Neurobiology

Gangliosides Trigger Inflammatory Responses via TLR4 in Brain Glia

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Gangliosides participate in various cellular events of the central nervous system and have been closely implicated in many neuronal diseases. However, the precise molecular mechanisms underlying the pathological activity of gangliosides are poorly understood. Here we report that toll-like receptor 4 (TLR4) may mediate the ganglioside-triggered inflammation in glia, brain resident immune cells. Gangliosides rapidly altered the cell surface expression of TLR4 in microglia and astrocytes within 3 hours. Using TLR4-specific siRNA and a dominant-negative TLR4 gene, we clearly demonstrate the functional importance of TLR4 in ganglioside-triggered activation of glia. Inhibition of TLR4 expression by TLR4-siRNA suppressed nuclear factor (NF)-κB-binding activity, NF-κB-dependent luciferase activity, and transcription of inflammatory cytokines after exposure to gangliosides. Transient transfection of dominant-negative TLR4 also attenuated NF-κB-binding activity and interleukin-6 promoter activity. In contrast, these activities were slightly elevated in cells with wild-type TLR4. In addition, CD14 was required for ganglioside-triggered activation of glia, and lipid raft formation may be associated with ganglioside-stimulated signal propagation. Taken together, these results suggest that TLR4 may provide an explanation for the pathological ability of gangliosides to cause inflammatory conditions in the brain. *(Am J Pathol 2006, 168:1619–1630; DOI: 10.2353/ajpath.2006.050924)*

Microglia and astrocytes are resident immunoeffector cells of the central nervous system. Although these cells are quiescent under normal conditions, they are rapidly activated in response to pathological stimuli. On activation, astrocytes and microglia change their morphology, immunophenotype, and expression pattern of inflammatory mediators, leading to immune and inflammatory responses.[1,2] Although transient activation of glial cells is beneficial for defense processes against pathogens, either chronic activation or overactivation can cause or exacerbate neuronal diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, ischemia, and human immunodeficiency virus encephalitis.[3,4]

Gangliosides are sialic acid-containing glycosphingolipids that are constituents of mammalian cell membranes. Increasing evidence suggests that gangliosides are not passive structural components of cell membranes but rather are modulators of important biological processes such as proliferation, adhesion, differentiation, and inflammation.[5–11] Neuronal cell membranes are particularly rich in gangliosides, which play important roles in brain physiology and pathology.[12,13] Some researchers have reported that brain injury can lead to the release of gangliosides from damaged neuronal cells into the extracellular space, possibly resulting in pathophysiological conditions.[14–16] Several studies have shown that exposure to gangliosides both *in vivo* and *in vitro* can induce the production of various inflammatory mediators, such as cytokines and inducible nitric oxide synthase in brain microglia and astrocytes.[11,17–19] Gangliosides have also been reported to play a role in Aβ toxicity as well as in the deposition of Aβ into senile plaques associated with Alzheimer's disease.[20–23] Thus, gangliosides have recently been recognized as being important contributors to pathological inflammatory conditions in the brain. However, little is known about the molecular mechanisms through which gangliosides trigger pathological immune responses.

Toll-like receptors (TLRs), which are the mammalian homologues of the *Drosophila* Toll receptor, act as primary sensors of various pathogens and trigger inflamma-

tory and immune responses.[24] TLRs are defined by the presence of the Toll/interleukin (IL)-1 receptor (TIR) domain in their cytosolic regions and an extracellular domain comprising leucine-rich repeats. To date, 13 TLRs have been described in mice and 10 in humans, and potential ligands have been identified for several of these receptors.[25,26] Each TLR recognizes a distinct pathogen and elicits different, but sometimes overlapping, immune responses.[27] TLR4, the first mammalian TLR identified in mammals, is involved in signaling responses elicited by various exogenous and endogenous molecules, including lipopolysaccharide (LPS).[28] Activation of TLRs initiates complex signal transduction cascades that activate many signaling proteins, such as nuclear factor (NF)-κB, thereby resulting in transcription of genes encoding inflammation-associated molecules and cytokines.[29] Different cell types express distinct combinations of TLRs. Moreover, TLR expression appears to be modulated based on the activation and maturation state of a cell.[30,31] That is, TLR expression levels are strictly regulated in both a cell type- and state-specific manner as well as in a stimulus-dependent manner.[32,33]

We have previously shown that gangliosides are capable of activating glia, thereby leading to inflammatory responses in the brain. Gangliosides can alter the morphology of microglia and trigger the production of inflammatory mediators through activation of various inflammation-associated signaling molecules including NF-κB, Janus kinase (JAK), signal transducer and activator of transcription (STAT), and mitogen-activated protein kinase (MAPK).[11,19,22,34] In the present report, we show that TLR4 expression levels rapidly change after treatment with gangliosides in rat primary glia and demonstrate that TLR4 is essential for ganglioside-triggered inflammatory signaling pathways using TLR4-specific siRNA and a dominant-negative TLR4 gene. These results suggest that TLR4 can function as an upstream sensor for gangliosides and provoke intracellular inflammatory signaling in the brain. Our data provide interesting insights into the understanding of the signaling pathways through which gangliosides cause pathological conditions in the brain.

Materials and Methods

Reagents

Purified ganglioside mixture (Gmix), monosialo-ganglioside (GM) 1, disialo-ganglioside (GD) 1a, and trisialo-ganglioside (GT) 1b from bovine brain were purchased from Matreya (Pleasant Gap, PA) and Sigma (St. Louis, MO). Endotoxin-tested GM1 and asialo-GM1 were obtained from Sigma. Rat interferon (IFN)-γ was obtained from Calbiochem (La Jolla, CA). *Salmonella typhimurium* LPS, polymyxin B sulfate, filipin, and methyl β-cyclodextrin (MβCD) were purchased from Sigma. Minimal essential medium, Lipofectamine plus, oligofectamine, and G418 antibiotics were obtained from Life Technologies, Inc. (Gaithersburg, MD). Dulbecco's modified Eagle's

medium and fetal bovine serum were purchased from Hyclone (Logan, UT).

Cell Culture

Primary microglia were cultured from the cerebral cortices of 1- to 3-day-old Sprague-Dawley rats. Briefly, cortices were triturated into single cells in minimal essential medium containing 10% fetal bovine serum and were plated in 75-cm^2 T-flasks (0.5 hemisphere/flask) for 2 weeks. The microglia were detached from the flasks by mild shaking and applied to a nylon mesh to remove astrocytes and cell clumps. Cells were plated in six-well plates (5 × 10^5 cells/well), 60-mm^2 dishes (8 × 10^5 cells/dish), or 100-mm^2 dishes (2 × 10^6 cells/dish). One hour later, the cells were washed to remove unattached cells before being used in experiments. After removal of the microglia, primary astrocytes were prepared using trypsinization. Cells were demonstrated to be more than 95% authentic microglia and astrocytes because of their characteristic morphology and the presence of the astrocyte marker glial fibrillary acidic protein and the microglia marker CD11b. For blocking of CD14 or LPS-binding protein (LBP), cells were preincubated for 30 minutes with 10 μg/ml of anti-CD14 monoclonal antibody (mAb), anti-LBP mAb, or isotype control mAb (HyCult Biotechnology, Liden, The Netherlands), and then the cells were treated with LPS or gangliosides for indicated times.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated using RNAzolB (Tel-Test Inc., Friendswood, TX), and cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (TaKaRa, Japan) according to the manufacturer's instructions. PCR was performed with 25 cycles of sequential reactions. Oligonucleotide primers were purchased from Bioneer (Seoul, Korea). The sequences for PCR primers were as follows: (forward) 5'-TCC CTC AAG ATT GTC AGC AA-3' and (reverse) 5'-AGA TCC ACA ACG GAT ACA TT-3' for GAPDH; (forward) 5'-TGA TGT TCC CAT TAG ACA GC-3' and (reverse) 5'-GAG GTG CTG ATG TAC CAG TT-3' for IL-1β; (forward) 5'-GTA GCC CAC GTC GTA GCA AA-3' and (reverse) 5'-CCC TTC TCC AGC TGG GAG AC-3' for tumor necrosis factor (TNF)-α; (forward) 5'-TTG AAG ACA AGG CAT GGC ATG G-3' and (reverse) 5'-TCT C CCC AAG ATC AAC CGA TG-3' for TLR4.

Flow Cytometric Analysis

Cells were treated with gangliosides or LPS in the presence of 5% serum for the indicated times. The cells were washed twice with phosphate-buffered saline containing 1% fetal bovine serum, collected, and stained with phycoerythrin-conjugated anti-mouse TLR4/MD2 antibody (eBioscience, CA) for 30 minutes at 4°C. After washing, the cells were analyzed with a FACS Vantage (BD Bio-

sciences, CA), and the data were processed using the CellQuest program and WinMDI. The mean fluorescence intensity (MFI) was analyzed by CellQuest software, and the change in MFI of cells incubated with TLR4/MD2 antibody was calculated for LPS- or ganglioside-treated and untreated cells after subtraction of the MFI obtained with the isotype control antibody (eBioscience).

