Activation of Toll-like Receptor 2 on Microglia Promotes Cell Uptake of Alzheimer Disease-associated Amyloid β Peptide*

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The human G-protein-coupled formyl peptide receptor-like 1 (FPRL1) and its mouse homologue mFPR2 mediate the chemotactic activity of a variety of polypeptides associated with inflammation and bacterial infection, including the 42-amino acid form of amyloid β peptide (Aβ42), a pathogenic factor in Alzheimer disease. Because mFPR2 was inducible in mouse microglial cells by proinflammatory stimulants, such as bacterial lipopolysaccharide, a ligand for the Toll-like receptor 4 (TLR4), we investigated the role of TLR2 in the regulation of mFPR2. We found that a TLR2 agonist, peptidoglycan (PGN) derived from Gram-positive bacterium Staphylococcus aureus, induced considerable mFpr2 mRNA expression in a mouse microglial cell line and primary microglial cells. This was associated with a markedly increased chemotaxis of the cells in response to mFPR2 agonist peptides. In addition, activation of TLR2 markedly enhanced mFPR2-mediated uptake of Aβ42 by microglia. Studies of the mechanistic basis showed that PGN activates MAPK and IkBα, and the effect of PGN on induction of mFPR2 was dependent on signaling pathways via ERK1/2 and p38 MAPKs. The use of TLR2 on microglial cells by PGN was supported by the fact that N9 cells transfected with short interfering RNA targeting mouse TLR2 failed to show increased expression of functional mFPR2 after stimulation with PGN. Our results demonstrated a potentially important role for TLR2 in microglial cells of promoting cell responses to chemotaxants produced in lesions of inflammatory and neurodegenerative diseases in the brain.

Alzheimer disease (AD)2 is a neurodegenerative disease with neuro-pathological hallmarks of intracellular neurofibrillary tangles and extracellular senile plaques composed primarily of amyloid β-peptides (Aβ) (1), in particular 42 amino acids (Aβ42). Senile plaques are considered the foci of local inflammatory responses in AD brain, as evidenced by the presence of increased levels of acute phase proteins, pro-inflammatory cytokines, complement components, and proteases (2–4). In addition, senile plaques are surrounded and often infiltrated by microglia, the monocyte phagocytes in the brain. Activated microglia in AD are thought to be major producers of proinflammatory mediators and neurotoxins (5). These cells have also been implicated in either promoting Aβ plaque formation or clearance under different experimental conditions (6–9). Aβ42 has been reported to interact with microglial cells through several purported cell surface receptors (5). Among those, the G protein-coupled formyl peptide receptor-like 1 (FPRL1) and its mouse homologue mFPR2 have been shown to mediate the chemotactic activity of Aβ42 for cells of the myeloid lineage, including microglia, and thus may be involved in the recruitment of microglial cells in lesions of AD brain (10, 11). In human macrophages, FPRL1 has also been shown to be crucial for Aβ42 uptake, a process important for Aβ42 aggregation and degradation (5, 10–15). Moreover, FPRL1, on cells from the neoroblastoma origin, appears to be responsible for the cytotoxicity of Aβ42 (15). Taken together, these observations suggest that FPRL1 may play an important role in the inflammatory amyloidosis and neurodegeneration in AD.

The expression of the FPRL1 counterpart mFPR2 in mouse microglial cells is subject to regulation by proinflammatory stimuli. We reported recently that microglial cells isolated from newborn mice expressed markedly enhanced levels of mFPR2 upon activation by tumor necrosis factor-α and LPS, suggesting that microbial infection and pro-inflammatory stress may profoundly affect the pathogenic process of AD via the up-regulation of mFPR2 in microglial cells (16, 17). Because TLR4 signals inducing mFPR2 in microglial cells play an important role in amplifying cell responses to Aβ42, the aim of the present study was to investigate whether TLR2, which is highly expressed by microglial cells, may exert stimulatory effects similar to TLR4. We report here that TLR2 in mouse microglial cells is activated by peptidoglycan (PGN), a cell wall component of Gram-positive bacteria. Activation of TLR2 in microglial cells induced the expression of mFPR2 by using ERK1/2 and p38 MAPK-dependent signaling pathways. In addition, microglial cells activated by TLR2 ligand exhibited a markedly increased capacity to endocytose Aβ42 through mFPR2.

