysaccharide in mice. To further assess the contribution of the NLRP3 inflammasome to the progression of depression, we evaluated the effects of NLRP3 signaling on levels of indoleamine 2,3-dioxygenase.
Results: *Nlrp3*-deficient mice exhibited significant attenuation of depressive-like behaviors and cerebral caspase-1 activation in a lipopolysaccharide-induced model of depression. Treatment with the antidepressant amitriptyline failed to block NLRP3-dependent activation of caspase-1, but inhibited lipopolysaccharide-promoted production of interleukin-1β mRNA via suppressing NF-κB signaling in mouse mixed glial cultures. Interestingly, lipopolysaccharide administration produced NLRP3-dependent increases in indoleamine 2,3-dioxygenase expression and activity of mouse brain. Furthermore, inflammasome-activating stimulations, but not treatment with the inflammasome product interleukin-1β, triggered *indoleamine 2,3-dioxygenase* mRNA induction in mixed glial cells.
Conclusions: Our data indicate that the NLRP3 inflammasome is significantly implicated in the progression of systemic inflammation-induced depression. NLRP3-dependent caspase-1 activation produced significant increases in indoleamine 2,3-dioxygenase levels, which may play a significant role in lipopolysaccharide-induced depression. Collectively, our findings suggest that indoleamine 2,3-dioxygenase is a potential downstream mediator of the NLRP3 inflammasome in inflammation-mediated depressive-like behaviors.

Keywords: NLRP3 inflammasome, depression, indoleamine 2,3-dioxygenase

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Significance Statement

Depression is a psychiatric disorder caused by multiple factors, including genetic or environmental influences. Given that conventional antidepressants are frequently ineffective for many patients with depression, further investigation regarding the pathogenic mechanism of depression is required. The NLRP3 inflammasome, which has gained recent attention for its potential role in inflammation-mediated depression, can be activated by a wide range of stimuli such as microbial infections, injured tissue-derived danger signals, and abnormal osmolarity. Thus, psychological or physical stress associated with depression may trigger certain environmental alterations, which in turn activate NLRP3 inflammasome signaling. In the present study, we demonstrated that the NLRP3 inflammasome is significantly implicated in systemic inflammation-mediated depression-like behaviors. Furthermore, we revealed that NLRP3 inflammasome activity is responsible for increases in IDO activity, which has also been implicated in depression. Our data thus highlight the importance of the cerebral NLRP3 inflammasome-IDO axis in the pathogenesis of inflammation-mediated depression.

Introduction

An inflammasome is a cytoplasmic caspase-1-activating multiprotein complex that is composed of a sensor protein such as NLRP3, an adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain, and an effector protein procaspase-1 (Schroder and Tschopp, 2010). Inflammasome components are assembled in the cytosol of myeloid cells only upon the detection of pathogen- or danger signal-derived molecular patterns and intracellular alterations by the sensor protein (Man and Kanneganti, 2016). The assembled inflammasome complex then leads to the activation of caspase-1, which in turn mediates the maturation and secretion of the proinflammatory cytokines interleukin (IL)-1β and IL-18 (Man and Kanneganti, 2016). Activation of inflammasome signaling primarily contributes to host protection against microbial infection (Yu and Lee, 2016). However, deregulated inflammasome activation has been increasingly implicated in the pathogenesis of metabolic or neurodegenerative disorders (Henao-Mejia et al., 2014; Heneka et al., 2014). Recently, much attention has focused on the NLRP3 inflammasome due to its potential association with cerebral inflammation-mediated disorders such as Alzheimer's disease and childhood cerebral adrenoleukodystrophy (Heneka et al., 2013; Jang et al., 2016).

Major depressive disorder is a neuropsychiatric disorder associated with multiple environmental risk factors, including inflammation (Kaufmann et al., 2017). The pathophysiology of major depressive disorder is still poorly understood, but potentially involved in diverse biological symptoms such as monoamine deficiency, an elevated level of cortisol and corticotrophin-releasing hormone, and increased production of cytokines (Belmaker and Agam, 2008). In detail, the production level of proinflammatory cytokines is frequently elevated in patients with major depression (Dowlati et al., 2010). Additionally, patients undergoing type 1 interferon therapy showed a higher risk for psychiatric symptoms such as depression (Kraus et al., 2003), indicating that peripheral inflammation is frequently associated with depressive disorder. On a mechanistic basis, cytokines produced via peripheral inflammation can cross the blood-brain barrier through diffusion or some transporters and activate afferent nerves, possibly contributing to the etiology of psychiatric disorders (Dantzer et al., 2008). Psychological or physical stress may also be involved in the pathophysiology of depression (McEwen, 2005). Of interest, NLRP3 inflammasome may act as a crucial sensor for psychological stress to trigger brain inflammation (Iwata et al., 2016).

Recent evidence further suggests that the NLRP3 inflammasome is implicated in depressive disorders (Alcocer-Gomez et al., 2014; Zhang et al., 2015; Xu et al., 2016). For example, one previous study reported that levels of inflammasome components (e.g., NLRP3 and caspase-1) and inflammasome products (e.g., IL-1β) were significantly increased in peripheral blood mononuclear cells (PBMC) or serum samples from patients with major depressive disorder (Alcocer-Gomez et al., 2014). Furthermore, selective inhibition of caspase-1 by VX-765 or YVAD significantly attenuates depressive-like behaviors triggered by diverse stimulations such as lipopolysaccharide (LPS) injection, chronic mild stress, and estrogen deficiency (Zhang et al., 2014, 2015; Xu et al., 2016). Caspase-1-deficient mice also exhibit a significant reduction in stress-induced depressive-like behaviors (Wong et al., 2016). Moreover, stress-induced depressive phenotypes were markedly attenuated in *Nlrp3*-deficient mice (Alcocer-Gomez et al., 2016), indicating that NLRP3-mediated inflammasome signaling plays a significant role in the progression of depressive-like behaviors. Supporting these findings, antidepressant amitriptyline treatment significantly reduced mRNA expression levels of inflammasome components, including NLRP3 and caspase-1, in PBMCs of patients with major depressive disorder (Alcocer-Gomez et al., 2014). Another study further demonstrated that amitriptyline suppresses the release of IL-1β in LPS-stimulated rat mixed glial cells (Obuchowicz et al., 2006). However, the underlying mechanism by which the NLRP3 inflammasome mediates depressive-like behaviors remains poorly understood. Therefore, in the present study, we examined the role of the NLRP3 inflammasome in LPS-induced depressive-like symptoms using *Nlrp3*-deficient mice.

Our findings indicate that indoleamine 2,3-dioxygenase (IDO) acts as a potent downstream mediator of the NLRP3 inflammasome. IDO catabolizes tryptophan (Trp) into kynurenine (Kyn), which lead to attenuation of neurotransmitter serotonin production (Moore et al., 2000; Walker et al., 2014). Previous studies intriguingly revealed that inflammatory contexts, including LPS-administered mice and interferon α-treated patients, promote the increase in the brain level or activity of IDO (Andre et al., 2008; Walker et al., 2014; Fischer et al., 2015). In addition to the monoaminergic system, IDO-mediated pathways may lead to the production of neurotoxic molecules such as quinolinic acid, which activates N-methyl-D-aspartate (NMDA) receptor and stimulates glutamate release, both of which have been implicated in depression (Walker et al., 2013, 2014; Miller and Raison, 2016). Given the possible involvement of IDO in the inflammation-related depressive disorder, we here present novel findings that NLRP3 inflammasome mediates the upregulation of IDO.