Plasmids

Wild-type TLR4 [pDisplay-*Tlr4*(*wt*)] and a dominant-negative mutant of TLR4 [pDisplay-*Tlr4*(*P712H*)], were gifts from Dr. Lynn Hajjar and C. Wilson (University of Washington, Seattle, WA). The 5×NF-κB-luciferase reporter construct and IL-6-luciferase reporter construct were from Dr. Seong Ho Jeon (Hanllym University, Korea).

Synthesis and Transfection of siRNAs

Chemically synthesized, double-stranded small interfering RNAs (siRNAs), with 19-nucleotide duplex RNA and 2-nucleotide 3′ dTdT overhangs, were purchased from Dharmacon Research (Lafayette, CO) in a deprotected and desalted form. To design TLR4-specific siRNA duplexes, the mRNA sequence for TLR4 was screened for unique 21-nucleotide sequences in the National Center for Biotechnology Information database using the BLAST search algorithm. The siRNA sequence targeting TLR4 in this study is 5′-ACG CUG UUC UGC UCA GGA GdTdT-3′. Forty to fifty percent of confluent cells were transfected with siRNA oligonucleotides using oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. All assays were performed at least 24 hours after RNA transfection.

Luciferase Assay

Transient transfections were performed in triplicate on 35-mm dishes using Lipofectamine plus reagents as instructed by the manufacturer (Life Technologies, Inc.). To normalize the variations in cell number and transfection efficiency, all cells were co-transfected with pCMV-β-GAL for 24 hours. Luciferase assays were performed according to the manufacturer's instructions (Promega, Madison, WI). Luciferase activity was measured using 20 μl of cell extract in 100 μl of assay buffer. Light intensity was measured for 30 seconds on a luminometer (Berthold Lumat LB9501). Luciferase activity was normalized by measuring β-galactosidase activity (in OD_{420}). All plasmid DNAs were prepared using endotoxin-free DNA isolation kit (Qiagen, Valencia, CA).

Electrophoretic Mobility Shift Assay

Cells were stimulated in the presence of 2.5% serum, and then the cell extracts were suspended in 9× packaged cell volume of a hypotonic solution (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethyl sulfonyl fluoride, 0.5% Nonidet P-40) and centrifuged at 5000 rpm for 10 minutes at 4°C. The pellet (nuclear fraction) was resuspended in 20 mmol/L HEPES (pH 7.9), 20% glycerol, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethyl sulfonyl fluoride, incubated on ice for 60 minutes with occasional gentle shaking, and centrifuged at 13,000 rpm for 20 minutes. The crude nuclear proteins in the supernatant were collected and stored at −70°C. Electrophoretic mobility shift assay was performed for 30 minutes on ice in a volume of 20 μl containing 2 μg of nuclear protein extract in a reaction buffer containing 8.5 mmol/L EDTA, 8.5 mmol/L EGTA, 8% glycerol, 0.1 mmol/L $ZnSO_4$, 50 μg/ml poly (dI-dC), 1 mmol/L dithiothreitol, 0.3 mg/ml bovine serum albumin, 6 mmol/L $MgCl_2$, and γ-^{32}P-radiolabeled oligonucleotide probe (3×10^4 cpm), with or without a 20- to 50-fold excess of unlabeled probe. DNA-protein complexes were separated on 6% polyacrylamide gels in Tris/glycine buffer, and the dried gels were exposed to X-ray film. The following double-stranded oligonucleotides were used in these studies: NF-κB gel shift oligonucleotides, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, 25 bp (sc-2505; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). 5′-End-labeled probes were prepared with 40 μCi of [γ-^{32}P]-ATP using T4 polynucleotide kinase (Promega) and purified on Sephadex G-25 quick spin columns (Roche Molecular Biochemicals, Indianapolis, IN).

Endotoxin Assay

All ganglioside preparations were tested for bacterial contamination using the chromogenic Limulus amebocyte lysate assay according to the manufacturer's specifications (WinKQCL, Bio-Whittaker). The purity of ganglioside mixture by thin layer chromatography was more than 98% according to the manufacturers' reports (Matreya and Sigma), and the purity of individual ganglioside was 93 to 98%.

Determination of Nitric Oxide (NO) Release

Media nitrite concentration was measured as an indication of NO release. After the indicated cell incubations, 50 μl of culture medium was removed and mixed with an equal volume of Griess reagent (0.1% naphthylethylene diamine, 1% sulfanilamide, 2.5% H_3PO_4), and absorbance of the mixture at 540 nm was measured.

Statistical Analysis

All data are expressed as the mean ± SD. Statistical analysis was performed using Student's *t*-test.

Results

Brain Gangliosides Rapidly Alter Cell Surface Expression of TLR4 in Primary Microglia from Cerebral Cortices

In an effort to address how gangliosides initiate inflammatory conditions in the brain, we examined whether TLR4 could function as an upstream sensor for gangliosides in rat primary microglia. First, we tested the engagement of TLR4 in ganglioside-induced inflammation by its expression level because TLR4 expression has been shown to be strictly regulated in a stimulus-dependent manner.[35,36] Rat primary microglia were treated with 50 µg/ml of brain ganglioside mixture (Gmix) for 18 hours, and TLR4 cell surface expression levels were analyzed by flow cytometric analysis using phycoerythrin-conjugated TLR4/MD2 (MTS510) antibody. Interestingly, we found that cell surface expression of TLR4 was markedly low in ganglioside-treated primary glia and that the reduced level of TLR4 in ganglioside-treated cells was similar to that in cells treated with 100 ng/ml of LPS, a representative TLR4 ligand (Figure 1A). We also observed that gangliosides significantly reduced TLR4 cell surface expression in BV2 mouse microglia (Figure 1A).

To evaluate further the effect of gangliosides on TLR4 surface expression, we examined the time- and dose-dependent effects of gangliosides in primary microglia. Cell surface expression of TLR4 was rapidly reduced within 3 hours of exposure to Gmix (Figure 1B). Moreover, TLR4 cell surface expression was slightly changed even in 10 µg/ml ganglioside-treated cells, and the increasing concentrations of gangliosides further diminished TLR4 expression (Figure 1C). These results indicate that gangliosides rapidly modulate TLR4 expression in primary microglia, suggesting the possibility that gangliosides may trigger inflammation through TLR4 in the brain.

Figure 1. Gangliosides rapidly modulate cell surface expression of TLR4 in microglia. Rat primary microglia and BV2 microglial cells (**A**) were treated with 50 µg/ml of brain ganglioside mixture (Gmix) or 100 ng/ml of LPS for 18 hours, and the TLR4 surface expression level was analyzed by flow cytometric analysis using phycoerythrin-conjugated TLR4/MD2(MTS510) antibody. Data shown are representative of at least four independent experiments. The MFI was analyzed by CellQuest software (BD Biosciences), and the change in MFI of cells incubated with TLR4 antibody was calculated for LPS- or Gmix-treated cells and untreated cells after subtraction of the MFI obtained with the isotype control antibody. The MFI values are mean ± SD of three independent experiments. *$P < 0.01$ when compared with control samples. **B:** Primary microglia were treated with 50 µg/ml of Gmix for the indicated times, and TLR4 surface protein expression was determined by FACS analysis. **C:** Primary microglia were treated with the indicated concentrations of Gmix for 18 hours. Data shown are representative of at least four independent experiments.

