TLR4, but Not TLR2, Signals Autoregulatory Apoptosis of Cultured Microglia: A Critical Role of IFN-β as a Decision Maker[1]

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TLRs mediate diverse signaling after recognition of evolutionary conserved pathogen-associated molecular patterns such as LPS and lipopeptides. Both TLR2 and TLR4 are known to trigger a protective immune response as well as cellular apoptosis. In this study, we present evidence that TLR4, but not TLR2, mediates an autoregulatory apoptosis of activated microglia. Brain microglia underwent apoptosis upon stimulation with TLR4 ligand (LPS), but not TLR2 ligands (Pam_3Cys-Ser-Lys_4, peptidoglycan, and lipoteichoic acid). Based on studies using TLR2-deficient or TLR4 mutant mice and TLR dominant-negative mutants, we also demonstrated that TLR4, but not TLR2, is necessary for microglial apoptosis. The critical difference between TLR2 and TLR4 signalings in microglia was IFN regulatory factor-3 (IRF-3) activation, followed by IFN-β expression: while TLR4 agonist induced the activation of IRF-3/IFN-β pathway, TLR2 did not. Nevertheless, both TLR2 and TLR4 agonists strongly induced NF-κB activation and NO production in microglia. Neutralizing Ab against IFN-β attenuated TLR4-mediated microglial apoptosis. IFN-β alone, however, did not induce a significant cell death. Meanwhile, TLR2 activation induced microglial apoptosis with help of IFN-β, indicating that IFN-β production following IRF-3 activation determines the apoptogenic action of TLR signaling. TLR4-mediated microglial apoptosis was mediated by MyD88 and Toll/IL-1R domain-containing adaptor-inducing IFN-β, and was associated with caspase-11 and -3 activation rather than Fas-associated death domain protein/caspase-8 pathway. Taken together, TLR4 appears to signal a microglial apoptosis via autocrine/paracrine IFN-β production, which may act as an apoptotic sensitizer. ***The Journal of Immunology,*** **2005, 174: 6467–6476.**

Toll-like receptors play a critical role in early innate immunity to invading pathogens by sensing microorganisms (1–4). TLRs are evolutionary conserved homologues of the *Drosophila* Toll gene, which recognize structural motifs only expressed on microbial pathogens called pathogen-associated molecular patterns (PAMPs)[3] (5). PAMPs include bacterial DNA, flagellin, and their cell wall components such as LPS, peptidoglycan, and lipopeptides. Stimulation of TLRs by these PAMPs initiates a signaling cascade that involves a number of Toll/IL-1R (TIR) domain-containing adaptor proteins (MyD88, TIR domain-containing adaptor protein (TIRAP)/MyD88 adaptor-like (Mal), TIR domain-containing adaptor-inducing IFN-β (TRIF), TRIF-related adaptor molecule), protein kinases (IL-1R-associated kinase-1, IL-1R-associated kinase-M, MAPK), and other signaling intermediates (TNFR-associated factor 6, Toll-interacting protein) (4, 6–8). This signaling cascade ultimately leads to the activation of NF-κB, which in turn induces the production of proinflammatory cytokines such as TNF-α, IL-1, and IL-12. Recognition of invading pathogens by TLRs and the ensuing production of proinflammatory cytokines are also important in the shaping of acquired immunity that follows. Of 10 mammalian TLRs that have been identified to date, TLR2 and TLR4 are unique in that they can signal apoptosis as well as protective immunity. TLR2 has been shown to play a critical role in the initiation and resolution of inflammation by inducing both activation and apoptosis of macrophages (9–12), whereas the role of TLR4 has been well demonstrated in the signaling of apoptosis in the bacteria-faced macrophages (13–15). TLR2 induced both NF-κB activation and apoptosis (9), and TLR2-initiated apoptosis of THP-1 human monocytic cells was mediated by MyD88, Fas-associated death domain protein (FADD), and caspase-8 (10). On the contrary, in macrophages that encountered a bacterial pathogen, it was TLR4 that acted as a potent inducer of apoptosis, and TRIF rather than MyD88-mediated macrophage apoptosis under this condition (14). In brain microglia, however, a role of TLR2 or TLR4 in the cellular apoptosis has not been studied, which is believed to be an important mechanism for the resolution of inflammation in CNS (16). Nevertheless, the expression of TLRs has been detected in microglia as well as astrocytes (17, 18), and their expression has been shown to be modulated by a variety of internal or external stimuli, including TLR agonists, hypoxia, and proinflammatory

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[3] Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; AICD, activation-induced cell death; BTG1, B cell translocation gene 1; DBD, DNA binding domain; FADD, Fas-associated death domain protein; iNOS, inducible NO synthase; IRF, IFN regulatory factor; LTA, lipoteichoic acid; Mal, MyD88 adaptor-like; PamCSK, (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-*N*-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys_4-OH trihydrochloride; PGN, peptidoglycan; TIR, Toll/IL-1R; TIRAP, TIR domain-containing adaptor protein; TRIF, TIR domain-containing adaptor-inducing IFN-β; UAS, upstream activator sequence; AMC, 7-amino-4-methylcoumarin.

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cytokines (19–22), suggesting an important role of TLRs in the regulation of CNS inflammation.

Microglia are CNS resident phagocytic cells, which function as brain macrophages (23). They migrate to area of injured nervous tissue, and they engulf and destroy microbes and cellular debris (24), as do macrophages in periphery. They also play a pivotal role in brain inflammation by producing a variety of inflammatory mediators (25). Activation of microglia may be intended to protect neurons at first. However, activation of microglial cells and inflammatory products derived from them has been also implicated in the neuronal destruction commonly observed in various neurodegenerative diseases (26). Therefore, the autoregulatory mechanisms that control the microglial activation may exist in vivo, and the failure of these autoregulatory mechanisms may be in part responsible for the deleterious effects of microglial activation. Thus, the elucidation of molecular mechanisms underlying the autoregulation of microglial activation may enhance our understanding of pathogenesis of neurodegenerative diseases. Recently, activated macrophages have been shown to undergo apoptosis (27–29). It has been suggested that the apoptosis of activated macrophages is one mechanism whereby an organism may regulate immune and inflammatory responses involving macrophages (29). We and others have demonstrated that a similar regulatory mechanism exists for microglial cells (30–32) and astrocytes (33) as well. We have previously shown that microglial cells and astrocytes undergo apoptosis upon inflammatory activation in a manner similar to activation-induced cell death (AICD) of lymphocytes (31, 33). Inflammatory stimuli such as LPS and IFN-γ played a multiple role in the microglial apoptosis: they not only induced cytotoxic NO production, but also initiated NO-independent apoptotic pathway via induction of caspase-11 (34) or antiproliferative B cell translocation gene 1 (BTG1) (35). Although downstream signaling events of autoregulatory microglial apoptosis have been partly elucidated, early signaling events proximal to plasma membrane are largely unknown. As increasing evidence indicates that TLRs are responsible for the initiation of cellular activation and apoptosis signaling in macrophages, we hypothesized that TLRs may also be involved in AICD of microglia. Among 10 TLRs identified, we focused on TLR2 and TLR4, because these two have been previously implicated in the macrophage apoptosis. In this study, we report that autoregulatory apoptosis of activated microglia is mediated through TLR4, but not TLR2. Also presented are the early signaling events following the apoptotic TLR4 activation and the important differences in the signaling pathways between TLR2 and TLR4 in microglia.