MATERIALS AND METHODS

Reagents and Cells—PGN was purchased from InvivoGen (San Diego, CA). fMLF and LPS were purchased from Sigma. Palmitoyl-Cys(RS)-2,3-di(palmitoyloxy)-propyl)-Ala-Gly-OH (Pam–CAG) was from Bachem, King of Prussia, PA. SB202190, PD098059, and BAY117082 were obtained from Calbiochem. Mouse SDF-1α (stromal cell-derived factor 1α) was purchased from PeproTech (Rocky Hill, NJ). The Aβ42 peptide was from California Peptide Research (Napa, CA). Antibodies specific for total ERK1/2, ERK1/2 phosphorylated at Tyr-204, phospho-p38 MAPK, total p38 MAPK, phosphorylated IkBα, and...
Induction of mFPR2 by TLR2 Activation in Microglial Cells

total 1xPBS were purchased from Cell Signaling Technology (Beverly, MA). Primary murine microglial cells were isolated from 1-day-old newborn C57BL/6 mice. The murine microglial cell line N9 was a kind gift from Dr. P. Ricciardi-Castagnoli (Università Degli Studi di Milano-Bicocca, Milan, Italy). The cells were grown in Iscove’s modified Dulbecco’s medium supplemented with 5% heat-inactivated FCS, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol.

Chemotaxis Assays—Chemotaxis assays for microglial cells were performed with 48-well chemotaxis chambers and polycarbonate filters (8-μm pore size) (NeuroProbe, Cabin John, MD) as described previously (15). The results are expressed as the mean ± S.D. of the chemotaxis index (CI), which represents the fold increase in the number of migrated cells, counted in three high power fields (×400), in response to chemoattractants over spontaneous cell migration (to control medium).

Calcium (Ca2+) mobilization—Ca2+ mobilization was measured by incubating 20 × 10⁶ N9 cells/ml in loading medium (RPMI 1640 containing 10% FCS, 2 mM glutamine) with 5 μl of Fura 2-AM (Molecular Probes, Eugene, OR) for 60 min at room temperature. The dye-loaded cells were washed and resuspended in saline buffer (138 mM NaCl, 1 mM KCl, 1 mM CaCl₂, 10 mM HEPES (pH 7.4), 5 mM glucose, 0.1% bovine serum albumin) at a density of 1 × 10⁵/ml. The cells were then transferred into cuvettes (2 × 10⁶ cells/ml) that were placed in a fluorescence spectrometer (PerkinElmer Life Sciences). Stimulants were added in a volume of 20 μl to each cuvette, and the Ca²⁺ mobilization was measured by recording the ratio of the fluorescence excited at 340 and 380 nm in response to stimulants.

RT-PCR—Total RNA was extracted from cells with an RNase free kit and dephlet of contaminating DNA with RNase-free DNase (Qiagen, Valencia, CA). For amplification of the mFpr2 gene, primers 5ʹ − TCTACCATCCTCACAGGTTCTGTTGG − 3ʹ (sense) and 5ʹ − TACATCTACACACATGTGAACATGAAT − 3ʹ (antisense) were designed to yield a 268-bp product. Specific primers for mouse CXCR4 were as follows: 5ʹ − GGGCTGAGCGACGTTGTCG − 3ʹ (sense) and 5ʹ − GGAGTGCAGTCGTAGCCTG − 3ʹ (antisense), which yield a product of 390 bp. Mouse β-actin primers were as follows: sense, TGTGATTGGGGATGTTGCA, and antisense, TTTGATGTGACGCACGGTTC. The cell lysate was centrifuged at 12,000 rpm (4 °C) for 5 min, and the protein concentration of the supernatant was measured by Micro BCA Protein Assay System (Pierce). Western blotting of phosphorylated p38, ERK1/2, and β-actin was performed according to the manufacturer’s instructions using phospho-specific antibodies. Briefly, proteins were electrophoresed on a 10% SDS-PAGE precast gel (Invitrogen) under reducing conditions and transferred onto ImmunoBlot polyvinylidene fluoride membrane (Bio-Rad). The membranes were blocked with 3% nonfat milk and then were incubated with primary antibodies overnight at 4 °C. After incubation with a horseradish peroxidase-conjugated secondary antibody, the protein bands were detected with a Super Signal Chemiluminescent Substrate (Pierce) and BIOMAX-MR film (Eastman Kodak Co.). For detection of total p38, ERK1/2, and β-actin, the membranes were stripped with Restore Western blot Stripping Buffer (Pierce) followed by incubation with specific antibodies.