The Sialic Acid Residue of Gangliosides Is Essential for Modulation of TLR4 Expression

The major types of gangliosides in the brain are GM1, GD1a, GD1b, GT1b, and GQ1b, each of which have a different number and position of carbohydrate-linked sialic acid residues.[37] We therefore investigated the role of the sialic acid residues in the regulatory effect of gangliosides on TLR4 expression. We first compared the effects of GM1, which has one sialic acid residue, with Gmix on TLR4 expression. As shown in Figure 2A, no significant differences in the surface expression level of TLR4 were observed between GM1- and Gmix-treated primary microglia. Reduced TLR4 surface expression was observed in cells treated with either GD1a, which has two sialic acid residues, or GT1b, which has three sialic acid residues; this decrease was also similar to that caused by Gmix (Figure 2, A and B). These results indicate that the number of sialic acid residues per ganglioside molecule has little effect on modulation of TLR4 expression at the cell surface expression level.
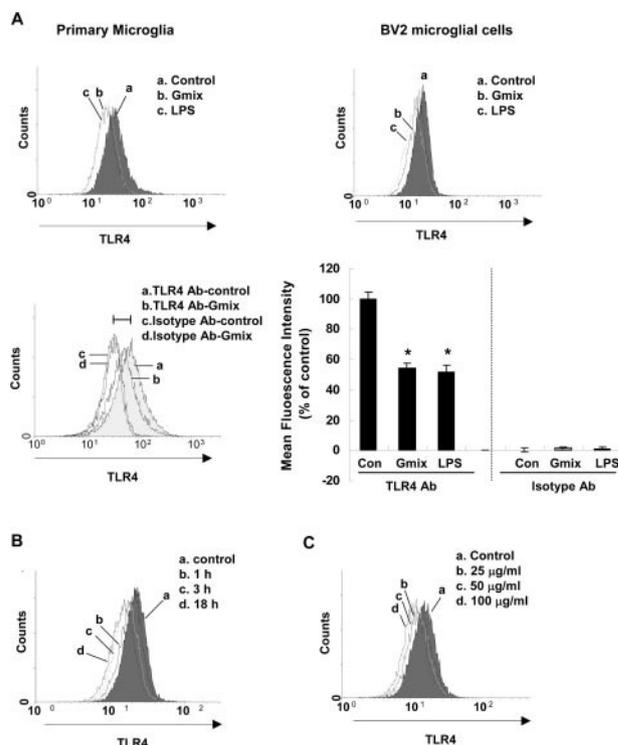
To further examine the role of sialic acid residue in ganglioside-induced changes of TLR4 expression, we compared the effects of Gmix and asialo-GM1, which has no sialic acid residue, on TLR4 expression. Compared to untreated control cells, cell surface expression of TLR4 was decreased in microglia treated with 25 µg/ml of Gmix, whereas transcription of IL-1β was markedly increased. In contrast, cell surface expression of TLR4 as well as IL-1β transcription was unchanged in cells treated with asilao-GM1 (Figure 2C). Taken together, these results suggest that the sialic acid residue is necessary for ganglioside-induced modulation of TLR4 expression, although the number of sialic residues per ganglioside molecule is not relevant.

Ganglioside-Dependent Modulation of TLR4 Is Not Attributable to LPS Contamination in Preparation of Gangliosides

To investigate whether ganglioside-dependent modulation of TLR4 could be attributable to LPS contamination in
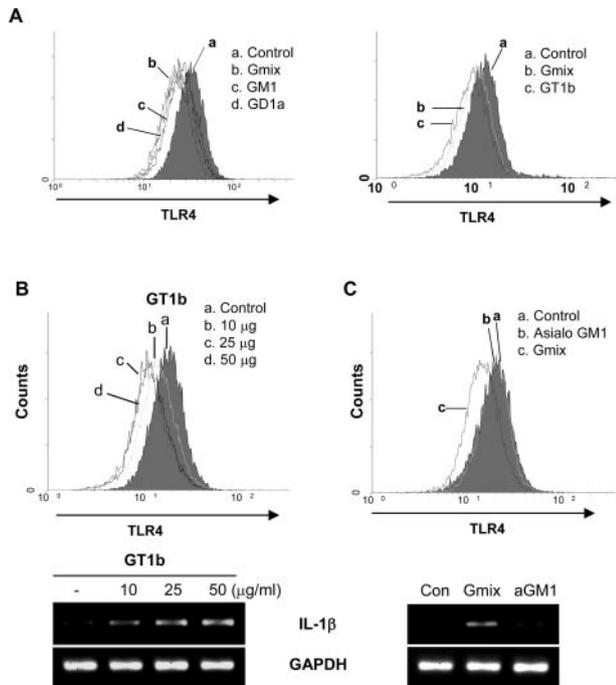
Figure 2. Sialic acid residue is essential for ganglioside-modulated TLR4 expression. **A:** Microglia were stimulated with GM1, GD1a, GT1b, or Gmix for 12 hours, and TLR4 expression was analyzed at the cell surface protein levels. **B:** Microglia were treated with the indicated concentrations of GT1b for 12 hours, after which expression of TLR4 and IL-1β was determined by FACS and RT-PCR analysis, respectively. **C:** TLR4 cell surface expression levels were determined in cells with 25 μg/ml of Gmix or asialo GM1. Data shown are representative of at least three independent experiments.

Figure 3. Polymyxin B, a LPS scavenger, does not affect the ganglioside-dependent expression of TLR4 and cytokine. **A:** Primary microglia were stimulated with Gmix in the presence or the absence of 10 μg/ml of poly-myxin B, after which TLR4 expression was analyzed at the cell surface protein level. **B:** Primary microglia were pretreated with polymyxin B for 1 hour, and stimulated with Gmix or LPS for 3 hours. mRNA expression of TNF-α and IL-1β was detected using a RT-PCR-based assay.

glioside-treated astrocytes. Primary astrocytes were treated with 50 μg/ml of Gmix or 100 ng/ml of LPS for 18 hours, and the TLR4 surface expression level was analyzed by flow cytometry. Consistent with the results in microglia, cell surface expression of TLR4 was significantly reduced on primary astrocytes treated with Gmix as well as LPS (Figure 4). Similar patterns of reductions in TLR4 expression were observed in astrocytes treated with GM1, GD1a, or GT1b (Figure 4). These results indicate that ganglioside treatment can alter TLR4 expression in both astrocytes and microglia, suggesting that TLR4 may play important roles in ganglioside-triggered inflammatory responses.

siRNA-Mediated Suppression of TLR4 Reduces Ganglioside-Induced Transcription of Inflammatory Cytokines

Because our results suggest a possible involvement of TLR4 in ganglioside-induced inflammatory events, we examined whether TLR4 could indeed contribute to ganglioside-triggered inflammatory signaling using TLR4-specific short interfering RNA (siRNA). To do this, we designed the synthetic siRNA that targets the TLR4 and chemically synthesized the small RNA duplexes corresponding to TLR4 (Dharmacon). After validating our siRNA as an inhibitory reagent capable of depleting TLR4 levels in rat primary astrocytes (Figure

preparation of gangliosides, we carefully tested all ganglioside preparations using chromogenic Limulus amebocyte lysate assay (WinKQCL, Bio-Whittaker). The average endotoxin level of Gmix was ~0.007 EU/ml (±0.002), which was not sufficient to induce LPS-induced inflammatory effects including modulation of TLR4 expression in glia. Endotoxin levels for GT1b, GD1a, GM1, and asialo-GM1 used for this study were 0.008 (±0.002), 0.009 (±0.002), 0.005 (±0.003), 0.009 (±0.002) EU/ml, respectively. To further eliminate the possibility for misinterpretation of our results by endotoxin contamination of gangliosides, we examined the effect of polymyxin B, a well-known pharmacological LPS scavenger, on the ganglioside-dependent change of TLR4 expression. As expected, pretreatment of polymyxin B did not affect ganglioside-induced modulation of TLR4 expression as well as ganglioside-triggered transcription of inflammatory cytokines (Figure 3). These results suggest that TLR4 may specifically recognize gangliosides in microglia.

Gangliosides Alter Cell Surface Expression of TLR4 Also in Primary Astrocytes

Astrocytes, which are another glial cell type vital to brain immune responses, express various cell surface receptors and produce inflammatory mediators such as cytokines. To define better the association of TLR4 with brain inflammatory responses, we investigated whether TLR4 expression levels were altered in gan-
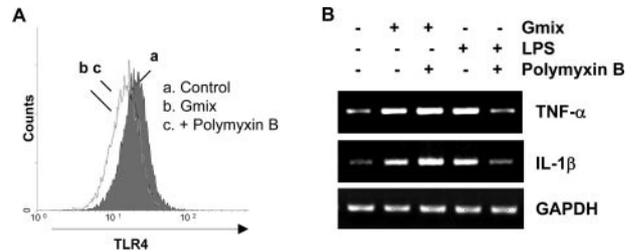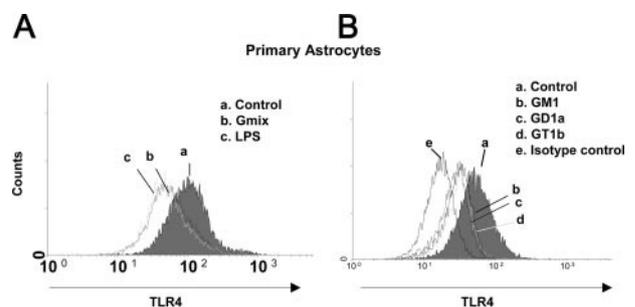