Materials and Methods

Reagents and plasmids

LPS from *Escherichia coli* 0111:B4 prepared by phenolic extraction and gel filtration chromatography was obtained from Sigma-Aldrich. A synthetic lipopeptide (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-*N*-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys_4-OH trihydrochloride (Pam_3Cys-Ser-Lys_4; PamCSK) was from Calbiochem. Peptidoglycan (PGN) from *Staphylococcus aureus* and lipoteichoic acid (LTA) from *Bacillus subtilis* were purchased from InvivoGen. Recombinant mouse IFN-β, mouse IFN-γ, neutralizing anti-mouse IFN-β Ab, and anti-mouse IFN-γ Ab were purchased from R&D Systems. Anti-Fas mAb (CH11) was purchased from Upstate Biotechnology. All other chemicals were obtained from Sigma-Aldrich, unless stated otherwise. Dominant-negative mutants of TLR2 (P681H) and TLR4 (P712H) in pDisplay (Invitrogen Life Technologies) were generously provided by L. Hajjar at University of Washington (Seattle, WA) (36–38). Dominant-negative mutant of MyD88 lacking death domain was generated by RT-PCR-based cloning, as described (39, 40), and dominant-negative mutant of TIRAP/Mal (P125H) was generated by RT-PCR-based cloning, followed by site-directed mutagenesis using QuickChange site-directed mutagenesis kit (Stratagene), as described (41). The occurrence of desired mutation was confirmed by nucleotide sequencing. Dominant-negative mutant of TRIF (P434H) and TRIF-ΔNΔC that lacks both N-terminal and C-terminal portion cloned in pEF-BOS (42) were gifts from S. Akira at Osaka University (Osaka, Japan) (43).

Mice and cells

Mice with a targeted mutation in the TLR2 gene were kindly provided by S. Akira at Osaka University (44), and bred in a virus-free facility at the Samsung Medical Center (Seoul, Korea). As TLR2-deficient mice were generated by microinjection of the targeted ES clones into C57BL/6 blastocysts, age-matched groups of C57BL/6 mice were used as wild-type control. C3H/HeJ mice with TLR4 point mutation to replace proline with histidine at position 712 were purchased from SLC. Primary microglial cells were prepared as previously described with minor modifications (31, 45). In brief, forebrains of newborn wild-type, TLR2-deficient, or C3H/HeJ mice were chopped and dissociated by trypsinization and mechanical disruption. The cells were seeded into poly(L-lysine)-coated flasks. After in vitro culture for 10 days, microglial cells were detached by rapid and gentle shaking of the culture flasks and seeded into plastic surfaces. After additional 1-h incubation, nonadherent cells were removed by replacing culture medium. The purity of microglial cultures was greater than 92%, as determined by isolectin B4 staining (data not shown). BV-2 mouse microglial cell line originally developed by V. Bocchini at University of Perugia (Perugia, Italy) (46) was generously provided by E. Choi at Korea University (Seoul, Korea). The cell line was maintained in DMEM supplemented with 5% FBS, 2 mM glutamine, and penicillin-streptomycin (Invitrogen Life Technologies). Jurkat human lymphoblastic T cell line was obtained from American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, and penicillin-streptomycin.

Assessment of cytotoxicity by MTT assay and TUNEL staining

For MTT assay, cells (3×10^4 cells in 200 μl/well) were seeded in 96-well plates and treated with various stimuli for the indicated time periods. After the treatment, the medium was removed and MTT (0.5 mg/ml) was added, followed by incubation at 37°C for 2 h in CO_2 incubator. Afterward, supernatants were carefully removed and DMSO was added to the cells. After insoluble crystals were completely dissolved, absorbance at 540 nm was measured using Thermomax microplate reader (Molecular Devices). Apoptosis of microglia was scored by TUNEL assay using a commercially available kit, according to the manufacturer's protocol (In Situ Cell Death Detection Kit, peroxidase; Roche Applied Science). The percentage of TUNEL-positive cells was quantitated by counting cells in 10 random microscope fields.

Nitrite quantification

After cells (3×10^4 cells in 200 μl/well) were treated with activating agents in 96-well plates, NO_2^- in culture supernatants was measured to assess NO production in microglial cells. A total of 50 μl of sample aliquots was mixed with 50 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) in 96-well plate and incubated at 25°C for 10 min. The absorbance at 550 nm was measured on a microplate reader. $NaNO_2$ was used as the standard to calculate NO_2^- concentrations.

RNA analysis

Total RNA was extracted from BV-2 cells or primary microglial cells by a sequential addition of 4 M guanidinium thiocyanate, 2 M sodium acetate, and acid phenol/chloroform. Reverse transcription was conducted using Superscript (Invitrogen Life Technologies) and oligo(dT) primer. PCR amplification using specific primer sets was conducted at 55°C annealing temperature for 30–40 cycles. Nucleotide sequences of the primers were based on published cDNA sequences (Table I).

Flow cytometry

Microglia that were treated with stimuli were detached with trypsin-EDTA and incubated with rat anti-mouse TLR4 mAb (Imgenex) for 30 min at 4°C. The cells were stained with 10 μg/ml FITC-conjugated anti-rat IgG (Zymed Laboratories). After washing in PBS containing 2% FCS and 0.02% sodium azide, flow cytometry was performed using FACSCalibur (BD Biosciences).

Native PAGE and Western blot analysis

The native PAGE assay was performed, as previously described, with slight modifications (47). In brief, after protein concentration in cell lysates was determined using Bio-Rad protein assay kit, an equal amount of protein for each cell lysate in native PAGE sample buffer (62.5 mM Tris-Cl,

Table I. *DNA sequences of the primers used for RT-PCR*

Mouse cDNAs	Primer Sequences	GenBank Accession Numbers	RT-PCR Product Size
TLR2	Forward 5′-ACA GCT ACC TGT GTG ACT CTC CGC C-3′ Reverse 5′-GGT CTT GGT GTT CAT TAT CTT GCG C-3′	AF165189	602 bp
TLR4	Forward 5′-ACC TGG CTG GTT TAC ACG TC-3′ Reverse 5′-CAG GCT GTT TGT TCC CAA AT-3′	AF110133	455 bp
MD2	Forward 5′-GTT CTG CAA CTC CTC CGA TG-3′ Reverse 5′-GCG GTG AAT GAT GGT GAA ATT C-3′	AB018550	400 bp
CD14	Forward 5′-CTG ATC TCA GCC CTC TGT CC-3′ Reverse 5′-GCA AAG CCA GAG TTC CTG AC-3′	X13987	454 bp
IRF-3	Forward 5′-AAC AAT GGG AGT TCG AGG TG-3′ Reverse 5′-TCC TTG TGG ACC TCT CCA TC-3′	MN_016849	340 bp
IFNβ	Forward 5′-TCC AAG AAA GGA CGA ACA TTC G-3′ Reverse 5′-TGA GGA CAT CTC CCA CGT CAA-3′	NM_010510	314 bp
Caspase-11	Forward 5′-CTT CAC AGT GCG AAA GAA CT-3′ Reverse 5′-GGT CCA CAC TGA AGA ATG TCT GGA GAA GCA TTT CA-3′	Y13089	275 bp
β-actin	Forward 5′-ATC CTG AAA GAC CTC TAT GC-3′ Reverse 5′-AAC GCA GCT CAG TAA CAG TC-3′	X03672	287 bp

pH 6.8, 15% glycerol, and 1% deoxycholate) was separated by 7.5% native PAGE and transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with 5% skim milk and sequentially incubated with primary Abs and HRP-conjugated secondary Ab (anti-rabbit IgG; Amersham Biosciences), followed by ECL detection (Amersham Biosciences). The primary Abs used were rabbit polyclonal anti-mouse IFN regulatory factor-3 (IRF-3) Ab (Zymed Laboratories), rabbit polyclonal anti-mouse/human caspase-3 Ab specific for the cleaved caspase-3 (Cell Signaling Technology), and rabbit polyclonal anti-mouse/human caspase-8 Ab (BD Pharmingen).