Flow Cytometry—Murine microglia stimulated with PGN and LPS were examined for expression of TLR2 and CD14 by labeling with phycoerythrin or FITC-conjugated monoclonal antibodies (Pharmingen). All staining procedures were completed at 4 °C in Dulbecco’s phosphate-buffered saline containing 5 mM EDTA and 1% FCS. After extensive washing, the cells were analyzed using a FACScan flow cytometer (BD Biosciences). Western Immunoblotting—N9 cells were grown in 60-mm dishes until subconfluency and then were cultured overnight in FCS-free medium. After treatment with PGN, the cells were lysed with 1 × SDS sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol), sonicated for 15 s, and then heated at 100 °C for 5 min. The cell lysate was centrifuged at 12,000 rpm (4 °C) for 5 min, and the protein concentration of the supernatant was measured by Micro BCA Protein Assay System (Pierce). Western blotting of phosphorylated p38, ERK1/2, and β-actin was performed according to the manufacturer’s instructions using phospho-specific antibodies. Briefly, proteins were electrophoresed on a 10% SDS-PAGE precast gel (Invitrogen) under reducing conditions and transferred onto ImmunoBlot polyvinylidene fluoride membrane (Bio-Rad). The membranes were blocked with 3% nonfat milk and then were incubated with primary antibodies overnight at 4 °C. After incubation with a horseradish peroxidase-conjugated secondary antibody, the protein bands were detected with a Super Signal Chemiluminescent Substrate (Pierce) and BIOMAX-MR film (Eastman Kodak Co.). For detection of total p38, ERK1/2, and β-actin, the membranes were stripped with Restore Western blot Stripping Buffer (Pierce) followed by incubation with specific antibodies.

FIGURE 1. TLR2 expression by microglia and the effect of PGN. N9 cells cultured in the presence of PGN (20 μg/ml) or LPS (300 ng/ml) for 24 h were examined for surface expression of TLR2 (A) and CD14 (B) by flow cytometry. The results are presented as percentage of positive cells and MFI.
Establishment of N9 Cells Stably Transfected with TLR2-Short Interference (si) RNA—The siRNA targeting mouse TLR2 in a mammalian cell expression plasmid was purchased from InvivoGen. A plasmid containing scrambled nucleotides (psiRNA-h75kg5Scr) was used as a control. N9 cells were transfected with TLR2-siRNA plasmid with Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions. Stable cells were selected and maintained by Zeocin (InvivoGen) at a concentration of 100 μg/ml.

Statistical Analysis—All experiments were performed at least three times, and the representative results were shown. For cell migration, the significance of the difference between test and control groups was analyzed with Student’s t test, and p values equal to, or less than, 0.05 were considered statistically significant.

RESULTS

Expression of TLR2 in Microglia and the Effect of PGN—We first examined the expression of TLR2 on murine microglial cell line N9 by flow cytometry. N9 cells constitutively expressed TLR2 on the cell surface, which was enhanced by treatment with PGN (Fig. 1A). PGN also increased N9 cell expression of CD14 (Fig. 1B), suggesting that PGN promotes the differentiation of N9 microglial cells into macrophage phenotype. Similarly LPS, a TLR4 ligand, also up-regulated TLR2 and CD14 expression by microglial cells (Fig. 1). These results are in agreement with previous reports (18, 19) and indicate the capacity of microglial cells to respond to pathogen-associated molecular patterns recognized by TLRs.

Expression of Functional mFPR2 by PGN-stimulated Murine Microglial Cells—We next examined whether activation of TLR2 on microglial cells might promote the expression of mFPR2. The mouse microglial cell line N9 and primary microglial cells treated with PGN increased the expression of mFpr2 mRNA. The effect of PGN was concentration- and time-dependent (Fig. 2, A and B), with optimal induction of mFpr2 mRNA by PGN at 20 μg/ml for 12 h. Although nonstimulated primary microglial cells expressed a relatively higher base-line level of mFpr2 mRNA as compared with nonstimulated N9 cells, PGN treatment also significantly enhanced the level of mFpr2 mRNA (Fig. 2C). In parallel experiments, we detected the expression of the gene encoding CXCR4, a G-protein-coupled receptor for the chemokine SDF-1α, in N9 cells and primary cells. However, unlike its effect on mFpr2 mRNA, PGN did not significantly affect the mRNA level of Cxcr4 in N9 cells and primary microglial cells, suggesting that PGN selectively up-regulated the mFpr2 gene.