Figure 4. Gangliosides modulate TLR4 expression also in primary astrocytes. **A:** Rat primary astrocytes were treated with 50 μg/ml of Gmix or 100 ng/ml of LPS for 18 hours, and the TLR4 surface expression levels were analyzed by flow cytometric analysis. **B:** Rat primary astrocytes were stimulated with 25 μg/ml of GM1, GD1a, or GT1b, after which TLR4 expressions were analyzed at cell surface protein levels.
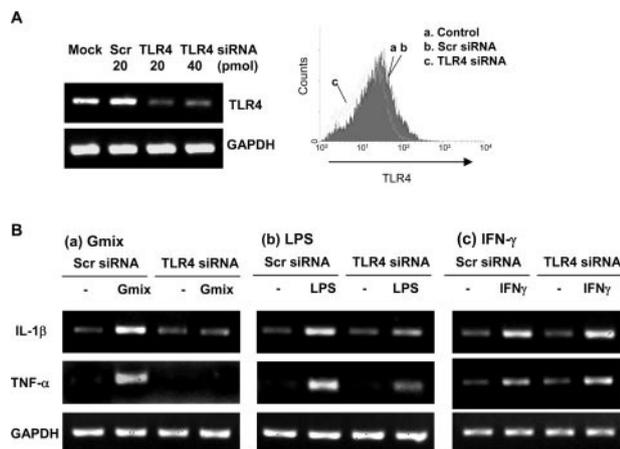
Figure 5. siRNA-mediated suppression of TLR4 reduces ganglioside-induced transcription of inflammatory cytokines. **A:** Rat primary astrocytes were transfected with a TLR4-specific 21-bp siRNA duplex or a nonsilencing control siRNA (Scramble 1 duplex, Dharmacon) using oligofectamine. Transcript levels of TLR4 were then detected by RT-PCR. Twenty-four hours after rat primary astrocytes were transfected with oligofectamine alone (mock), 20 pmol of Scramble 1 (Scr)-siRNA or 20 pmol of TLR4-siRNA, TLR4 cell surface expression levels were measured by FACS analysis. **B:** Rat primary astrocytes were transfected with either 20 pmol of TLR4-siRNA or 20 pmol of Scr control-siRNA, and the cells were treated with 50 μg/ml of brain ganglioside mixture (**a**), 100 ng/ml of LPS as a positive control (**b**), or 10 U/ml of IFN-γ as a negative control (**c**) for 3 hours. Total RNA was then extracted for RT-PCR analysis.

5A), we tested whether siRNA-mediated repression of TLR4 could specifically inhibit the ganglioside-stimulated transcription of inflammatory cytokines. Rat primary astrocytes were transfected with either TLR4-siRNA or nonsilencing control siRNA, and the cells were treated with Gmix, LPS as a positive control, or IFN-γ as a negative control for 3 hours. As shown in Figure 5B, Gmix as well as LPS significantly increased the message level of proinflammatory cytokines, including IL-1β and TNF-α, in scramble-siRNA-transfected cells but not in TLR4-siRNA-transfected cells. Transfection of TLR4-siRNA consistently suppressed TNF-α transcription induced by both Gmix and LPS, although the reduced extent was slightly different in experimental conditions such as transfection efficiency. By contrast, IFN-γ-induced transcription of IL-1β and TNF-α in TLR4-siRNA-transfected cells was similar to that in scramble-siRNA-transfected cells. These results indicate that TLR4 is required for ganglioside-induced transcription of IL-1β and TNF-α but not for IFN-γ-induced transcription of these genes.

siRNA-Mediated Inhibition of TLR4 Also Reduces Ganglioside-Induced NF-κB Promoter Activity

To further address the functional importance of TLR4 in ganglioside-induced inflammatory signaling, we assayed whether siRNA-mediated repression of TLR4 could influence ganglioside-stimulated NF-κB promoter activity in rat primary astrocytes. Eighteen hours after transfection with TLR4-siRNA or scramble control-siRNA, the cells were transfected with luciferase reporter plasmids con-
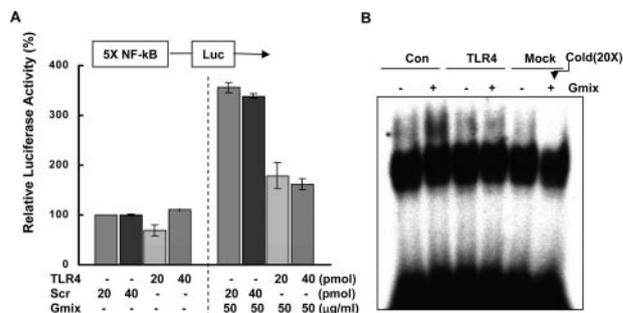

Figure 6. siRNA-mediated inhibition of TLR4 also suppresses ganglioside-induced NF-κB activity. **A:** Eighteen hours after transfection with TLR4-siRNA or Scramble control-siRNA in rat primary astrocytes, cells were transfected with luciferase reporter plasmids containing 5$\times$NF-κB binding elements. After incubation for 24 hours, the cells were stimulated with 50 μg/ml of Gmix for 6 hours. Luciferase activity was then determined, and the result was normalized for transfection efficiency by comparing β-galactosidase activity. Values are mean $\pm$ SD of triplicates. Data are representative of three independent experiments. **B:** Eighteen hours after transfection with 20 pmol of control siRNA, 20 pmol of TLR4-siRNA, or oligofectamine alone (mock) in rat primary astrocytes, cells were serum-starved for 24 hours and then treated with 50 μg/ml of Gmix for 30 minutes in the presence of 2.5% serum. Nuclear extracts were then prepared and analyzed using a γ-^{32}P-labled consensus NF-κB binding elements oligonucleotide probe.

taining 5$\times$NF-κB binding elements. Gmix significantly enhanced NF-κB luciferase activity in scramble-siRNA-transfected control cells, but this enhancement was markedly attenuated in TLR4-siRNA-transfected cells (Figure 6A). The ganglioside-induced binding activity of nuclear factors to NF-κB binding elements was also reduced in TLR4-siRNA-transfected cells compared to that in scramble control-siRNA-transfected cells (Figure 6B). These results indicate that TLR4 contributes to the ganglioside-stimulated activation of NF-κB, thus provoking inflammatory signaling cascades in brain resident immune cells.

Transient Transfection of Wild-Type or Dominant-Negative TLR4 Affects the Nuclear Factors Binding Activity to NF-κB Binding Elements and IL-6 Promoter Activity

Genetic studies in C3H/HeJ mice, which are endotoxin-hyporesponsive, have shown that a P712H substitution in the related TLR4 acts as a dominant-negative mutation of the receptor and blocks endotoxin signaling.[37,38] To further evaluate the contribution of TLR4 to ganglioside-induced inflammatory responses, we examined the effect of wild-type TLR4 gene [pDisplay-*Tlr4*(*wt*)] or a dominant-negative TLR4 mutant gene [pDisplay-*Tlr4*(*P712H*)] on transcriptional activation of various inflammatory mediators.[27,38,39] After confirming the expressions of wild-type and mutant TLR4 genes in rat primary astrocytes (Figure 7A), we examined whether transient transfection of these genes could influence the ganglioside-induced binding of nuclear factors to NF-κB binding elements using an electrophoretic mobility shift assay. Gmix markedly stimulated nuclear factor binding within 5 minutes in vector-transfected cells, and the binding activity was significantly reduced in mutant TLR4-transfected cells [Figure 7B(a)]. In contrast, the binding activity was slightly en-
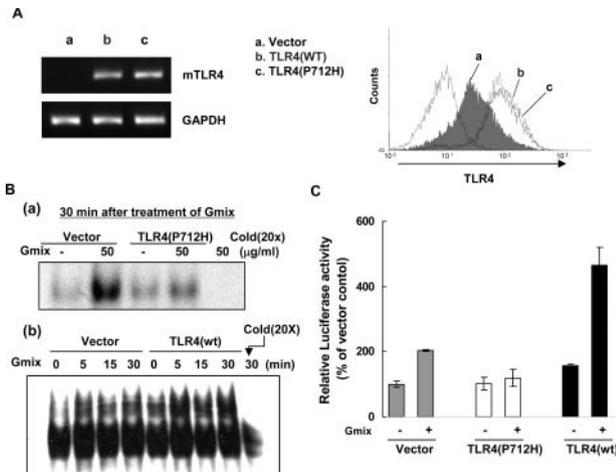
Figure 7. Transient transfection of wild-type or mutant TLR4 genes affects the ganglioside-dependent nuclear factor binding activity to NF-κB binding elements as well as IL-6 promoter activity. **A:** Rat primary astrocytes were transiently transfected with wild-type TLR4 [pDisplay-*Tlr4*(*wt*)], dominant-negative TLR4 [pDisplay-*Tlr4*(*P712H*)], or vector alone, and their expressions were determined at the transcript and surface protein expression level using RT-PCR and FACS analysis. The sequences for mouse-specific TLR4 primer used in this study were (forward) 5′-GAACAAACAGCCTGAGAC-3′ and (reverse) 5′-GACTGGTCAAGCCAAGAA-3′. **B:** Rat primary astrocytes were transiently transfected with pDisplay-*Tlr4*(*P712H*) (**a**), pDisplay-*Tlr4*(*wt*) (**b**), or vector, and then incubated for 18 hours. The cells were serum-starved for 24 hours, and treated with 50 μg/ml of Gmix for the indicated times in the presence of 2.5% serum. Nuclear extracts were then prepared and analyzed using a γ-³²P-labled consensus NF-κB binding elements oligonucleotide probe. **C:** Eighteen hours after transfection with luciferase reporter plasmids containing IL-6 promoter elements and pDisplay-*Tlr4*(*wt*), pDisplay-*Tlr4*(*P712H*), or control vector, cells were serum-starved for 24 hours and stimulated with 50 μg of Gmix for 6 hours. Luciferase activity was then determined, and the result was normalized for transfection efficiency by comparison with β-galactosidase activity. Values are mean ± SD of triplicates. Data shown are representative of at least three independent experiments.

hanced in cells transfected with wild-type TLR4 compared to that in cells transfected with control vector [Figure 7B(b)].