Materials and Methods

Mice

C57BL/6 and *Nlrp3*$^{-/-}$ (C57BL/6 background) mice were obtained from Jackson Laboratory and bred at Yonsei University College of Medicine. All mice were maintained under specific

pathogen-free conditions. Seven- to 10-week-old male mice were used for all experiments. Protocols for the animal experiments were approved by the Institutional Ethical Committee, Yonsei University College of Medicine (2014-0257). All experiments were performed in accordance with the approved guidelines of the Institutional Ethical Committee.

Reagents and Antibodies

LPS, ATP, nigericin, amitriptyline, glibenclamide, and poly dA:dT were purchased from Sigma-Aldrich. Z-VAD-fmk was obtained from Bachem. Recombinant mouse interferon-γ (IFN-γ) and IL-1β were purchased from PeproTech. Recombinant mouse IL-18 was purchased from Sino Biological. Anti-mouse caspase-1 (1:2000) and anti-NLRP3 (1:1000) antibodies were obtained from Adipogen. Anti-mouse IL-1β (1:4000) antibody was from R&D Systems. Anti-IL-18 antibody (1:500) was obtained from Santa Cruz. Anti-phospho-IκB and IκB antibodies (1:1000) were obtained from Cell Signaling.

Depression-Related Behavioral Tests

To induce depressive-like symptoms, mice were i.p. injected with LPS (1.8 mg/kg) or PBS as a control. Systemic administration of LPS causes not only depression-like behavior but also results in cytokine-induced sickness behavior (Dantzer et al., 2008). To minimize the experimental bias caused by sickness response, we performed all the behavioral tests after 24 hours post LPS administration (Remus and Dantzer, 2016). At 24 hours post injection, body weights of mice were measured, and the tail suspension test (TST) was then performed as previously described (Steru et al., 1985; O'Connor et al., 2009). Briefly, mice were suspended by the tail with adhesive tape such that their heads remained approximately 10 cm above the floor for 6 min, and the procedure was recorded using a video camera. Time spent immobile during the last 4 min was counted. At 28 h post-injection, the forced swim test (FST) was then conducted as previously described (Porsolt et al., 1977; O'Connor et al., 2009). Briefly, mice were individually placed in a cylinder (diameter: 15 cm; height: 22 cm) containing 15 cm of tap water maintained at 24°C. Then, mice were video recorded for 6 minutes, and the time spent immobile was analyzed during the last 4 minutes. Immobility was defined as no motion or movement, with the exception of the minimal movement required to float. The sucrose preference test was conducted as follows: Mice were group-housed (2 mice/cage) and habituated to drink 1.5% sucrose solution for 48 hours. After adaptation, mice were deprived of water for 24 hours. Mice were then injected with LPS or PBS and were given both normal water and 1.5% sucrose solution. Consumption of normal and sucrose-containing water was measured per cage at 24 hours post-LPS/PBS injection, and the sucrose preference was calculated as follows: (consumed sucrose water)/(consumed sucrose water + normal water) × 100. Sucrose preference was evaluated in 5 or 6 cages. To measure locomotor activity in the open field, mice were placed individually in an open field arena (43.5 × 43.5 × 30.5 cm) and allowed to explore freely for 20 minutes. Activity was recorded using a camera mounted above the open field arena and analyzed by SMART 2.5 software. The total distance traveled was used to represent locomotor activity.

Cell Cultures

Mouse brain mixed glial cells were prepared from the whole brains of mouse pups on the first postnatal day as previously described (Kim et al., 2013) and cultured in DMEM/F-12 medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin for 2 to 3 weeks. Mouse bone marrow cells were isolated from mouse femurs and differentiated into bone marrow-derived macrophages (BMDMs) with L929-conditioned medium (Fernandes-Alnemri et al., 2010). BMDMs were maintained in L929-conditioned DMEM supplemented with 10% FBS and antibiotics. Mouse hippocampal HT-22 cell lines were maintained in DMEM supplemented with 10% FBS and antibiotics.

Assay of Cerebral Inflammation

After 24 hours post injection, mice were killed by CO_2 asphyxiation. To evaluate levels of central nervous system (CNS)-associated phagocytes, hippocampal slices were isolated from mouse brains, suspended in DMEM supplemented with 10% FBS, and dissociated to single cells using a Pasteur pipette. Cells were then filtrated using a cell strainer, following which they were resuspended in PBS containing 10% FBS and stained with anti-CD45-BV421 (1:50, BD Biosciences) and anti-CD11b-PE antibodies (1:50, eBioscience). Cells were then analyzed via flow cytometry, and $CD45^+CD11b^+$ cells were considered as CNS-associated phagocytes (Cazareth et al., 2014). To quantify the levels of proinflammatory cytokines in the brain, hippocampal slices or whole brain tissues were homogenized using 25 mM Tris-Cl (pH 7.5) containing 1% NP-40, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors. The samples were then sonicated and centrifuged. The soluble fractions were normalized based on protein concentration using Bradford assay and then used to quantify the levels of proinflammatory cytokines via ELISA.

Quantification of mRNA Expression

Quantitative real-time PCR was performed to quantify levels of mRNA expression in the brain. Briefly, total cellular RNA was prepared from mouse brains using a TRIzol reagent (Invitrogen) with homogenization and sonication and reverse transcribed using PrimeScript RT Master Mix (Takara) in accordance with the manufacturer's instructions. Template DNA was amplified by quantitative real-time PCR using SYBR *Premix Ex Taq* II (Takara). The following primers were used: 5′-GGC TAG AAA TCT GCC TGT GC-3′ and 5′-AGA GCT CGC AGT AGG GAA CA-3′ (mouse *Ido*); 5′-GCC CAT CCT CTG TGA CTC AT-3′ and 5′-AGG CCA CAG GTA TTT TGT CG-3′ (mouse *Il-1b*); 5′-AGT TGC CTT CTT GGG ACT GA-3′ and 5′-TCC ACG ATT TCC CAG AGA AC-3′ (mouse *Il-6*); 5'-ATG CTG CTT CGA CAT CTC CT-3' and 5'-AAC CAA TGC GAG ATC CTG AC-3' (mouse *Nlrp3*).

Assay of IDO Activity

To determine the IDO activity of mouse brain, the concentration of kynurenine and tryptophan was measured by high performance liquid chromatography (HPLC) analysis. At 24 hours post injection, mouse brain homogenates were prepared in the lysis buffer containing 25 mM Tris-Cl (pH 7.5), 1% NP-40, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors. Homogenates were normalized by Bradford assay and used to quantify the level of kynurenine and tryptophan by HPLC-MS/MS analysis. Liquid-liquid extraction using water-saturated ethyl acetate was performed, and aqueous layer was injected into HPLC-Tandem mass spectrometry (MS/MS) on phenomenex Kinetex C18 column to separate kynurenine and tryptophan in selective reaction monitoring mode (kynurenine *m/z* 209 > 192;

tryptophan *m/z* 205 > 118). To further assess the enzyme activity of IDO in the hippocampal homogenates of mouse brain, IDO1 activity assay kit (BioVision) was used according to the manufacturer's instruction. One unit of IDO1 activity is the amount of enzyme that generates 1 μmol of detected *N*-formylkynurenine per minute by oxidation of 1 μmol L-tryptophan at 37°C.