Transient transfection

BV-2 cells in six-well plates were cotransfected with 1 μg of various dominant-negative constructs together with 0.2 μg of *lac*Z gene (pCH110; Pharmacia) using lipofectAMINE reagent (Invitrogen Life Technologies), according to the supplier's instructions. At 24 h after the transfection, the cells were treated with LPS for 48 h. The cells were then fixed with 0.5% glutaraldehyde for 10 min at room temperature and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal; 1 mg/ml) in 4 mM potassium ferricyanide/4 mM potassium ferrocyanide/2 mM magnesium chloride at 37°C for the detection of blue cells expressing *lac*Z. Alternatively, in some experiments, Jurkat cells were cotransfected with 1 μg of dominant-negative mutant of FADD lacking its N-terminal 79 aa (provided by D. Goeddel at Tularik, South San Francisco, CA) (48, 49) along with 0.2 μg of *lac*Z gene. At 48 h after the transfection, the cells were treated with anti-Fas Ab (CH11) for 12 h. The cells were then fixed and stained, as described above. At least 250 blue cells were counted for each experiment, and transfection efficiency was 16–23%. The percentage of apoptotic cells was based on the morphology of blue cells coexpressing dominant-negative mutants and *lac*Z. Dark blue and condensed cells were considered to be apoptotic. In all experiments, empty vectors that do not harbor the dominant-negative mutant cDNAs of interest were used as a control, and the total amounts of plasmids transfected remained constant.

Reporter assays

NF-κB reporter activity was measured using dual-luciferase reporter assay system (Promega). In brief, BV-2 cells in 12-well plates were cotransfected with 0.5 μg of NF-κB-responsive reporter gene construct carrying two copies of κB sequences linked to firefly luciferase gene (IgGκ NF-κB luciferase; generously provided by G. Rosen, Stanford University, Stanford, CA) (50) together with 0.1 μg of *Renilla* luciferase gene under HSV thymidine kinase promoter (pRL-TK; Promega) using LipofectAMINE reagent (Invitrogen Life Technologies). At 24 h after the transfection, cells were treated with stimuli. After 5 h, activities of firefly luciferase and *Renilla* luciferase in transfected cells were measured sequentially from a single sample using dual-luciferase reporter assay system (Promega). Results were presented as firefly luciferase activity normalized to *Renilla* luciferase activity. For the measurement of IRF-3 reporter activity, a hybrid protein (GAL4/IRF-3) consisting of yeast GAL4 DNA binding domain (DBD) fused to IRF-3 lacking its own DBD was used. Reporter gene expression from the GAL4 upstream activator sequence (UAS) in this system requires IRF-3 activation. BV-2 cells were cotransfected with 0.5 μg of p55UAS$_G$Luc carrying GAL4 UAS linked to firefly luciferase gene, 0.5 μg of pEF-GAL4/IRF-3 (kindly provided by T. Fujita at Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) (47, 51), and 0.1 μg of pRL-TK. At 48 h after the transfection, cells were treated with stimuli for 4 h. Activities of firefly luciferase normalized to *Renilla* luciferase in transfected cells were measured. In some experiments, BV-2 cells were cotransfected with p55UAS$_G$Luc and pEF-GAL4/IRF-3 along with dominant-negative TLR2 or TLR4.

EMSAs

Nuclear extracts were prepared from microglial cells, as previously described (52). Synthetic double-strand oligonucleotides of consensus NF-κB-binding sequence, GAT CCC AAC GGC AGG GGA (Promega), were end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extract was incubated with the labeled probe in the presence of poly(dI-dC) in a binding buffer containing 20 mM HEPES at room temperature for 30 min. DNA-protein complexes were resolved by electrophoresis in a 5% nondenaturing polyacrylamide gel, dried, and visualized by autoradiography.

Assessment of caspase activity

Caspase-3- or -8-like activities were measured using a caspase assay kit (BD Pharmingen), according to the supplier's instruction. In brief, caspase-3 or -8 fluorogenic substrates (Ac-DEVD-7-amino-4-methylcoumarin (AMC) or Ac-IETD-AMC) were incubated with LPS-treated cell lysates for 1 h at 37°C, then AMC liberated from Ac-DEVD-AMC or Ac-IETD-AMC was measured using a fluorometric plate reader with an excitation wavelength of 380 nm and an emission wavelength of 420–460 nm.

Statistical analysis

All data were presented as means ± SEM from three or more independent experiments. Statistical comparison between different treatments was done by either Student's *t* test or one-way ANOVA with Dunnett's multiple comparison tests using GraphPad Prism program (GraphPad). Differences with p value <0.05 were considered statistically significant.

Results

The expression of TLR2, TLR4, MD2, and CD14 in microglia

The expression of TLRs in BV-2 mouse microglia and primary microglia cultures was assessed by RT-PCR (Fig. 1). Both TLR2 and TLR4 were expressed in BV-2 cells as well as in primary microglia cultures. The levels of TLR2 or TLR4 expression were not significantly influenced by LPS or IFN-γ treatment within 6 h (Fig. 1*A*). This was confirmed by the flow cytometric analysis, in which the surface expression of TLR4 proteins was not significantly affected by LPS or IFN-γ (Fig. 1*B*). Only the combination of LPS and IFN-γ enhanced the TLR4 expression after 24-h treatment, which is known to cause a massive cell death (31, 34). Both BV-2 cells and primary microglia cultures also expressed MD2 and CD14, which are known to play an important role in TLR4 signaling. The expression of MD2 or CD14 was not significantly affected by LPS or IFN-γ either (Fig.

1*A*). Although LPS and a major macrophage-activating lymphokine IFN-γ have been previously shown to modulate the expression of TLRs in macrophages, endothelial cells, and other cell types (21, 22, 53, 54), the expression of TLR2, TLR4, MD2, or CD14 was not affected by these stimuli alone in microglia under the current experimental condition.