Next, we examined whether expression of wild-type or mutant TLR4 influenced the promoter activity of IL-6, which has many proinflammatory roles and contains NF-κB-binding sites in its promoter. Rat primary astrocytes were transiently transfected with luciferase reporter plasmids containing IL-6 promoter elements and pDisplay-*Tlr4*(*wt*), pDisplay-*Tlr4*(*P712H*), or control vector. Compared to cells transfected with vector alone, Gmix-induced IL-6 promoter activity was increased in cells transfected with wild-type TLR4 but reduced in cells transfected with mutant TLR4 (Figure 7C). These results further demonstrate that TLR4 can transduce ganglioside-derived signals to downstream molecules, thus leading to inflammatory responses in brain glial cells.

CD14 Also Contributes to Ganglioside-Induced Activation of Glia

CD14 has been shown to recognize several microbial and cellular products including TLR ligands and plays essential roles in certain TLR4-mediated inflammatory signaling events. Thus, we examined whether CD14 could contribute to ganglioside-induced activation of glia

using blocking antibodies. First, we investigated the effect of CD14 on ganglioside-induced NO release. Primary microglia were preincubated with 10 μg/ml of anti-CD14 mAb, or isotype control mAb, and then the cells were treated with 50 μg/ml of Gmix or 100 ng/ml of LPS as a control for 24 hours. The effect of LBP, an essential molecule for recognition of TLR4 by LPS, was also tested using anti-LBP mAb as a control. The amount of NO was determined by measuring the amount of nitrite converted from NO in the media. Interestingly, treatment of CD14-blocking antibody significantly suppressed NO release by gangliosides (Figure 8A). Compared to cells incubated with isotype control antibody, Gmix-induced NO release was reduced to 51.9 ± 3.2% and LPS-induced NO release was reduced to 46.2 ± 5% in cells with CD14 antibody. However, LBP blockade had little effect on NO release by Gmix while it remarkably reduced NO release by LPS (Figure 8A). Similar patterns of inhibitory effects on NO release were observed in GM1-, GD1a-, and GT1b-treated cells (Figure 8B and data not shown).

To examine events further, we tested the effect of CD14 or LBP blockade on ganglioside-induced transcription of TNF-α and IL-1β. As shown in Figure 8C, the result was consistent with that from NO measurement. Transcription of TNF-α and IL-1β induced by Gmix was reduced in cells with CD14 antibody compared to cells with isotype control. However, treatment with LBP antibody had little effect on Gmix-induced transcription of TNF-α and IL-1β. These results suggest that CD14 has an essential role in TLR4-mediated activation of glia by gangliosides.

Raft-Disrupting Drugs Inhibit Ganglioside-Induced NO Release

Lipid raft formation has recently been shown to be important for the dynamic association of multiprotein receptor complex involved in immune responses.[40–42] Because CD14 and TLR4 have been found in lipid rafts after ligand stimulation, we examined if lipid rafts could be involved in ganglioside-stimulated immune responses. To gain insight into this, we measured the amount of ganglioside-induced NO release in the presence or absence of cholesterol-depleting agents, such as methyl-β-cyclodextrin (MβCD) and filipin. Primary microglia were preincubated with 1 mmol/L MβCD or 1 μg/ml of filipin for 30 minutes, and then the cells were treated with 25 μg/ml of Gmix for 24 hours. Interestingly, pretreatment with MβCD or filipin markedly reduced the ganglioside-stimulated NO release compared to untreated controls (Figure 9). These results indicate that lipid raft formation is necessary for ganglioside-stimulated production of NO, suggesting that lipid rafts may act as a platform for recruitment and association of signaling molecules involved in ganglioside-induced immune responses.

Discussion

Several lines of evidence underscore the importance of brain-derived gangliosides in immune responses and pa-
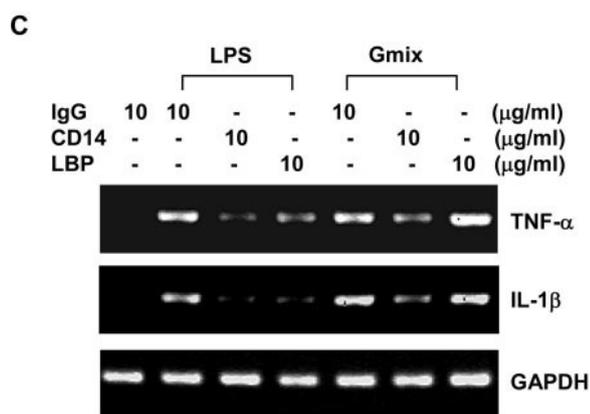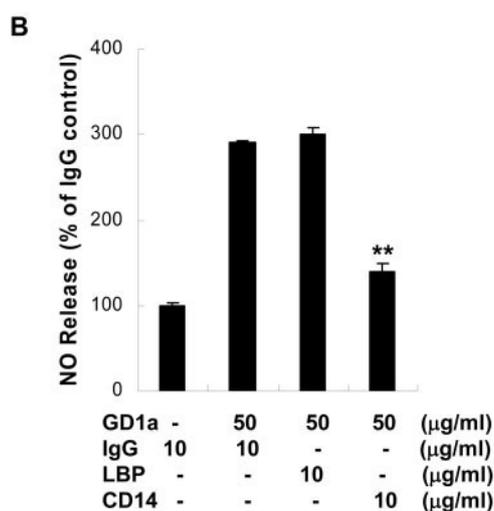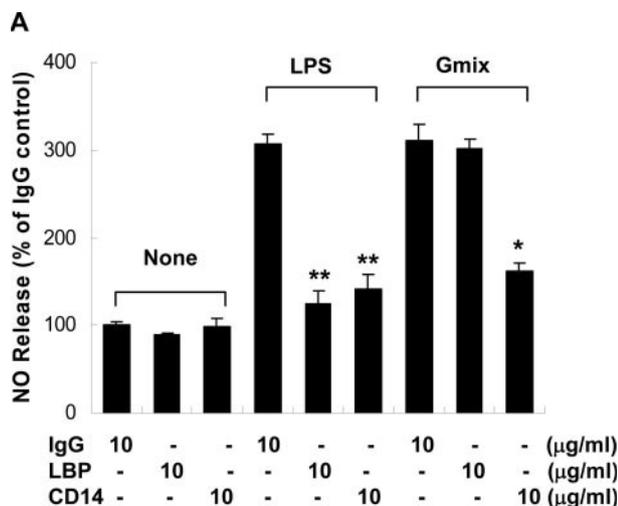
A

B

C

Figure 8. CD14 is required for ganglioside-induced inflammatory response. **A:** Primary microglia were preincubated with 10 μg/ml of anti-CD14 mAb, anti-LBP mAb, or isotype control mAb, and then the cells were treated with 50 μg/ml of Gmix or 100 ng/ml of LPS as a control for 24 hours. **B:** Primary microglia with 10 μg/ml of anti-CD14 mAb, anti-LBP mAb, or isotype control mAb were treated with 50 μg/ml of GD1a for 24 hours. The amount of NO produced was determined by measuring the amount of nitrite converted from NO in the media. Values are mean ± SD of triplicates. **P < 0.001 when compared with isotype mAb-treated control samples; *P < 0.005 when compared with isotype mAb-treated control samples. **C:** Cells were treated with 50 μg/ml of Gmix or 100 ng/ml of LPS for 3 hours. Total RNA was then extracted for RT-PCR analysis.
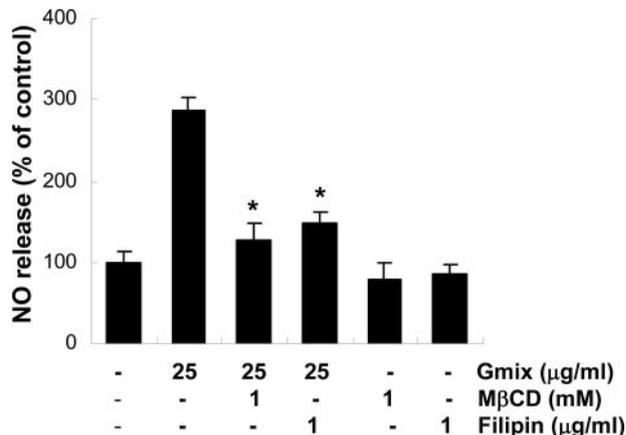

Figure 9. Ganglioside-induced NO release is significantly inhibited by raft-disrupting drugs, such as MβCD and filipin. Primary microglia were incubated with MβCD or filipin for 30 minutes and then stimulated with the ganglioside mixture for 24 hours. The amount of NO produced was determined by measuring the amount of nitrite converted from NO in the media. Values are mean ± SD of triplicates. *P < 0.001 when compared with Gmix alone-treated samples.

thologies of the brain.[11,16–23] However, little is known about the molecular mechanisms underlying the action of gangliosides as a leading cause for pathological immune responses in the brain. One intriguing question is how gangliosides can initiate inflammatory signaling cascades. In the present study, we demonstrate that TLR4, an important sensor for pathogens, can recognize ganglioside-derived stimuli and transduce inflammatory signaling cascades in brain immune cells. These findings have helped elucidate the molecular mechanisms by which gangliosides trigger inflammatory signaling responses in the brain.