Assay of Inflammasome Activation

To activate NLRP3 inflammasome, cells were primed with LPS (0.25 μg/mL) for 3 hours, followed by treatments with ATP (2 mM, 45 minutes) or nigericin (5 μM, 45 minutes). To stimulate absent in melanoma 2 (AIM2) inflammasome, cells were transfected with poly dA:dT (1 μg/mL) using a Lipofectamine 2000 (Invitrogen) for the indicated times according to the manufacturer's protocol. Inflammasome activation was determined by the detection of active caspase-1 p20 and active IL-1β in culture supernatants using immunoblots and by quantification of extracellular IL-1β using the mouse IL-1β Quantikine ELISA kit (R&D Systems). To assess the presence of active caspase-1 in the brain, hippocampal slices were prepared from mouse brains and dissociated into single cells as described above. Cells were then stained with an active caspase-1-specific FAM-FLICA (ImmunoChemistry Technologies) probe in accordance with the manufacturer's protocol. FLICA-positive cells were then assayed via flow cytometry.

Immunoblotting

Cells were lysed in 20 mM HEPES (pH 7.5) buffer containing 0.5% Nonidet P-40 (NP-40), 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and protease inhibitors. Soluble lysates were subjected to SDS-PAGE and then immunoblotted with the appropriate antibodies. For some experiments, culture supernatants from BMDMs or mixed glial cells were collected after inflammasome stimulations, and proteins were precipitated by the addition of a methanol/chloroform mixture as reported previously, following which they were immunoblotted. All blots presented in figures are representative images of at least 3 independent experiments.

Statistical Analysis

All values were expressed as the mean and SE of individual samples. Data were analyzed using 1-way ANOVA followed by Dunnett's posthoc test for comparison of all groups with control group or 2-way ANOVA with Bonferroni posthoc test for comparison between wild-type and *Nlrp3*-deficient mice group. The level of statistical significance was set at $P \leq .05$. Analyses were performed with GraphPad Prism.

Results

NLRP3 Deficiency Attenuates LPS-Induced Depressive-Like Symptoms

To evaluate the role of the NLRP3 inflammasome in inflammation-mediated depression, we i.p. injected LPS into wild-type or *Nlrp3*-knockout mice and conducted behavioral tests as summarized in Figure 1A. Consistent with the findings of previous studies, LPS injection resulted in a significant increase in depression-like behaviors in wild-type mice at 24 and 28 hours post injection, as assessed via the TST ($P < .001$; Figure 1B) and FST ($P < .001$; Figure 1C) (O'Connor et al., 2009; Aguilar-Valles et al., 2014). Compared with wild-type mice, *Nlrp3*-deficient mice exhibited a significant reduction in the time spent immobile in both behavioral tests following LPS administration ($P < .01$, Figure 1B; $P < .001$, Figure 1C). However, sucrose preference decreased in both mouse groups following LPS administration, although this preference was significantly higher in LPS-treated *Nlrp3*-deficient mice than in wild-type mice ($P < .05$; Figure 1D), indicating that inflammation-mediated reductions in sucrose preference may be attenuated by deficiencies in NLRP3. On the other hand, both groups of mice showed a significant impairment of locomotor activity as measured by the total distance traveled via open field test ($P < .001$, wild-type; $P < .001$, *Nlrp3*-knockout) (Figure 1E). Furthermore, there was no significant difference in locomotor activity between wild-type and *Nlrp3*-knockout mice following PBS or LPS injection ($P > .05$). Supporting these observations, both wild-type and *Nlrp3*-knockout mice showed a similar loss in the body weight at 24 hours post LPS treatment ($P < .001$) (Figure 1F). These findings collectively demonstrate that systemic inflammation-induced depressive-like behaviors were significantly attenuated in *Nlrp3*-knockout mice.

NLRP3 Deficiency Attenuates LPS-Induced Caspase-1 Activation in the Brain

To examine whether the observed attenuation in depression-like behaviors in *Nlrp3*-deficient mice resulted from reduced brain inflammation, we first assessed the population of $CD45^+CD11b^+$ cells in the hippocampal region of the mouse brain. $CD45^+CD11b^+$ cells represent CNS-associated phagocytes such as neutrophils or monocytes and are the primary mediators of LPS-induced cerebral inflammation (Cazareth et al., 2014). Indeed, we observed that LPS administration caused a slight but significant increase in the population of CNS-associated phagocytes in the hippocampal region of wild-type mice ($P < .05$) (Figure 2A-B). However, no such increases were observed in *Nlrp3*-deficient mice ($P > .05$). We then measured levels of the proinflammatory cytokine IL-1β, a final product of the inflammasome signaling pathway, in mouse brain homogenates via ELISA. Systemic administration of LPS induced a significant increase in levels of IL-1β in the mouse brain homogenates after 6 and 24 hours of administration ($P < .001$, 6 hours; $P < .05$, 24 hours) (Figure 2C). However, there was no significant difference in the level of IL-1β between the whole brain homogenates of wild-type and *Nlrp3*-deficient mice following LPS administration ($P > .05$) (Figure 2D). On the other hand, the level of IL-1β was significantly elevated in the hippocampal region of wild-type mice ($P < .05$), but not that of *Nlrp3*-knockout mice ($P > .05$) after LPS injection (Figure 2E). Unlike other proinflammatory cytokines, the production of active IL-1β requires unique posttranslational processing of inactive precursor pro-IL-1β by active caspase-1 (Black et al., 1988). As the quantification of IL-1β by ELISA cannot distinguish active IL-1β from immature pro-IL-1β, the detection of active caspase-1 is a much more significant indication of inflammasome activation. We thus examined the activation of caspase-1 in the hippocampal region using an active caspase-1-specific FLICA probe. Intriguingly, LPS administration caused a marked increase in FLICA-positive cells from the hippocampal area of wild-type mice ($P < .001$) (Figure 2F-G). In contrast, the number of active caspase-1-containing cells in the hippocampal region did not significantly increase in *Nlrp3*-deficient mice following LPS administration ($P > .05$) (Figure 2F-G). These findings demonstrate that systemic LPS administration promoted robust cerebral caspase-1 activation in a NLRP3-dependent manner possibly contributing to depression-like behavior.