Induction of microglial apoptosis by TLR4, but not TLR2, agonists

As we have previously shown on the basis of nuclear condensation and DNA ladder that LPS induces microglial cell death by apoptosis (31, 34), the viability of microglia was first assessed to compare the apoptosis-inducing effects of the TLR2 and TLR4 agonists. In contrast to TLR4-activating LPS, which induced microglial cell death, TLR2 agonists such as synthetic lipopeptide PamCSK, PGN, or LTA did not induce a significant cell death (Fig. 2*A*). LPS-induced microglial apoptosis was confirmed by TUNEL staining (Fig. 2*C*), in which the extent of microglial apoptosis correlated well with the reduction of the viability measured by MTT assay. These TLR agonists, however, all induced NO production in a concentration-dependent manner, indicating that these agonists can activate microglia with varying magnitude (Fig. 2*B*). Studies using TLR2-deficient or TLR4 mutant mice confirmed that LPS-induced microglial apoptosis is indeed mediated by TLR4. LPS-induced cell death occurred in microglia isolated from TLR2-deficient mice, but not from TLR4 mutant C3H/HeJ mice (Fig. 2*D*). The results obtained from primary microglia cultures were reassessed in BV-2 cells, a pure microglial cell line, to rule out the effects of possibly contaminated astrocytes or oligodendrocytes in our microglia culture preparation. BV-2 cell death was induced by LPS, but not by PamCSK. Both LPS and PamCSK strongly induced NO production (Fig. 3*A*). Transfection of dominant-negative mutant of TLR4, but not TLR2, inhibited LPS-induced cell death, further supporting that TLR4 signals LPS-induced microglial apoptosis (Fig. 3*B*). The dominant-negative mutant form of TLR2 (P681H) or TLR4 (P712H) contains a single missense mutation that converts a cytoplasmic proline residue to histidine. These mutants have been previously demonstrated to act in a dominant-negative fashion (36, 38). We next investigated whether TLR2 or TLR4 agonist induces NF-κB activation in microglia, because many TLRs commonly induce proinflammatory cytokine production via NF-κB activation. Both LPS and PamCSK strongly induced NF-κB activation in BV-2 cells, as demonstrated by NF-κB-responsive reporter assays (Fig. 3*C*) and EMSAs (Fig. 3*D*). Inhibition of NF-κB by a proteasome inhibitor MG-132 (34) attenuated the LPS-induced microglial apoptosis (47.6% inhibition), indicating that NF-κB is proapoptotic: LPS (100 ng/ml for 48 h), 34.7% apoptosis; LPS + MG-132 (0.5 μM), 18.2% apoptosis as determined by TUNEL staining.

TLR4-mediated activation of IRF-3 and expression of IFN-β in microglia: a critical role of IFN-β in TLR4-mediated microglial apoptosis

Next, we sought to determine the differences between TLR2 and TLR4 signaling in microglia to better understand the mechanisms

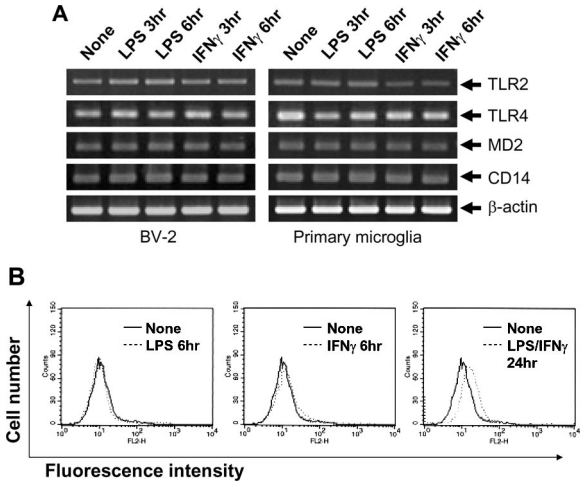

FIGURE 1. The expression of TLR2, TLR4, MD2, and CD14 in microglia. *A*, The expression of TLRs, MD2, and CD14 in BV-2 mouse microglia cells and primary microglia cultures was assessed by RT-PCR. All of them were expressed in microglia, which were not significantly influenced by LPS (100 ng/ml) and IFN-γ (5 U/ml) treatment within 6 h. In primary microglia cultures, the expression of TLR4 seemed slightly decreased by LPS at 3 h in this particular experiment. However, the difference was not consistently observed in the repeated experiments. The results shown are representative from four independent experiments. Mouse β-actin was used as an internal control in RT-PCR. The amplified products were not observed in RT-PCR without reverse transcriptase (data not shown). *B*, The expression of TLR4 protein was analyzed by flow cytometry. BV-2 cells were treated with LPS (100 ng/ml) or IFN-γ (5 U/ml) for 6 h, and then the surface expression of TLR4 protein was measured using flow cytometer. Cells treated with LPS plus IFN-γ for 24 h are shown for comparison. Treatment of microglia with LPS or IFN-γ alone for 6 h did not affect the TLR4 expression. The combination of LPS and IFN-γ induced the up-regulation of TLR4 after 24 h; however, this results in a marked cell death.

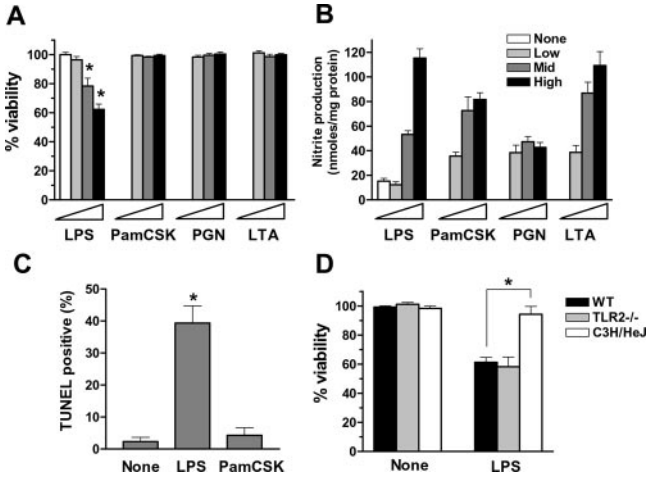

FIGURE 2. Induction of microglial apoptosis by TLR4, but not TLR2, agonists. *A* and *B*, After primary microglia cultures were incubated with increasing concentrations of various stimuli for 72 h, cell viability (*A*) or nitrite production (*B*) was assessed by MTT assay or Griess reaction, respectively. The low, mid, and high concentrations of the stimuli were: 1, 10, and 100 ng/ml for LPS; 10, 100, and 1000 ng/ml for PamCSK; 1, 10, and 100 μg/ml for PGN; and 0.1, 1, and 10 μg/ml for LTA. The viability of untreated cells was set to 100%. The results in this and all similar experiments were repeated several times, and one representative done in triplicates is shown. Values represent mean ± SEM. All treatments except for 1 ng/ml LPS induced a statistically significant production of nitrite ($p < 0.05$). *C*, LPS-induced cytotoxicity was due to apoptosis, as determined by TUNEL staining. After primary microglia cultures were treated with either LPS (100 ng/ml) or PamCSK (1000 ng/ml) for 72 h, the extent of apoptotic cell death was determined by TUNEL staining. *D*, Primary microglia cultures prepared from wild-type (WT), TLR2-deficient ($TLR2^{-/-}$), or C3H/HeJ mice were similarly treated with LPS (100 ng/ml) for 72 h, and then the viability was assessed by MTT assay. Asterisks indicate statistically significant differences from untreated control (*A* and *C*) or significant difference between the two treatments (*D*) ($p < 0.05$).