TLRs are a family of proteins involved in the initial phase of host defense against various pathogens. TLRs have been found in diverse cell types, including peritoneal macrophages, dendritic cells, intestinal epithelial cells, endothelial cells, and hepatic stellate cells.[31,43–45] These cells have preferential expression of TLRs, and TLR expression levels differ even in the same cell type according to the cell state. For example, whereas immature dendritic cells express TLRs 1, 2, 4, and 5, on maturation expression of these molecules is down-regulated and expression of TLR3 is up-regulated.[31,46,47] In addition, the level of TLR expression changes on ligand binding, although this differs depending on cell type and environmental state. Thus, analysis of TLR expression profiles provides valuable information about the functions of TLRs in certain immune responses. Recently, several reports have shown that microglia and astrocytes also express TLRs and that TLR activation can lead to activation of inflammatory signaling and neurodegeneration.[32,33,48,49] We have also observed that TLR4 is present in rat primary microglia and astrocytes and that its expression is regulated on exposure to TLR4 ligands. We therefore hypothesized that TLRs could respond to gangliosides and trigger ganglioside-stimulated inflammatory signaling in rat brain and tested this hypothesis by examining TLR expression patterns after exposure to gangliosides. Interestingly, we found that TLR4 expres-

sion was rapidly changed after ganglioside treatment in a manner similar to that induced by LPS treatment (Figure 1). In contrast, IFN-γ, a well known glial activator, had no effect on surface expression level of TLR4 in rat primary microglia and astrocytes. TLR4 expression profiles of ganglioside-treated cells provide a clue that TLR4 may be involved in inflammatory responses by gangliosides.

Although relatively little is known regarding the amounts of gangliosides released from injured cells into the extracellular space (ECS), studies have shown that the ganglioside content of brain tissue is ~1.5 to 24.1 μg per mg dry weight.[50–52] The ECS, which forms the microenvironment of neurons and glia, is in contact with the cerebrospinal fluid. Thus, it is notable that the concentration of GD3 was found to range from 24 ± 9 to 64.6 ± 13 nmol/L, and the concentration of GM1 was ~19 nmol/L in cerebrospinal fluid from normal individuals.[16,53] Under pathological states such as ischemia, injury, gliosis, and demyelination, the compositions and volume of the ECS have been found to be changed, which may cause molecular crowding in the ECS. This may slow the movement of various molecules and increase their local concentrations at injured sites.[54] Thus, the concentration of gangliosides in the ECS at injured sites may be much higher than the reported level of gangliosides from cerebrospinal fluid. Furthermore, *in vitro* studies have shown that ganglioside concentrations ranging from 16 to 100 μmol/L are required for protein phosphorylation and/or modulation of several signaling events.[55–59] Based on these *in vivo* and *in vitro* findings, we chose to use ganglioside concentrations of 1 to 100 μg/ml (~0.7 to 70 μmol/L) for the present study. These doses induced the production of inflammatory signaling molecules such as NO and cytokines but were nontoxic to the used cells.

Gangliosides contain sialic acid residues (*N*-acetylneuraminic acids) linked to the sugar residues of a ceramide oligosaccharide. Several types of gangliosides exist. The approximate percentages of each ganglioside present in the brain ganglioside mixture (Gmix) used in the current study are as follows: 18% GM1, 55% GD1a, 15% GD1b, 10% GT1b, and 2% others. We investigated whether the structural diversity of gangliosides affected their modulation of TLR4 expression and compared the effects of GM1, GD1a, GT1b, and Gmix on TLR4 surface expression. Despite containing different numbers of sialic acid residues per molecule, GM1, GD1a, and GT1b influence TLR4 expression to a similar extent, which was similar to that caused by Gmix (Figure 2A). Although the number of sialic acid residues on gangliosides does not appear to influence TLR4 expression, the presence of sialic acids was critical, because asialo-GM1 did not modulate TLR4 expression (Figure 2C). Thus, it appears that the sialic acid residues of gangliosides are required for TLR4 ligation and that one residue per molecule is sufficient for this process. These results are consistent with our previous report that the presence of sialic acid residues is important for ganglioside-stimulated JAK-STAT inflammatory signaling, although the number of sialic residues per ganglioside molecule does not influence phosphorylation.[11]

Our above results raised the question of whether TLR4 could function as an upstream signal transducer in inflammatory pathways activated by gangliosides. As a first step to addressing it, we tested the function of TLR4 in ganglioside-stimulated intracellular signaling using siRNA and a dominant-negative gene for TLR4. TLR3 recognizes not only viral double-stranded (ds) RNA but also synthetic dsRNAs.[60] Astrocytes have been reported to express preferentially TLR3 and produce cytokines on TLR3 ligation.[61] Thus, we used scrambled siRNA as a control for the effects of TLR4 siRNA and tested the basal transcript level of inflammatory cytokines in siRNA-transfected cells (Figures 5 and 6). In transfection analysis with TLR4-wild-type or its mutant plasmid, we used vector alone as a control because TLR9 can recognize bacterial DNA and can be activated as a result of the plasmid transfection.[62] Several experimental approaches show that TLR4 is required for activation of various ganglioside-induced signaling events including NF-κB and expression of proinflammatory cytokines (Figures 5, 6, and 7). These results convincingly demonstrate that TLR4 indeed plays a role in ganglioside-stimulated intracellular inflammatory signaling.

In addition, we observed that CD14 also participates in recognition of gangliosides using blocking antibodies (Figure 8). Increasing evidence has suggested that immune responses are often regulated by dynamic associations among multiple receptors and signaling molecules rather than by a single receptor, and these associations may occur within lipid rafts.[40–42] It has been shown that the membrane form of CD14 is constitutively present in lipid rafts, and TLR4 is recruited to rafts after stimulation. Furthermore, gangliosides, especially GM1, are considered as representative components of lipid rafts. We therefore asked if lipid raft formation might be associated with ganglioside-triggered inflammatory responses, and tested whether ganglioside-induced NO release, a ganglioside-triggered inflammatory response, could be influenced by raft-disrupting agents. Indeed, we found that lipid raft formation appeared to be necessary for ganglioside-stimulated release of NO, indicating that this response might require lipid raft formation. Mechanistically, it is possible that ligation of CD14 by gangliosides triggers recruitment of TLR4 to lipid rafts and subsequent TLR4-activated intracellular signaling. However, further studies will be needed to ascertain this issue.

It has been reported that the downstream effects after TLR4 ligand binding are elicited via the TIR domain. On stimulation, TLR4 recruits adaptor molecules containing the TIR domain, which activates inflammatory signaling cascades and ultimately leads to activation of NF-κB and transcription of immunologically relevant genes such as proinflammatory cytokines.[26,29] These adaptors include myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal, also known as TIRAP), TIR-containing adaptor molecule (TRIF, also known as TICAM-1), and TRIF-related adapter molecule (TRAM, also known as TICAM-2). The interaction of TLRs with distinct combinations of adapter molecules creates a platform to which additional kinases, *trans*-acting factors, and possibly other molecules are recruited, events that ultimately
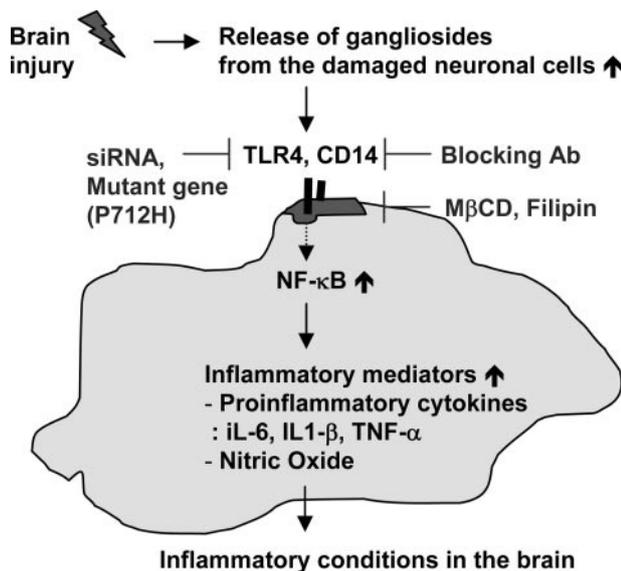
Figure 10. Schematic diagram depicting possible mechanisms for ganglioside-triggered inflammation in the brain. Brain injury causes damage to neuronal cells, which release gangliosides into the extracellular space. TLR4 and CD14 recognize these gangliosides within lipid rafts and activate downstream inflammatory signaling cascades in glia, leading to pathological conditions in the brain.

lead to gene expression.[63] We are therefore currently investigating ganglioside-engaged adaptor molecules and kinases, which will provide additional information about the question of how TLR4 can stimulate specific inflammatory signaling cascades in response to gangliosides and provide a foundation for the future development of therapeutic methods for inflammation-associated neuronal diseases including Alzheimer's disease and Parkinson's disease.