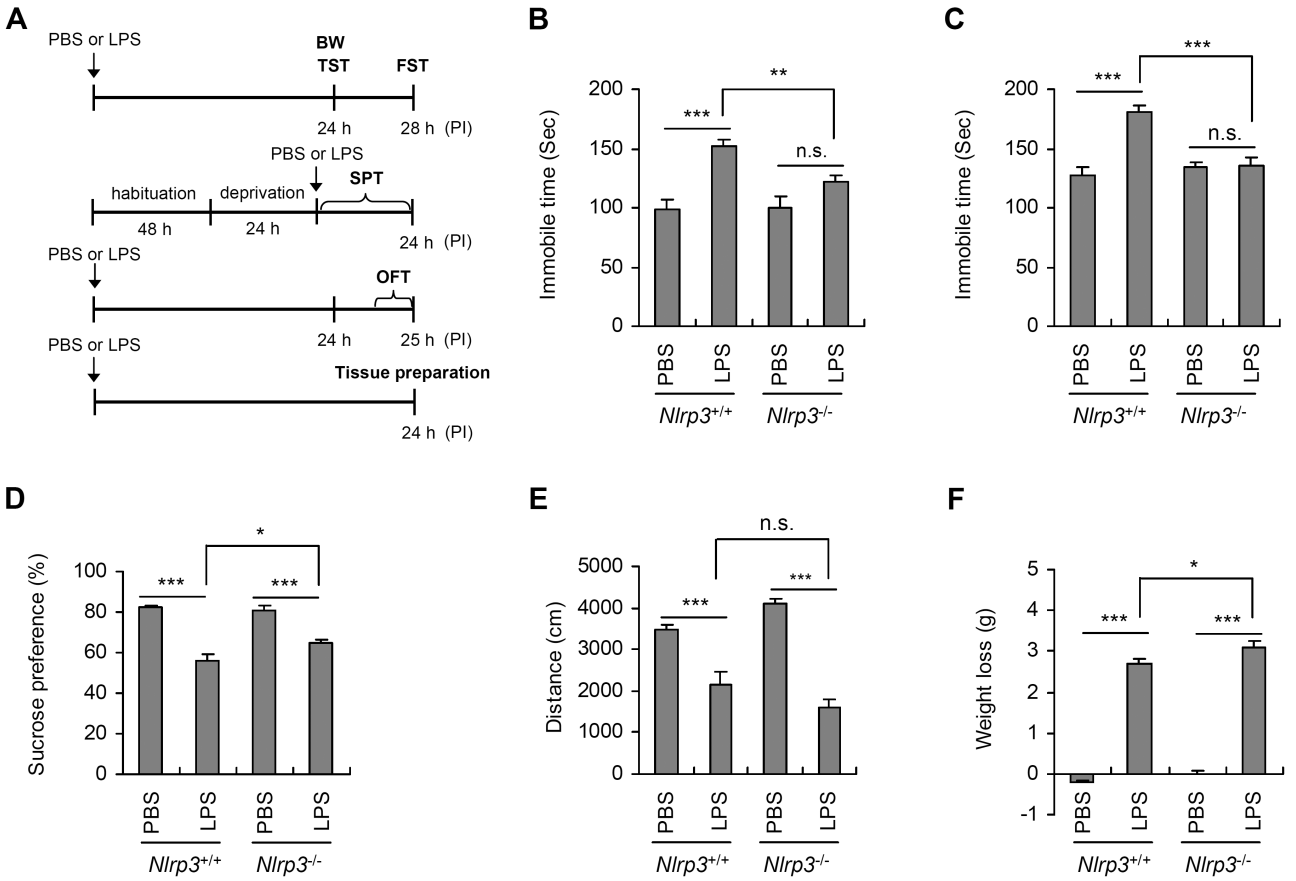

Figure 1. Depression-related behavioral tests in wild-type and *Nlrp3*-deficient mice upon lipopolysaccharide (LPS) administration. (A) Time schedule for behavioral tests of mice injected with PBS or LPS. (B-C) The tail suspension test (TST) (B) and forced swim test (FST) (C) were conducted with PBS- or LPS-administered mice at 24 hours (B) and 28 hours (C) post injection and analyzed. PBS (n = 11), LPS (n = 13). (D) The sucrose preference test (SPT) was conducted with PBS- or LPS-administered mice at 24 hours post administration of PBS (n = 5 cages) or LPS (n = 6 cages). (E) The open field test (OFT) was conducted with PBS- or LPS-administered mice at 24 hours post injection. PBS (n = 6), LPS (n = 6). (F) Body weights (BW) of mice were measured at 24 hours post administration, and the weight loss was displayed. PBS (n = 11), LPS (n = 13). (B-F) Data were analyzed by 2-way ANOVA with Bonferroni posthoc test. Asterisks indicate significant differences. (*P<.05; **P<.01; ***P<.001; n.s., not significant).

Antidepressant Amitriptyline Does Not Inhibit NLRP3-Dependent Caspase-1 Activation but Suppresses LPS-Triggered *IL-1β* mRNA Production

To further elucidate the pathophysiological role of NLRP3-dependent caspase-1 activation in depression, we examined the effects of the tricyclic antidepressant amitriptyline on NLRP3 inflammasome activation. Amitriptyline did not attenuate caspase-1 activation upon stimulation with NLRP3-activating LPS/ATP and LPS/nigericin treatments in mouse mixed glial cells (Figure 3A). Consistent with this observation, amitriptyline failed to inhibit the activation of caspase-1 in mouse BMDMs stimulated with NLRP3 agonists (Figure 3B). Nevertheless, amitriptyline markedly reduced the level of IL-1β in the culture supernatants of mixed glial cells and BMDMs upon NLRP3-activating stimulations as determined by immunoblotting (Figure 3A-B) and ELISA (P<.001) (Figure 3C-D). Indeed, amitriptyline produced a robust inhibition of *IL-1β* mRNA induction in mixed glial cells and BMDMs in response to LPS stimulation (P<.001) (Figure 3E-F). This reduction of *IL-1β* mRNA levels by amitriptyline may explain the observed attenuation in IL-1β secretion from mixed glial cultures or BMDMs upon NLRP3 inflammasome stimulation. Supporting these data, amitriptyline clearly dampened the phosphorylation and degradation of IκB, which are typical indications of NF-κB signaling activation, in BMDMs upon LPS stimulation (Figure 3G). These data demonstrate that the antidepressant amitriptyline may inhibit LPS-triggered *IL-1β* mRNA induction leading to attenuated IL-1β secretion, without influencing NLRP3-mediated caspase-1 activation.

NLRP3 Inflammasome Contributes to the Induction of IDO

Consistent with the findings of a recent study that utilized a chemical stress condition (Alcocer-Gomez et al., 2016), our data also indicate that NLRP3 deficiency attenuated depression-like symptoms in mice upon systemic LPS administration. However, the underlying mechanisms by which NLRP3-mediated inflammation mediates depressive-like behaviors remain poorly understood. Given that IDO may be a potent mediator between inflammation and depression (Andre et al., 2008; O'Connor et al., 2009; Miller and Raison, 2016), we assessed levels of *Ido* mRNA expression in the mouse brain. Intriguingly, LPS administration induced a significant increase in levels of IDO in the brains of wild-type mice (P<.01), but not in those of *Nlrp3*-deficient mice (Figure 4A). Systemic administration of LPS also increased levels of *Nlrp3* mRNA in the brains of wild-type mice (P<.001) (Figure 4B). To determine IDO activity in brain, we calculated the Kyn/Trp ratio in the brain homogenates via HPLC analysis. Consequently, LPS administration led to a significant increase in