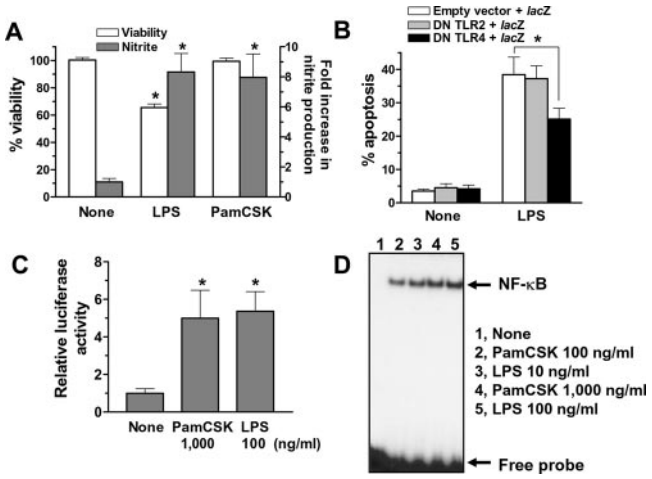

FIGURE 3. TLR-mediated apoptosis and NF-κB activation in BV-2 microglial cells. *A*, After BV-2 cells were treated with either LPS (100 ng/ml) or PamCSK (1000 ng/ml) for 48 h, cell viability or nitrite production was assessed by MTT assay or Griess reaction, respectively. *B*, BV-2 cells were transiently cotransfected with dominant-negative (DN) TLR2 or TLR4 along with *lacZ*, and then treated with LPS for 48 h before the assessment of apoptosis by counting and morphological evaluation of *lacZ*-expressing blue cells. The pDisplay was used as an empty vector. *C* and *D*, NF-κB-activating activity of LPS or PamCSK was measured by NF-κB reporter assay (*C*) or EMSA (*D*) after 5- or 1-h treatment, respectively. Asterisks indicate statistically significant differences from untreated control (*A* and *C*) or significant difference between the two treatments (*B*) ($p < 0.05$).

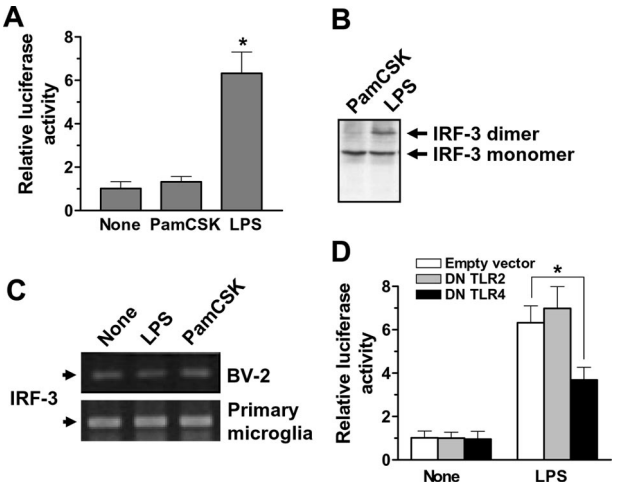

FIGURE 4. TLR4-mediated activation of IRF-3 and expression of IFN-β in microglia. *A*, After treatment of BV-2 cells with LPS (100 ng/ml) or PamCSK (1000 ng/ml) for 4 h, GAL4/IRF-3 reporter activity was measured to assess IRF-3 activation. *B*, LPS-induced IRF-3 activation was confirmed by native PAGE, followed by the detection of IRF-3 dimer at 2 h after the stimulation of primary microglia cultures by Western blot analysis. *C*, Both BV-2 cells and primary microglia cultures expressed IRF-3 gene, which was not significantly influenced by treatment with LPS or PamCSK for 2 h. *D*, LPS-induced IRF-3 reporter activity was inhibited by cotransfection of BV-2 cells with dominant-negative (DN) TLR4, but not DN TLR2. The pDisplay was used as an empty vector. Asterisks indicate statistically significant differences from untreated control (*A*) or significant difference between the two treatments (*D*) ($p < 0.05$).

of TLR4-mediated microglial apoptosis. First, we used IRF-3 reporter system to evaluate the effects of TLR stimulation on IRF-3 activation. The reporter system uses a hybrid protein GAL4-IRF-3 consisting of the yeast GAL4 DBD fused to IRF-3 lacking its own DBD (47, 51). Reporter gene expression from the GAL4 upstream activation sequence in this assay requires IRF-3 activation. LPS stimulation resulted in the activation of GAL4/IRF-3 fusion protein in BV-2 microglia, whereas PamCSK treatment was without effect (Fig. 4*A*). LPS-induced IRF-3 activation was also demonstrated in primary microglia cultures by native PAGE assay, followed by Western blot analysis, which detected dimerized endogenous IRF-3 representing specific serine phosphorylation (Fig. 4*B*). When activated, IRF-3 forms a dimer with a slowly migrating pattern in native PAGE (47). Although LPS markedly induced IRF-3 dimerization, the effect of PamCSK was minimal. The expression of endogenous IRF-3 gene was shown by RT-PCR in BV-2 cells as well as in primary microglia cultures (Fig. 4*C*). The levels of IRF-3 mRNA were not significantly affected by LPS or PamCSK. The experiments using dominant-negative mutant forms of TLR2 or TLR4 also confirmed that LPS-specific IRF-3 activation is mediated by TLR4 (Fig. 4*D*). Transfection of dominant-negative TLR4, but not TLR2, inhibited LPS-induced IRF-3-responsive reporter activity. When IFN-β production was next analyzed, TLR4-specific response was again observed. TLR4 agonist LPS, but not TLR2 agonist PamCSK, strongly induced IFN-β gene expression in BV-2 cells as well as in primary microglia cultures (Fig. 5*A*). Taken collectively, TLR4, but not TLR2, appears to induce IRF-3 activation and IFN-β production in microglia, and this may be the basis of TLR4-specific induction of microglial apoptosis. To further test this possibility, rIFN-β and neutralizing Ab against IFN-β were used to evaluate the role of IFN-β in microglial apoptosis. IFN-β Ab partly abolished the apoptosis-inducing TLR4 signaling: LPS-induced reduction of cell viability was reversed by IFN-β Ab treatment (Fig. 5*B*). Neutralizing Ab against IFN-γ, which was used as a control, did not have such an effect. More importantly, addition of exogenous rIFN-β rendered microglia sensitive to TLR2-mediated cell death (Fig. 5*C*). When microglia were treated with PamCSK in the presence of rIFN-β, a significant cell death took place, indicating that IFN-β production plays a critical role in TLR-mediated microglial apoptosis. IFN-β alone, however, did not induce a significant cell death.

MyD88 and TRIF as adaptors involved in apoptotic signaling of TLR4

TLR signal transduction is mediated by adaptor proteins (6, 7). MyD88, which harbors a TIR domain and a death domain, has been shown to link TLRs and MyD88-dependent downstream events leading to proinflammatory cytokine production. Recent studies, however, revealed that other adaptors exist to mediate MyD88-independent pathways (6, 7). TIRAP/Mal and TRIF have been found to mediate TLR signaling independent of MyD88. Thus, in the current study, we examined which adaptor molecule(s) mediates the apoptosis-inducing TLR signaling in microglia using dominant-negative mutant forms of these adaptor genes (Fig. 6). Transfection of BV-2 cells with dominant-negative MyD88 or TRIF significantly inhibited the TLR4-induced apoptosis, suggesting that both MyD88-dependent and -independent pathways mediate the TLR4-induced microglial apoptosis, and TRIF plays an important role in the MyD88-independent pathway. In contrast, transfection of the cells with dominant-negative TIRAP did not significantly affect the TLR4-mediated apoptosis. Dominant-negative activities of all of these adaptor mutants used have been previously reported (41, 43).