TLR4 was first identified as the signal transducer for LPS in 1998.[38] Since then, several other structurally unrelated molecules have been reported to activate TLR4 signaling, including microbial components from bacteria, fungi, and viruses,[64–66] as well as endogenous molecules such as heat-shock proteins, fibronectin, fibrinogen, hyaluronan, and lectin surfactant protein A.[67–70] However, the molecular basis underlying these ligand-receptor interactions is not yet clear, and in many cases the stimulatory capacities of these ligands await confirmation *in vivo*.[71] Here we report for the first time that gangliosides may also function as an endogenous ligand for TLR4. Recent studies using knockout mice have suggested that danger-induced organ injury, including ischemia, is closely associated with TLR4-dependent signaling. For example, TLR4-deficient mice exhibited reduced inflammation after myocardial and hepatic ischemia-reperfusion injury,[72–73] indicating that TLR4 likely plays a proinflammatory role in ischemia-reperfusion injury.[74] In addition, TLR4-deficient mice showed inadequate chemokine gradient and decreased activation of NF-κB after lung injury, leading to decreased survival.[74] Several endogenous TLR4 ligands, such as HMGB1, may be released from injured tissues, providing a critical link between tissue injury and activation of TLR4-mediated immune responses.[72–74] On the basis of these reports

and our results, we propose the following scenario. Brain injury damages neuronal cells, which release gangliosides into the extracellular space. TLR4 and CD14 recognize the abnormally released gangliosides within lipid rafts and activate downstream inflammatory signaling cascades, leading to pathological conditions in the brain (Figure 10).

Inflammation has emerged as a leading cause of various brain diseases, and defining the inflammatory mechanisms that operate in the brain is thus of great importance. To date, these mechanisms are not completely understood. The recent discovery of TLRs has led to a remarkable increase in our knowledge of the immune system, leading to rapid strides to explain many biological events including inflammation-associated diseases. Here we report that TLR4 may provide an explanation for ganglioside-dependent inflammatory events in brain immune cells. Our data provide the first clue regarding the involvement of TLR4 in ganglioside-stimulated inflammation in the brain. Because gangliosides have been closely implicated as important endogenous molecules in diverse neuronal diseases, further study of ganglioside-dependent inflammatory signaling through TLR4 should contribute to a better understanding of brain inflammation and provide interesting insights into potential therapeutic interventions for inflammation-associated neuronal diseases.

Acknowledgments

References

1. Ridet JL, Malhotra SK, Privat A, Gage FH: Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci 1997, 20:570–577
2. Matyszak MK: Inflammation in the CNS: balance between immunological privilege and immune responses. Prog Neurobiol 1998, 56:19–35
3. Streit WJ, Walter SA, Pennell NA: Reactive microgliosis. Prog Neurobiol 1999, 57:563–581
4. Aloisi F, Ria F, Adorini L: Regulation of T-cell responses by CNS antigen-presenting cells: different roles for microglia and astrocytes. Immunol Today 2000, 21:141–147
5. Mutoh T, Tokuda A, Miyadai T, Hamagucci M, Fujiki N: Ganglioside GM1 binds to the Trk protein and regulates receptor function. Proc Natl Acad Sci USA 1995, 92:5087–5041
6. Fukumoto S, Mutoh T, Hasegawa T, Miyazaki H, Okada M, Goto G, Furukawa K, Urano T: GD3 synthase gene expression in PC12 cells results in the continuous activation of TrkA and ERK1/2 and enhanced proliferation. J Biol Chem 2000, 275:5832–5838
7. Yamashita T, Hashiramato A, Haluzik M, Mizukami H, Beck S, Norton N, Kono M, Tsuji S, Daniotti JL, Werth N, Sandhoff R, Sandhoff K, Proia RL: Enhanced insulin sensitivity in mice lacking ganglioside GM3. Proc Natl Acad Sci USA 2003, 100:3445–3449
8. Miljan EA, Bremer EG: Regulation of growth factor receptors by gangliosides. Sci STKE 2002, 160:RE15
9. Mitsuda T, Furukawa K, Fukumoto S, Miyazaki H, Urano T: Overexpression of ganglioside GM1 results in the dispersion of platelet-

derived growth factor receptor from glycolipid-enriched microdomains and in the suppression of cell growth signals. J Biol Chem 2002, 277:11239–11246

10. Zurita AR, Crespo PM, Koritschoner NP, Daniotti JL: Membrane distribution of epidermal growth factor receptors in cells expressing different gangliosides. Eur J Biochem 2004, 271:2428–2437

11. Kim OS, Park EJ, Joe E, Jou I: JAK-STAT signaling mediates gangliosides-induced inflammatory responses in brain microglial cells. J Biol Chem 2002, 277:40594–40601

12. Derry DM, Wolfe LS: Gangliosides in isolated neurons and glial cells. Science 1967, 158:1450–1452

13. Sonnino S, Chigorno V: Ganglioside molecular species containing C18- and C20-sphingosine in mammalian nervous tissues and neuronal cell cultures. Biochim Biophys Acta 2000, 1469:63–77

14. Michikawa M, Gong J, Fan Q, Sawamura N, Yanagisawa K: A novel action of Alzheimer's amyloid beta-protein (Abeta): oligomeric Abeta promotes lipid release. J Neurosci 2001, 21:7226–7235

15. Blennow K, Davidsson P, Walin A, Fredman P, Gottfries CG, Karlsson I, Mansson JE, Svennerholm L: Gangliosides in cerebrospinal fluid in 'probable Alzheimer's disease.' Arch Neurol 1991, 48:1032–1035

16. Gisslen M, Hagberg L, Norkransn G, Lekman A, Fredman P: Increased cerebrospinal fluid ganglioside GM1 concentrations indicating neuronal involvement in all stages of HIV-1 infection. J Neurovirol 1997, 3:148–152

17. Kanda N, Watanabe S: Gangliosides GD1b, GT1b, and GQ1b enhance IL-2 and IFN-γ production and suppress IL-4 and IL-5 production in phytohemagglutinin-stimulated human T cells. J Immunol 2001, 166:72–80

18. Pyo H, Joe E, Jung S, Lee SH, Jou I: Gangliosides activate cultured rat brain microglia. J Biol Chem 1999, 274:34584–34589

19. Ryu JK, Shin WH, Kim J, Joe E, Lee YB, Cho KG, Oh YJ, Kim WU, Jin BK: Trisialoganglioside GT1b induces in vivo degeneration of nigral dopaminergic neurons: role of microglia. Glia 2002, 38:15–23

20. Yanagisawa K, Okada A, Suzuki N, Ihara Y: GM1 ganglioside-bound amyloid beta-protein (A beta): a possible form of preamyloid in Alzheimer's disease. Nat Med 1995, 1:1062–1066

21. Ledesma MD, Silva JSD, Cassaerts K, Delacourte A, Strooper BD, Dotti CG: Brain plasmin enhances APP alpha-cleavage and Abeta degradation and is reduced in Alzheimer's disease brains. EMBO Rep 2000, 6:530–535

22. Mclaurin J, Franklin T, Fraser PE, Chakrabartty A: Structural transitions associated with the interaction of Alzheimer β-amyloid peptides with gangliosides. J Biol Chem 1998, 273:4506–4515

23. Wang SS, Rymer DL, Good TA: Reduction in cholesterol and sialic acid content protects cells from the toxic effects of β-amyloid peptides. J Biol Chem 2001, 276:42027–42034

24. Medzhitov R, Preston-Hurlburt P, Janeway Jr CA: A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997, 388:394–397

25. Aderem A, Ulevitch RJ: Toll-like receptors: critical proteins linking innate and acquired immunity. Nature 2000, 406:782–787

26. Akira S: Toll-like receptors. Curr Opin Immunol 2003, 15:5–11

27. Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, Bassetti M, Aderem A: The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 1999, 401:811–815