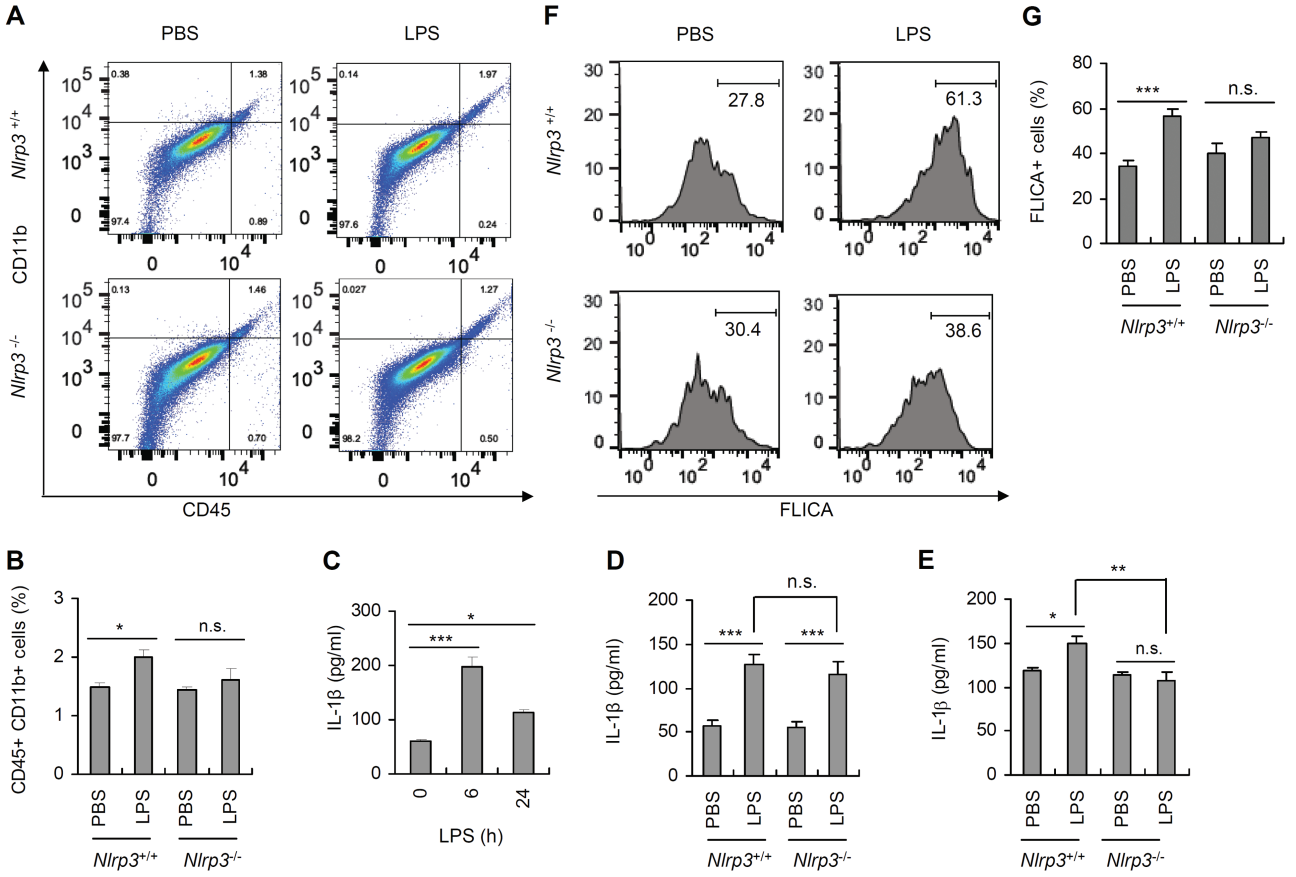

Figure 2. Brain inflammation in wild-type and *Nlrp3*-deficient mice upon lipopolysaccharide (LPS) administration. (A-B) Hippocampal regions were isolated from PBS- or LPS-administered mice at 24 hours post injection and dissociated into single cell suspensions. Cells were then stained with CD11b- and CD45-specific antibodies and analyzed via flow cytometry. The relative percentages of $CD11b^{+}CD45^{+}$ cells are plotted in B ($n = 4$). (C-D) Levels of IL-1β were quantified by ELISA in the brain homogenates of untreated mice and LPS-injected mice after 6 or 24 hours of administration (C, $n = 4$) and of PBS- or LPS-injected mice after 24 hours of administration (D, $n = 8$). (E) Levels of interleukin (IL)-1β were quantified by ELISA in the hippocampal homogenates of PBS- or LPS-injected mice after 24 hours post injection ($n = 4$). (F-G) Hippocampal regions were dissociated into single cell suspension as in (A). Cells were also stained with anti-CD11b antibody, anti-CD45 antibody, and active caspase-1-specific FLICA reagent (carboxyfluorescein-YVAD-FMK), following which they were analyzed via flow cytometry ($n = 4$). (B-E, G) Data were analyzed by 1-way ANOVA with Dunnett's posthoc test (C) or 2-way ANOVA with Bonferroni posthoc test (B, D, E, G). Asterisks indicate significant differences. (*$P < .05$; **$P < .01$; *** $P < .001$; n.s., not significant).

Kyn/Trp ratio in brains of wild-type mice ($P < .001$), while no significant change was observed in *Nlrp3*-deficient mice following LPS injection (Figure 4C). Furthermore, IDO1 enzymatic activity of brain hippocampal homogenates was significantly increased in the LPS-administered wild-type mice ($P < .05$), but not in the *Nlrp3*-deficient mice (Figure 4D).

To determine whether the NLRP3 inflammasome is involved in the induction of *Ido*, we measured levels of *Ido* mRNA in mouse brain mixed glial cultures. Interferon-γ (IFN-γ) was used as a positive control to induce *Ido* mRNA expression ($P < .001$) (Figure 4E). Interestingly, LPS/ATP stimulation produced a significant induction of *Ido* mRNA in mixed glial cultures ($P < .01$, Figure 4E; $P < .001$, Figure 4F), while neither LPS alone nor ATP alone caused the upregulation of Ido ($P > .05$) (Figure 4E-F). This LPS/ATP-mediated induction of *Ido* was not observed in *Nlrp3*-deficient mixed glial cells (Figure 4G), suggesting that the NLRP3 inflammasome is responsible for the induction of *Ido*. On the other hand, antidepressant amitriptyline showed no inhibition on the LPS/ATP-induced upregulation of *Ido* mRNA expression in mixed glial cells (Figure 4H).

Inhibition of Inflammasome/Caspase-1 Suppresses the Production of IDO

To further confirm whether NLRP3-mediated caspase-1/inflammasome activity is required for *Ido* induction, we examined the effect of zVAD, a pan caspase inhibitor, on the expression of *Ido* mRNA. Consequently, zVAD treatment abolished LPS/ATP-promoted *Ido* induction in mouse mixed glial cells ($P < .001$) (Figure 5A). Similarly, treatment with glibenclamide, a chemical blocker of potassium efflux, also significantly reduced the induction of *Ido* mRNA expression triggered by LPS/ATP stimulation ($P < .01$) (Figure 5B). Both inhibitors clearly decreased inflammasome-mediated caspase-1 activation and IL-1β or IL-18 secretion in mixed glial cells (Figure 5C). Indeed, the caspase inhibitor zVAD reduced inflammasome-dependent IL-1β secretion ($P < .001$) but not IL-6 secretion (Figure 5D-E). We thus examined the possibility that IL-1β or IL-18 mediates *Ido* induction. Unlike LPS/ATP stimulation, neither IL-1β nor IL-18 treatment induced the expression of *Ido* mRNA in mixed glial cells (Figure 5F). These data demonstrate that inflammasome-dependent caspase-1 activation, but not the subsequent secretion of IL-1β or IL-18, is a prerequisite for *Ido* induction in mixed glial cells. Next, we examined whether NLRP3 inflammasome-activating stimulation influences the expression of *Ido* in mouse hippocampal neuronal HT-22 cells, as *Ido* induction in neurons may lead to impaired production of serotonin. However, neither NLRP3-stimulating LPS/ATP treatment nor treatment with inflammasome-mediated proinflammatory cytokines induced mRNA expression of *Ido* in HT-22 cells (Figure 5G), indicating that NLRP3 inflammasome activation may induce IDO