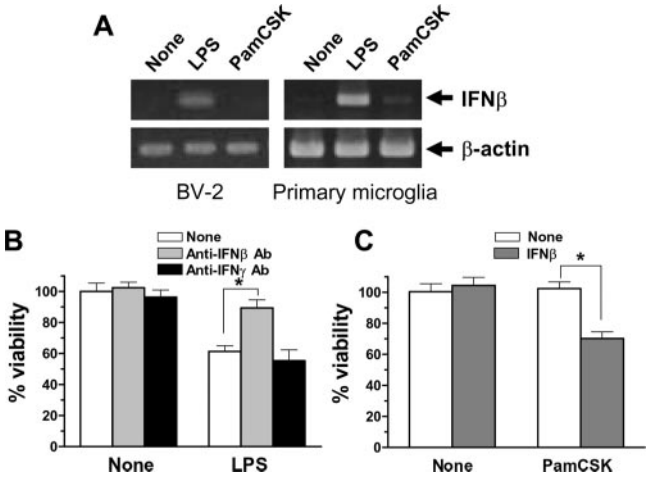

FIGURE 5. Critical role of IFN-β in TLR4-mediated microglial apoptosis. *A*, The expression of IFN-β was induced by LPS (100 ng/ml), but not PamCSK (1000 ng/ml), in BV-2 cells as well as in primary microglia cultures after 2-h treatment. *B*, Primary microglia cultures were treated with LPS in the presence of either neutralizing anti-IFN-β Ab (10 μg/ml) or anti-IFN-γ Ab (10 μg/ml) for 72 h, and then cell viability was measured by MTT assay. *C*, After primary microglia cultures were treated with PamCSK (1000 ng/ml) for 72 h in the absence or presence of mouse rIFN-β (100 U/ml), cell viability was similarly assessed. Asterisks indicate statistically significant differences between the two treatments ($p < 0.05$).

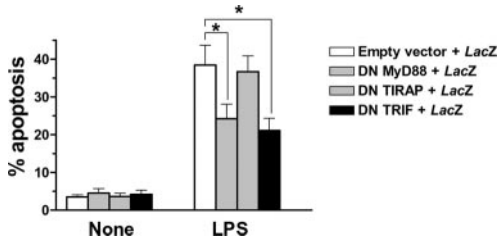

FIGURE 6. MyD88 and TRIF as adaptors involved in apoptotic signaling of TLR4. BV-2 cells that were cotransfected with dominant-negative (DN) mutants of MyD88 lacking death domain, TIRAP (P125H), or TRIF-ΔNΔC harboring only the TIR domain, along with *lacZ* were treated with LPS (100 ng/ml) for 48 h, and then the extent of apoptosis was assessed by counting *lacZ*-expressing blue cells with apoptotic morphology. *, Statistically significant differences between the two treatments ($p < 0.05$). The pEF-BOS was used as an empty vector.

Involvement of caspase-11/caspase-3 pathway in TLR4-mediated microglial apoptosis

We have previously shown that inflammatory stimuli initiate two separate apoptotic pathways in microglia: they induced production of autocrine toxic mediator (NO) in an IRF-1-dependent manner, and they concurrently initiated NO-independent apoptotic pathways through caspase-11 induction (34). Up-regulation of caspase-11 expression alone was sufficient for initiation of apoptotic pathways in microglial cells through autoactivation of caspase-11, followed by the activation of downstream executioner caspase (caspase-3) without involving caspase-8. Recent studies, however, have shown that both TLR2 and TLR4 signal for apoptosis in monocytes/macrophages via a pathway involving FADD and caspase-8 (10, 14). Thus, we examined whether caspase-11 or FADD (or both) is involved in the TLR4-mediated microglial apoptosis. TLR4 agonist LPS, but not TLR2 agonist PamCSK, induced caspase-11 expression in microglia (Fig. 7*A*). LPS-induced caspase-11 induction was not observed in microglia isolated from C3H/HeJ mice with TLR4 mutation. TLR2 deficiency, however, did not affect this response. In addition, caspase-3, but not caspase-8, was activated by TLR4 in microglia, as determined by the cleavage of fluorogenic substrates specific for either caspase-3 (Ac-DEVD-AMC) or caspase-8 (Ac-IETD-AMC) (Fig. 7*B*). The results were confirmed by Western blot analysis, in which a cleaved caspase-3, but not cleaved caspase-8, was detected in the LPS-treated microglia (Fig. 7*C*). Microglia from TLR4 mutant C3H/HeJ mice were used as a control, in which TLR4-mediated caspase-3 activation was markedly suppressed (Fig. 7*B*). In the next set of experiments, possible involvement of FADD in TLR4-mediated microglial apoptosis was evaluated (Fig. 7*D*). Transfection of dominant-negative mutant of FADD did not significantly inhibit TLR4-mediated microglial apoptosis. The FADD-inhibiting effect of the dominant-negative mutant was validated by the inhibition of Fas-mediated apoptosis in Jurkat cells. Taken together, these results indicate that TLR4 signals microglial apoptosis through caspase-11/caspase-3 rather than FADD/caspase-8.

Discussion

AICD is an autoregulatory mechanism for the immune system to remove unwanted activated immune cells after making appropriate use of them. Although the mechanism of AICD has been first found in lymphocytes (55, 56), recent works indicated that both microglial cells (31) and astrocytes (33) in CNS might be under the control of a similar regulatory mechanism. Elimination of activated microglial cells by apoptosis is believed to be a mechanism of the microglial population control in CNS (16). In contrast to AICD of T lymphocytes, in which Fas-Fas ligand interaction plays a central role, neither Fas-Fas ligand interaction nor TNF-α is important in AICD of microglial cells. Instead, NO produced by activated microglial cells themselves is one of the major cytotoxic mediators (31). However, the presence of NO-independent cytotoxic mechanism has been also reported. Our previous work indicated that inflammatory stimuli play a multiple role in AICD of microglial cells (34) and astrocytes (57). They not only induced the indirect apoptotic pathway via autocrine/paracrine production of NO, but also initiated the direct apoptotic pathway through the induction of caspase-11 (34) or antiproliferative BTG1 gene (35). Although IRF-1 and NF-κB were involved in NO-dependent apoptosis of microglial cells mainly by mediating NO synthesis (via inducible NO synthase (iNOS) induction), caspase-11 induction and its subsequent activation were required for NO-independent apoptotic pathway. Up-regulated caspase-11 is autoactivated in microglia and triggers an activation cascade of downstream caspases, which ultimately leads to cellular apoptosis. Meanwhile, BTG1 not only suppressed microglial proliferation, but also participated in the AICD of microglia by lowering the threshold for apoptosis: BTG1 increased the sensitivity of microglia to apoptogenic action of autocrine cytotoxic mediator NO, indicating an important link between the proliferative state of microglia and their sensitivity to apoptogenic agents.

In contrast to the downstream signaling events of microglial apoptosis that are partly elucidated as described above (31, 34, 35), little is known about the early signaling events proximal to the plasma membrane. Recently, TLRs have been shown to trigger both cellular activation and apoptosis of monocytes/macrophages upon recognition of bacterial products (4, 9, 12, 14, 15). TLRs as membrane proteins are known to initiate diverse signaling pathways, which include activation of NF-κB, double-stranded RNA-activated protein kinase, STAT1, and IRFs. Some of these signaling pathways are also known to be involved in the microglial apoptosis, as discussed above. In particular, TLR2 and TLR4 initiated the activation as well as the apoptosis of macrophages whose structure and function are closely related to microglia in brain.