28. Sabroe I, Read RC, Whyte MKB, Dockrell DH, Vogel SN, Dower SK: Toll-like receptors in health and disease: complex questions remain. J Immunol 2003, 171:1630–1635

29. O'Neill LA: Signal transduction pathways activated by the IL-1 receptor/toll-like receptor superfamily. Curr Top Microbiol Immunol 2002, 270:47–61

30. Weiss DS, Raupach B, Takeda K, Akira S, Zychlinsky A: Toll-like receptors are temporally involved in host defense. J Immunol 2003, 172:4463–4469

31. Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, Segal DM: Regulation of Toll-like receptors in human monocytes and dendritic cells. J Immunol 2001, 166:249–255

32. Bowman CC, Rasley A, Tranguch SL, Marriott I: Cultured astrocytes express toll-like receptors for bacterial products. Glia 2003, 43:281–291

33. Olson JK, Miller SD: Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J Immunol 2004, 173:3916–3924

34. Yang MS, Park EJ, Sohn S, Kwon HJ, Shin WH, Pyo HK, Jin B, Choi S, Jou I, Joe E: Interleukin-13 and -4 induce death of activated microglia. Glia 2002, 38:273–280

35. Rehli M: Of mice and men: species variations of Toll-like receptor-expression. Trends Immunol 2002, 23:375–378

36. Weiss DS, Raupach B, Takeda K, Akira S, Zychlinsky A: Toll-like receptors are temporally involved in host defense. J Immunol 2004, 172:4463–4469

37. Dreyfus H, Guerold B, Freysz L, Hicks D: Successive isolation and separation of the major lipid fractions including gangliosides from single biological samples. Anal Biochem 1997, 249:67–78

38. Poltorak A, He X, Smiranova I, Liu M, Huffel CV, Du X, Birdwell D, Alejos E, Ricciardi-Castagnoli P, Layton B, Beutler B: Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998, 282:2085–2088

39. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S: Toll-like receptor 4-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the LPS gene product. J Immunol 1999, 162:3749–3752

40. Triantafilou M, Miyake K, Golenbock DT, Triantafilou K: Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J Cell Sci 2002, 115:2603–2611

41. Dustin ML: Membrane domains and the immunological synapse: keeping T cells resting and ready. J Clin Invest 2002, 109:155–160

42. Rajendran L, Simons K: Lipid rafts and membrane dynamics. J Cell Sci 2005, 118:1099–1102

43. Nomura F, Akashi S, Sakao Y, Sato S, Kawai T, Matsumoto M, Nakanish K, Kimoto M, Miyake K, Takeda K, Akira S: Endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface Toll-like receptor 4 expression. J Immunol 2000, 164:3476–3479

44. Hornef MW, Normark BH, Vandewalle A, Normark S: Intracellular recognition of lipopolysaccharide by toll-like receptor 4 in intestinal epithelial cells. J Exp Med 2003, 198:1225–1235

45. Paik YH, Schwabe RF, Bataller R, Russo MP, Jobin C, Brenner DA: Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. Hepatology 2003, 37:1043–1055

46. Reis e Sousa C: Toll-like receptors and dendritic cells: for whom the bug toll. Semin Immunol 2004, 16:27–34

47. Iwasaki A, Medzhtov R: Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004, 10:987–995

48. Lehnardt S, Lachance C, Patrizi S, Lefebvre S, Follett PL, Jensen FE, Rosenberg PA, Volpe JJ, Vartanian T: The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. J Neurosci 2002, 22:2478–2486

49. Vogel SN, Fitzgerald KA, Fenton MJ: TLRs: differential adapter utilization by toll-like receptors mediates TLR-specific patterns of gene expression. Mol Interv 2003, 3:466–477

50. DeVries GH, Zetusky WJ, Zmachinski C, Calabrese VP: Lipid composition of axolemma-enriched fractions from human brains. J Lipid Res 1981, 22:208–216

51. Saito M, Sugiyama K: Characterization of nuclear gangliosides in rat brain: concentration, composition, and developmental changes. Arch Biochem Biophys 2002, 398:153–159

52. Trindade VMT, Brusque AM, Raasch JR, Pettenuzzo LF, Rocha HP, Wannmacher CMD, Wajner M: Ganglioside alterations in the central nervous system of rats chronically injected with methylmalonic and propionic acids. Metab Brain Dis 2002, 17:93–102

53. Lekman A, Fredman P: A new procedure for determining gangliosides GD3 a potential glial cell activation marker in cerebrospinal fluid. Neurochem Int 1998, 33:103–108

54. Sykova E: Glia and volume transmission during physiological and pathological states. J Neural Transm 2005, 112:137–147

55. Chan K: Ganglioside-modulated protein phosphorylation. J Biol Chem 1987, 262:5248–5255

56. Liu Y, Li R, Ladisch S: Exogenous ganglioside GD1a enhances epidermal growth factor receptor binding and dimerization. J Biol Chem 2004, 279:36481–36489

57. Rabin SJ, Bachis A, Mocchetti I: Gangliosides activate Trk receptors by inducing the release of neurotrophins. J Biol Chem 2002, 277:49466–49472

58. Duchemin A, Ren Q, Mo L, Neff NH, Hadjiconstantinou M: GM1

ganglioside induces phosphorylation and activation of Trk and Erk in brain. J Neurochem 2002, 81:696–707

59. Tagami S, Inokuchi J, Kabayama K, Yoshimura H, Kitamura F, Uemura S, Ogawa C, Ishii A, Saito M, Ohtsuka Y, Sakaue S, Igarashi Y: Ganglioside GM3 participates in the pathological conditions of insulin resistance. J Biol Chem 2002, 277:3085–3092

60. Kariko K, Bhuyan P, Capodici J, Weissman D: Small interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signaling through Toll-like receptor 3. J Immunol 2004, 172:6545–6549

61. Farina C, Krumbholz M, Giese T, Hartmann G, Alosi F, Meinl E: Preferential expression and function of Toll-like receptor 3 in human astrocytes. J Neuroimmunol 2005, 159:12–19

62. Bauer S, Kirschning C, Hacker H, Redecke V, Hausmann S, Akira S, Wagner H, Lipford GB: Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci USA 2001, 98:9237–9242

63. Lehnardt S, Massillon L, Follett P, Jenson FE, Ratan R, Rosenberg PA, Volpe JJ, Vartanian T: Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. Proc Natl Acad Sci USA 2004, 100:8514–8519

64. Shoham S, Huang C, Chen JM, Golenbock DT, Levitz SM: Toll-like receptor 4 mediates intracellular signaling without TNF-α release in response to Cryptococcus neoformans polysaccharide capsule. J Immunol 2001, 166:4620–4626

65. Wang JE, Warris A, Ellingsen EA, Jorgensen PF, Flo TH, Espevik T, Solberg R, Verweij PE, Aasen AO: Involvement of CD14 and Toll-like receptors in activation of human monocytes by Aspergillus fumigatus hyphae. Infect Immun 2001, 69:2402–2406

66. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, Walsh EE, Freeman MW, Golenbock DT, Anderson LJ, Finberg RW: Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol 2000, 1:398–340

67. Ohashi K, Burkart V, Flohe S, Kolb H: Cutting edge: heat shock protein 60 is a putative endogenous ligand of the Toll-like receptor-4 complex. J Immunol 2000, 164:558–561

68. Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rose J, Chow JC, Strauss III JF: The extra domain A of fibronectin activates Toll-like receptor 4. J Biol Chem 2001, 276:10229–10233

69. Smiley ST, King JA, Hancock WW: Fibrinogen stimulates macrophage chemokine secretion through Toll-like receptor 4. J Immunol 2001, 167:2887–2894

70. Guillot L, Balloy V, McCormack FX, Golenbock DT, Chignard M, Si-Tahar M: Cutting edge: the immunostimulatory activity of the lung surfactant protein-A involves Toll-like receptor 4. J Immunol 2002, 168:5989–5992

71. Backhed F, Hornef M: Toll-like receptor 4-mediated signaling by epithelial surfaces: necessity or threat? Microbes Infect 2003, 5:951–959

72. Oyama J, Blais C, Liu X, Pu M, Kobzik L, Kelly RA, Bourcier T: Reduced myocardial ischemia-reperfusion injury in toll-like receptor 4-deficient mice. Circulation 2004, 109:784–789

73. Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, Yang H, Li J, Tracey KJ, Geller DA, Billiar TR: The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med 2005, 201:1135–1143

74. Jiang D, Liang J, Fan J, Yu S, Chen S, Lou Y, Prestwich G, Mascarenhas M, Garg H, Quinn D, Homer R, Goldstein DR, Bucala R, Lee PJ, Medzhitov R, Noble PW: Regulation of lung injury and repair by toll-like receptors and hyaluronan. Nat Med 2005, 11:1173–1179