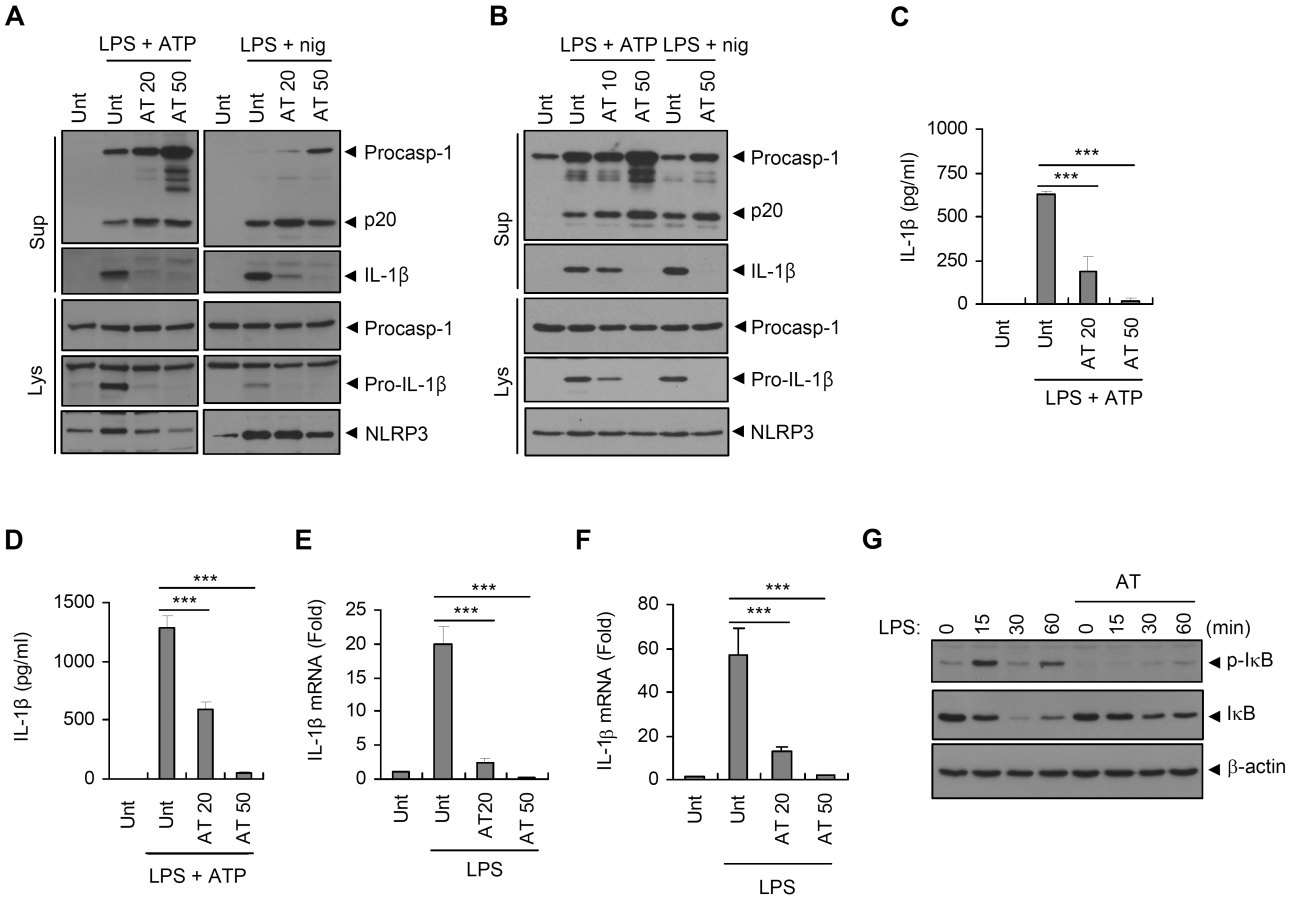

Figure 3. Effect of amitriptyline on NLRP3 inflammasome activation. (A) Mouse brain mixed glial cells were pretreated with amitriptyline (AT, 20 or 50 μM) for 1 hour, then primed with lipopolysaccharide (LPS) (0.25 μg/mL, 3 hours), followed by treatment with ATP (2 mM, 45 minutes) or nigericin (nig, 5 μM, 45 minutes). (B) Mouse bone marrow-derived macrophages (BMDMs) were pretreated with amitriptyline (AT, 10 or 50 μM) for 1 hour, then primed with LPS (0.25 μg/mL, 3 h), followed by treatment with ATP (2 mM, 45 minutes) or nigericin (nig, 5 μM, 45 minutes). (A-B) Culture supernatants (Sup) or cellular lysates (Lys) were immunoblotted with the indicated antibodies. (C-F) Mouse mixed glial cells (C, E) or BMDMs (D, F) were pretreated with amitriptyline (AT, 20 or 50 μM) for 1 hour, then primed with LPS (0.25 μg/mL, 3 hours), followed by treatment with ATP (2 mM, 45 minutes). (C-D) Culture supernatants were assayed for the quantification of IL-1β via ELISA. (E-F) Cellular lysates were assayed for the quantification of IL-1β mRNA via quantitative real-time PCR. (C-F) Data were analyzed by 1-way ANOVA with Dunnett's posthoc test. Asterisks indicate significant differences ($n = 5$, C; $n = 3$, D; $n = 3$, E; $n = 4$, F; ***$P < .001$). (G) Mouse BMDMs were pretreated with amitriptyline (AT, 50 μM) for 30 minutes, followed by the treatment with LPS (0.5 μg/mL) for the indicated times. Cellular lysates were immunoblotted with the indicated antibodies. Unt: untreated.

expression exclusively in mixed glial cells but not in neurons. Of note, the activation of AIM2 inflammasome by poly dA:dT transfection also caused a significant increase in *Ido* mRNA expression in mixed glial cells ($P < .05$, Figure 5H), proposing that other inflammasome activation rather than NLRP3 could induce the upregulation of IDO. Our data thus highlight that IDO may be a downstream mediator of caspase-1/inflammasome pathways in the pathophysiology of depression.

Discussion

Accumulating evidence has demonstrated that the NLRP3 inflammasome is closely implicated in the pathogenesis of many chronic inflammatory or metabolic disorders (Wen et al., 2012; Henao-Mejia et al., 2014). In addition, recent studies have also proposed that NLRP3 inflammasome activation may be implicated in neuroinflammation, neurodegenerative disorders (Heneka et al., 2013; Jang et al., 2016; Zhang et al., 2016), and neuropsychiatric disorders such as depression (Alcocer-Gomez et al., 2014). Intriguingly, a recent study revealed that NLRP3 deficiency clearly abolishes depressive behaviors in mice subjected to immobilization stress (Alcocer-Gomez et al., 2016). Given that diverse stress signals including microbial toxins, particulate matters, and osmotic stress can stimulate the NLRP3 inflammasome (Yu and Lee, 2016), it is highly likely that psychological stress, a major cause of depression, activates NLRP3-dependent inflammasome signaling (Iwata et al., 2016).