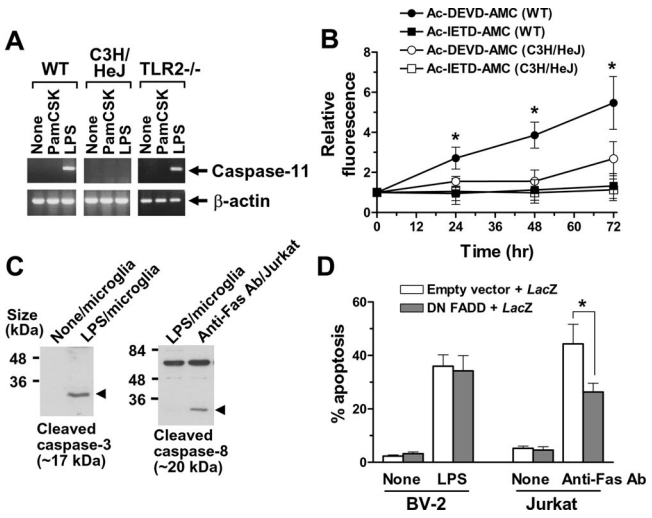

FIGURE 7. Involvement of caspase-11/caspase-3 pathway in TLR4-mediated microglial apoptosis. *A*, Induction of caspase-11 was evaluated by RT-PCR in primary microglia cultures from wild-type (WT), C3H/HeJ, or TLR2-deficient mice (TLR2$^{-/-}$), after treatment with LPS (100 ng/ml) or PamCSK (1000 ng/ml) for 6 h. *B*, Activities of caspase-3 or -8 were measured using specific fluorogenic substrates (Ac-DEVD-AMC for caspase-3; Ac-IETD-AMC for caspase-8) following LPS stimulation in wild-type (WT) or C3H/HeJ microglia cultures. *C*, Microglia were left untreated or treated with LPS for 16 h, and then cleaved caspase-3 (*left*) or a full-length and cleaved caspase-8 (*right*) was detected using Abs specific for the cleaved caspase-3 or total caspase-8. Jurkat cell lysates that were treated with anti-Fas Ab (CH11, 25 ng/ml) for 3 h were used as a positive control for the detection of the cleaved caspase-8. *D*, Dominant-negative (DN) FADD- and *lac*Z-cotransfected BV-2 cells were treated with LPS for 48 h, or similarly cotransfected Jurkat cells were stimulated with anti-Fas Ab (CH11, 25 ng/ml) for 12 h. The percentage of apoptotic cells was similarly analyzed. Asterisks indicate statistically significant differences from the value at 0 h (*B*) or significant difference between the two treatments (*D*) ($p < 0.05$).

These previous findings led us to hypothesize that TLR2 or TLR4 may mediate AICD of microglia. Our results indicate that autoregulatory apoptosis of activated microglia is mediated through TLR4, but not TLR2. The critical differences between TLR2 and TLR4 signalings in microglia were TLR4-specific activation of IRF-3 and IFN-β expression. Although both TLR2 and TLR4 agonists induced NF-κB activation and NO production in microglia, only TLR4 agonist was able to induce IRF-3 activation, followed by IFN-β expression. This seems to be an important difference in terms of the TLR-induced microglial apoptosis, because neutralization of IFN-β using the specific Ab blocked the TLR4-mediated apoptosis, and TLR2 agonist can induce microglial apoptosis in the presence of exogenous IFN-β (Fig. 5). The activation of NF-κB and the production of NO by TLR2 agonist alone did not seem to be sufficient for the induction of apoptosis. Concurrent activation of IFN-β signaling by exogenously added rIFN-β was required for the efficient induction of apoptosis by TLR2. This is consistent with our previous findings that LPS-induced microglial apoptosis is greatly enhanced by IFN-γ treatment that is also known to activate NO-producing STAT1/IRF-1 signaling pathway (34).

Unlike TLR2 agonist, TLR4 agonist appears to activate an additional IFN-β signaling pathway that renders microglia sensitive to the apoptotic TLR signaling. TLR4-specific IFN-β signaling was also found in macrophages in which autocrine/paracrine IFN-β mediated LPS-induced activation of STAT1 (58, 59), suggesting that IFN-β may also induce STAT1 activation in microglia. Then, how does IFN-β signaling act as an apoptotic sensitizer in microglia? To answer this question, the regulation of caspase-11 gene expression first needs to be considered. The expression of caspase-11 gene is controlled by NF-κB and STAT1 (60). Analysis of caspase-11 gene promoter revealed an essential role for NF-κB and STAT1 binding in the up-regulation of caspase-11 in response to LPS and IFN-γ, respectively. In TLR4-mediated microglial apoptosis, it may be the cooperation between NF-κB activation and IFN-β-induced STAT1 activation that is responsible for the efficient caspase-11 induction. This may be an event in which IFN-β acts as an apoptotic sensitizer in microglia. In addition to caspase-11 induction, IFN-β signaling may also participate in NO production via STAT1/IRF-1 pathway. It has been previously reported that, in primary human microglia, IFN-β activates STAT1 and IRF-1 (61), supporting our hypothesis. As our results indicate that IFN-β alone is not apoptotic in microglia, IFN-β-induced NO alone does not seem to be sufficient to induce apoptosis (Fig. 5*C*). It may require concurrent NF-κB-inducing TLR signaling for the induction of apoptosis (Fig. 8). The same seems to be true for TLR2 signaling that induces NF-κB activation alone, failing to induce apoptosis: it requires IFN-β signaling. This may be another step of cooperation between IFN-β signaling and NF-κB-inducing TLR signaling for the induction of microglial apoptosis.

Studies using MyD88-deficient mice have shown that TLR signaling is mediated by at least two pathways: a MyD88-dependent pathway that leads to the production of proinflammatory cytokines, and a MyD88-independent pathway associated with the induction of IFN-inducible genes and maturation of dendritic cells (1). Our current results indicate that both MyD88-dependent and -independent pathways are involved in microglial apoptosis: 1) TLR4-mediated microglial apoptosis was attenuated by dominant-negative MyD88 as well as TRIF; 2) both NF-κB and IFN-β signalings were necessary for the induction of microglial apoptosis. The role of TRIF in microglial apoptosis is in agreement with the results in

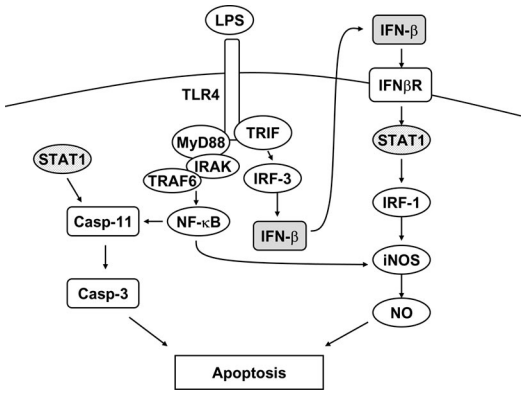

FIGURE 8. Schematic diagram of apoptotic TLR4 signaling in microglia. Microglial apoptosis signaling initiated by TLR4 consists of at least two pathways, with one being MyD88-mediated NF-κB activation and the other being TRIF-mediated IRF-3 activation. Activated IRF-3 induces IFN-β expression, which in turn initiates STAT1 signaling. Both pathways appear to be required for the TLR4-mediated apoptosis of microglia (or the induction of apoptotic molecules). Caspase-11 or iNOS may be an example of those apoptotic molecules that require both pathways for the efficient induction of the gene expression. Caspase-11 induction/activation leads to caspase-3 activation, whereas iNOS induction results in apoptogenic NO production. The difference between TLR2 and TLR4 signaling in microglial apoptosis is that TLR4 agonist initiates these two pathways (NF-κB activation plus IFN-β signaling), but TLR2 agonist induces only one of them (NF-κB activation), failing to induce apoptosis. Not all signaling components are shown. The proposed signaling events downstream of IFN-β, such as the involvement of STAT1 and NO, are not based on the experimental data.