In accordance with this finding, our data here indicate that *Nlrp3*-deficient mice were significantly protected from the depressive effects of systemic LPS administration. Previous studies showed that LPS administration to mice causes acute or long-term depression-like behaviors as well as acute sickness behaviors (O'Connor et al., 2009; Lawson et al., 2013a; Zhu et al., 2017). To minimize the overlap with sickness response, we performed behavioral tests after 24 hours of LPS injection similar to experimental designs from previous reports (Dantzer et al., 2008; Remus and Dantzer, 2016). In our data, both wild-type and *Nlrp3*-deficient mice exhibited a similar reduction in the locomotion and weight loss at 24 hours post-LPS administration. Moreover, there was no significant difference in the basal locomotion between these groups, indicating that the different locomotion or sickness behavior may not account for the attenuated depression-like behaviors of *Nlrp3*-knockout mice. Of interest, we found that active caspase-1-containing cells in

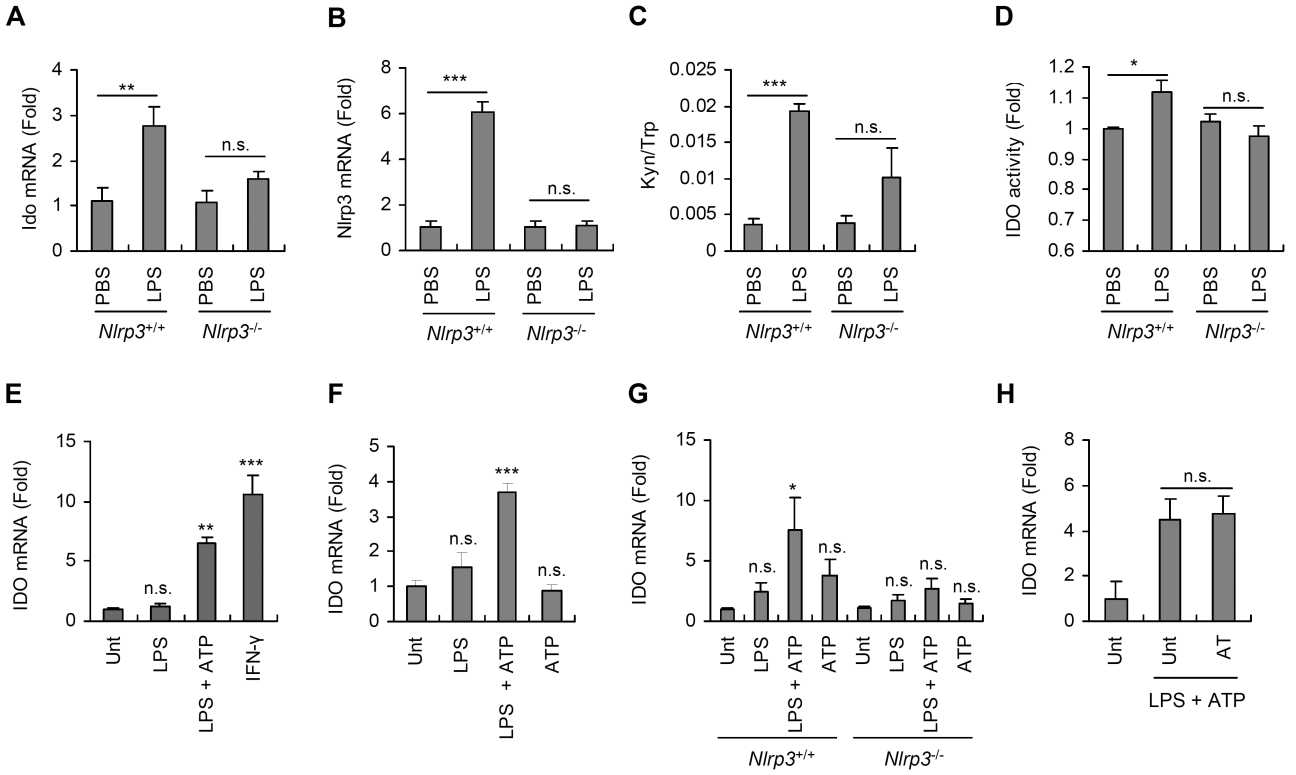

Figure 4. Effect of NLRP3 inflammasome activation on indoleamine 2,3-dioxygenase (IDO) production. (A-C) After 24 hours of PBS or lipopolysaccharide (LPS) administration, mouse brain homogenates were prepared and assayed for *Ido* (A) or *Nlrp3* (B) mRNA levels via quantitative real-time PCR ($n = 4$) or employed for the quantification of kynurenine (Kyn) and tryptophan (Trp) via HPLC analysis (C) ($n = 4$). (D) At 24 hours post PBS or LPS injection, mouse brain hippocampal homogenates were prepared and assayed for IDO1 enzyme activity. IDO1 specific activity of samples was determined as the amount of produced *N*-formylkynurenine per minute per mg protein and expressed as the relative fold value compared with PBS-injected wild type mice ($n = 3$). (E) Mouse mixed glial cells were untreated or treated with LPS alone (0.5 μg/mL, 5 hours), LPS (0.5 μg/mL, 3 hours) followed by ATP (2 mM, 14 hours), or IFN-γ (20 ng/mL, 14 hours) ($n = 4$, LPS alone; $n = 6$). (F) Mouse mixed glial cells were untreated or treated with LPS alone (0.25 μg/mL, 3 hours), LPS (0.25 μg/mL, 3 hours) followed by ATP (2 mM, 30 minutes), or ATP alone (2 mM, 30 minutes). ($n = 3$) (G) Wild-type or *Nlrp3* $^{-/-}$ mice mixed glial cells were treated with LPS alone, LPS followed by ATP, or ATP alone (2 mM, 10 hours) ($n = 6$). (H) Mouse mixed glial cells were pretreated with amitriptyline (AT, 50 μM) for 30 minutes, treated with LPS (0.25 μg/mL, 3 hours), followed by ATP treatment (2 mM, 30 minutes). ($n = 3$) (E-H) Cellular lysates were assayed for the quantification of IDO mRNA via quantitative real-time PCR. Data were analyzed by 1-way ANOVA with Dunnett's posthoc test (E-F, H) or 2-way ANOVA with Bonferroni posthoc test (A-D, G). Asterisks indicate significant differences. (*$P < .05$; **$P < .01$; ***$P < .001$; n.s., not significant). Unt, untreated.

the hippocampus were significantly increased in wild-type but not in *Nlrp3*-knockout mice after LPS administration. These data further provide important evidence that NLRP3 inflammasome activation in the hippocampus actively occurred in our experimental settings. In this regard, the NLRP3 inflammasome may represent a relevant physiological target for the treatment of depressive symptoms. Nevertheless, the mechanism underlying the contribution of NLRP3 inflammasome activation to depressive-like behaviors remains poorly understood.

Emerging evidence has suggested that IDO acts as a central mediator of cytokine-induced depression (O'Connor et al., 2009; Walker et al., 2014; Miller and Raison, 2016) and that systemic LPS administration induces a manifest upregulation of IDO in the hippocampal region (Andre et al., 2008; Walker et al., 2013). Moreover, selective inhibition of IDO1 by 1-methyl-tryptophan significantly attenuates depressive-like behaviors in mice treated with LPS (O'Connor et al., 2009; Lawson et al., 2013b). Given this context, our data provide novel evidence that brain IDO expression and activity is elevated in an NLRP3 inflammasome-dependent manner. Furthermore, NLRP3 inflammasome-activating stimulations remarkably increased the expression of *Ido* in mixed glial cultures. However, this *Ido* induction was not observed following treatment with IL-1β or IL-18, indicating that inflammasome-dependent caspase-1 activation is responsible for the upregulation of IDO. Of note, the transfection of poly dA:dT increased the mRNA expression of *Ido* in mixed glial cells in our data, suggesting that AIM2 inflammasome could also mediate the upregulation of IDO. Further studies are required to more fully elucidate the contribution of caspase-1 signaling to *Ido* induction.

Previous studies have revealed that tricyclic antidepressant amitriptyline attenuates *Nlrp3* mRNA expression in PBMCs and serum IL-1β level in patients with major depressive disorder (Alcocer-Gomez et al., 2014; Alcocer-Gomez et al., 2017). Furthermore, amitriptyline treatment also inhibits the extracellular release of IL-1β from LPS-triggered mixed glial or microglial cells (Obuchowicz et al., 2006). Thus, we examined whether the antidepressant amitriptyline impairs NLRP3-dependent caspase-1 activation. However, we observed that amitriptyline did not abolish ATP or nigericin-triggered NLRP3-mediated caspase-1 activation in BMDMs and mixed glial cells. Consistent with this finding, amitriptyline showed no suppressive effect on the induction of IDO in mixed glial cells in response to NLRP3-activating stimulations. On the other hand, amitriptyline significantly reduced LPS-stimulated *IL-1β* mRNA induction in mixed glial cells. Supporting this finding, we found that amitriptyline clearly inhibited LPS-induced phosphorylation of IκB, a robust indication of NF-κB activation. Our data are consistent with the previous study showing that amitriptyline inhibits toll-like receptor 4 signaling (Hutchinson et al., 2010). These

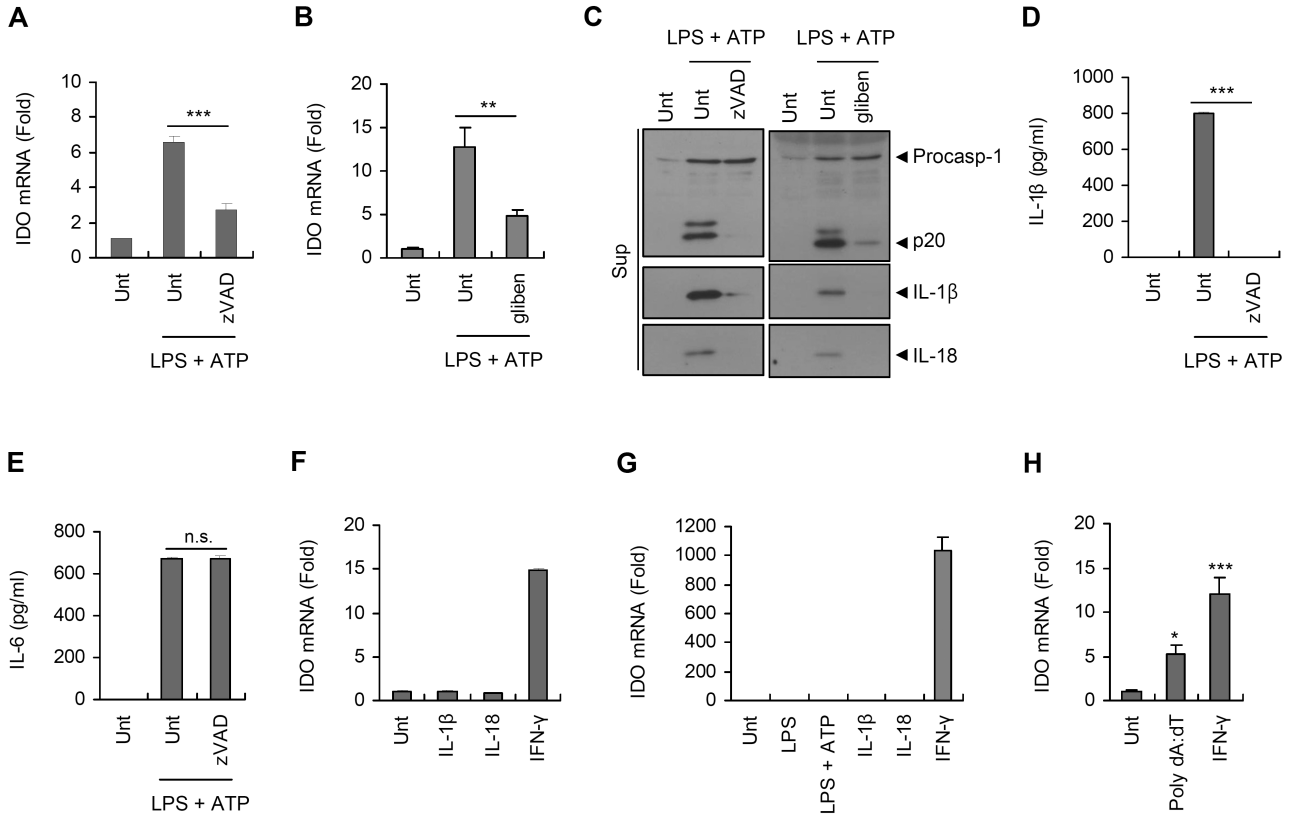

Figure 5. Inhibition of caspase-1/inflammasome attenuates indoleamine 2,3-dioxygenase (IDO) production. (A-C) Mouse mixed glial cells were treated with LPS (0.5 μg/mL) in the presence of zVAD-fmk (40 μM) or glibenclamide (100 μM) for 3 hours followed by ATP (2 mM, 15 hours). (A-B) Cellular lysates were assayed for the quantification of *Ido* mRNA via quantitative real-time PCR ($n = 3$). (C) Culture supernatants were immunoblotted with the indicated antibodies. (D-E) Mouse mixed glial cells were treated with lipopolysaccharide (LPS) (0.5 μg/mL) in the presence of zVAD-fmk (40 μM) for 3 hours followed by ATP (2 mM, 15 hours). Culture supernatants were assayed for the quantification of interleukin (IL)-1β or IL-6 via ELISA ($n = 4$). (F) Mouse mixed glial cells were treated with IL-1β (20 ng/ml), IL-18 (20 ng/ml), or interferon (IFN)-γ (20 ng/mL) for 20 hours ($n = 3$). (G) Mouse hippocampal neuronal HT-22 cells were treated with LPS alone (0.5 μg/mL, 20 hours), LPS (0.5 μg/mL, 3 hours) followed by ATP (2 mM, 20 hours), IL-1β (20 ng/mL, 20 hours), IL-18 (20 ng/mL, 20 hours), or IFN-γ (20 ng/mL, 20 hours) ($n = 3$). (H) Mouse mixed glial cells were transfected with poly dA:dT (1 μg/mL, 14 hours) or treated with IFN-γ (20 ng/mL) for 14 hours ($n = 3$). (F-H) Cellular lysates were assayed for the quantification of *Ido* mRNA via quantitative real-time PCR. Data were analyzed by 1-way ANOVA with Dunnett's posthoc test. Asterisks indicate significant differences (*$P < .05$; **$P < .01$; ***$P < .001$; n.s., not significant). Unt, untreated.

findings thus provide a molecular explanation for the reduced IL-1β production by amitriptyline observed in the previous literature. Interestingly, a recent paper by Alcocer-Gomez et al. (Alcocer-Gomez et al., 2017) suggested that 9 common antidepressants, including amitriptyline, decreased Nlrp3 mRNA level in the blood mononuclear cells of patients with major depressive disorder, possibly via inducing autophagy. It will be interesting to examine whether other antidepressants such as selective serotonin reuptake inhibitors directly inhibit NLRP3-dependent caspase-1 activation and the subsequent IDO induction in the future study. In conclusion, our present data suggest that the NLRP3 inflammasome contributes substantially to inflammation-mediated depressive disorders via increases in brain IDO expression or activity. In terms of the effects on IDO induction, selective inhibition of NLRP3 or caspase-1, rather than blocking of IL-1R, will be a more effective strategy to attenuate inflammation-mediated depressive-like symptoms.

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Statement of Interest

None.

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