macrophages in which a crucial function of TRIF as a proapoptotic signal transducer has been demonstrated (14). In that study, however, a role of MyD88 was not prominent. In another report, MyD88 mediated TLR2-induced macrophage apoptosis via a pathway involving FADD and caspase-8 (10). A role of MyD88 seems to be different in microglial apoptosis than in macrophages. Our results suggested that TLR4-initiated microglial apoptosis does require MyD88, but it involves caspase-11 activation rather than FADD/caspase-8 pathway (Fig. 7). Induction of caspase-11 and activation of caspase-3, but not caspase-8, was demonstrated in the TLR4-mediated microglial apoptosis (Fig. 7, *A* and *B*), and this is in agreement with our previous findings that a peptide inhibitor of caspase-3, but not caspase-8, suppressed the apoptosis of activated microglia (34). Autoactivation of caspase-11 following the induction of the gene expression is well documented in microglia (34) as well as in other cell types (62, 63). Caspase-11 has been proposed to play an important regulatory role in both apoptosis and inflammatory responses (64). Activation of caspase-11 was crucial not only for the activation of caspase-1 (64), but also for the activation of caspase-3 under pathological conditions (63). Caspase-11 also has been shown to carry out an essential function in apoptotic death of neuronal cells and oligodendrocytes (63, 65, 66). Caspase-11-deficient mice were partly resistant to the induction of experimental allergic encephalomyelitis (66), and showed reduced number of apoptotic cells after middle cerebral artery occlusion (63), further supporting the central role of this caspase in CNS cellular apoptosis. The very caspase is now implicated in TLR4-mediated microglial apoptosis.

Compared with IFN-γ-induced STAT1 activation occurring at 30 min, LPS induces STAT1 activation in microglia as late as 4 h (H. Lee and K. Suk, unpublished data) and in astrocytes at 2 h (67), indicating that the response is not direct. Also, STAT1 activation in microglia was specific for TLR4: the phosphorylation of STAT1 at tyrosine residue 701 was induced by TLR4 agonist, but not TLR2 agonist (H. Lee and K. Suk, unpublished data). The results are in accordance with the previous report in macrophages, in which TLR4, but not TLR2, indirectly activates STAT1 via IFN-β production (59). Taken together, these findings support our hypothesis that microglial apoptosis signaling initiated by TLR4 may consist of at least two pathways, with one being MyD88-mediated NF-κB activation and the other being TRIF-mediated IRF-3 activation, followed by autocrine/paracrine IFN-β production, which may in turn initiate STAT1 pathway. Both pathways appear to be required for the TLR4-mediated apoptosis of microglia (Fig. 8). The signaling events downstream of IFN-β proposed in this work are not based on the experimental data. In AICD of microglia, NF-κB is proapoptotic: inhibition of NF-κB suppressed AICD of microglia, and activation of NF-κB via transfection with p65 subunit of NF-κB did not protect microglia from NO toxicity (34). NF-κB rather appeared to mediate cytotoxic NO production in microglia. Moreover, activation of NF-κB as an early event in TLR-mediated apoptosis has been reported in THP-1 cells treated with lipoproteins of *Mycoplasma fermentans* (12), suggesting that NF-κB may play a role in TLR-mediated macrophage apoptosis as well. NF-κB can be either proapoptotic or antiapoptotic, depending on the timing of the modulating NF-κB activity relative to the death stimulus (68, 69).

The expression of TLR2 and TLR4 has been previously detected in BV-2 microglial cells (18, 70, 71) as well as in mouse primary microglia cultures (72), which is consistent with our results (Fig. 1). TLR2 and TLR4 expression was increased by LPS; however, their expression levels were not affected by IFN-γ following 24 h of incubation in mouse primary microglia cultures (72). In the current study, the expression was analyzed within 6 h of incubation, which may reflect the discrepancies in the results. Previously, LPS either increased or decreased TLR4 expression. In human monocytes, LPS increased TLR4 mRNA expression after 2 h of treatment (21). However, treatment of mouse peritoneal macrophages or RAW264.7 cells with LPS under similar conditions decreased the gene expression (73, 74). TLR2 expression was consistently increased by LPS both in human monocytes and mouse macrophages (21, 53). IFN-γ has been shown to enhance TLR2 as well as TLR4 expression in human endothelial cells (54). In all of these works, the expression of TLR genes was evaluated within 6 h of stimulation, indicating that the regulation of most TLR gene expression occurs within this time frame. The LPS-induced increase in TLR2 or TLR4 expression in primary microglia cultures was an exceptional case, in which the relative levels of TLR gene expression were detected after 24 h of stimulation by real-time PCR (72).

It has yet to be determined whether TLR molecules are true receptors for the agonists that are known to activate the corresponding TLRs. Even though LPS is a widely used agonist of TLR4, a direct interaction between LPS and TLR4 has never been demonstrated (3). It has been suggested that the interaction of TLR agonists and cells may take place over a large contact area involving mutimeric protein complexes in lipid rafts, containing a variety of pattern recognition receptors and other proteins (75, 76). Thus, a direct contact of the TLRs with the agonists may not be always required, but TLR extracellular domains may rather play a role in stabilization of signaling complexes. In addition, combinations of different TLR molecules, for instance, TLR2/6 and TLR2/1 heterodimers, are often used to generate additional diversity in the recognition of bacterial products (36, 77). There is also a report in which LPS response occurs independently of TLR4 (74). In this report, authors have claimed that the reduction of cell surface TLR4 expression after LPS treatment is observed even in peritoneal macrophages of C3H/HeJ mice that harbor nonfunctional TLR4 mutation. Moreover, earlier studies indicated that TLR2 might be involved in LPS signaling, even though it has been shown later that the activation of TLR2 was due to low concentrations of highly bioactive contaminants such as bacterial lipoproteins in the LPS preparations (78). Although we used highly purified LPS throughout the experiments, we sought to confirm that LPS-induced microglial apoptosis is indeed mediated through TLR4. Our results obtained from TLR2-deficient mice and TLR4 mutant mice confirmed this: LPS-induced apoptosis occurred in microglia isolated from TLR2-deficient mice, but not from TLR4 mutant mice (Fig. 2*D*). Our hypothesis was further supported by transfection of dominant-negative mutant of either TLR2 or TLR4, in which inhibition of TLR2 by transfection of dominant-negative mutant did not affect LPS-induced microglial apoptosis, but TLR4 inhibition did (Fig. 3*B*).

In conclusion, TLR4, but not TLR2, signals apoptosis of microglia following inflammatory activation. Although there is some similarity between TLR2 and TLR4 signaling in microglia, IRF-3 activation and IFN-β expression are unique in TLR4 signaling. More importantly, IFN-β appears to act as an apoptotic sensitizer in determining TLR-mediated microglial apoptosis, as TLR2 agonist in the presence of IFN-β also induces microglial apoptosis. Further elucidation of TLR-mediated microglial apoptosis mechanisms will help understand the autoregulatory processes of microglial activation and the CNS disorders associated with pathological activation of microglia.

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Disclosures

The authors have no financial conflict of interest.

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