The increased mFpr2 mRNA expression in PGN-stimulated microglial cells is associated with the acquirement of cell responsiveness to chemotactic agonist peptides for mFPR2. As shown in Fig. 3, nonstimulated N9 cells did not migrate toward a wide concentration range of fMLF (Fig. 3, A and B), a peptide derived from Gram-negative bacteria and W peptide (Fig. 3A), a well-defined synthetic mFPR2 agonist (20). In contrast, these cells showed a potent chemotactic response to the chemokine SDF-1α (data not shown). However, both fMLF and W peptide induced the migration of N9 cells treated with PGN (Fig. 3, A and B), whereas PGN treatment reduced the migration of these cells to SDF-1α (data not shown). We also observed a markedly increased migration induced by Aβ42 in N9 cells treated with PGN, and the results were confirmed with primary murine microglial cells isolated from the brains of newborn mice (Fig. 3C). Similarly, primary murine microglial cells treated with PGN showed significantly increased chemotactic response to fMLF, W peptide, and Aβ42 but reduced migration to SDF-1α (Fig. 3D).

To examine further the function of mFPR2 in PGN-activated microglial cells, we measured Ca2+ mobilization. The nonstimulated N9 cells only responded to the chemokine SDF-1α with increased Ca2+ flux, but not to the mFPR2 agonists. In contrast, although Ca2+ flux induced by SDF-1α was diminished in PGN-treated N9 cells, these cells showed significantly increased responses to challenge by fMLF and W peptide (Fig. 3E). To ascertain if the stimulatory activity of PGN is independent of LPS contamination, polymyxin B, a LPS inhibitor, was tested. We found that polymyxin B (10 μg/ml) did not affect PGN-induced mFpr2 mRNA expression (data not shown) and mFPR2-mediated migration of N9 cells to mFPR2 agonists, including fMLF and W peptide. In contrast, polymyxin B inhibited LPS-induced mFpr2 mRNA expression and mFPR2-mediated migration of N9 cells to mFPR2 agonists (Fig. 3F).
FIGURE 3. mFPR2 function in PGN-activated microglia. A, N9 cells were incubated with different concentrations of PGN at 37 °C for 24 h and then were examined for migration in response to fMLF (10⁻⁵ M) and W peptide (W pep) (10⁻⁶ M). B, N9 cells treated with 20 μg/ml PGN for the indicated time intervals were examined for migration in response to fMLF (10⁻⁵ M). C, N9 cells were stimulated with PGN (20 μg/ml) or LPS (300 ng/ml) at 37 °C for 24 h and then were examined for migration in response to Aβ42 (75 μg/ml) or fMLF (10⁻⁵ M). D, primary mouse microglial cells stimulated with 20 μg/ml PGN were examined for migration in response to fMLF (10⁻⁵ M), W peptide (10⁻⁶ M), Aβ42 (75 μg/ml), or SDF-1α (100 ng/ml). The results were expressed as CI representing fold increase in cell migration in response to chemotactants over the base-line migration (to medium). * indicates a statistically significant (p < 0.05) increase in cell migration compared with unstimulated cells. E, N9 cells cultured at 37 °C for 24 h with PGN (20 μg/ml) were examined for Ca²⁺ mobilization induced by SDF-1α (50 ng/ml), W peptide (10⁻⁶ M), and fMLF (10⁻⁵ M). F, LPS (100 ng/ml) or PGN (20 μg/ml) was preincubated with polymyxin B (Poly B) (10 μg/ml) for 1 h at 37 °C. Then the mixture was used to stimulate N9 cells for 24 h at 37 °C. The cells were examined for migration in response to fMLF (10⁻⁵ M). * indicates statistically significant (p < 0.05) decrease in cell migration compared with cells treated with LPS only.

Aβ42 uptake by PGN-activated microglia. A, N9 cells placed on chamber slides were cultured in the presence or absence of PGN (20 μg/ml) at 37 °C for 24 h and then were incubated with Aβ42 (50 μg/ml) at 37 °C for 5–30 min. The slides were stained using a monoclonal antibody against Aβ42 (green) and propidium iodide for nuclei (red) and evaluated by confocal microscopy. B, N9 cells on chamber slides were cultured in the presence or absence of PGN (20 μg/ml) for 24 h at 37 °C. The cells were then incubated with PTX (500 ng/ml), cholera toxin (CTX) (500 ng/ml), or W peptide (W pep, 1 μM) for 1 h before further incubation with Aβ42 for 24 h. The slides were stained by anti-Aβ42 antibody (green) and propidium iodide for nuclei (red). The intensity of green fluorescence detected for Aβ42 was analyzed with ImageJ (NIH software) (B), and the numbers on the x axis of the schematic correspond to the numbers in the pictures in panel B.
Induction of mFPR2 by TLR2 Activation in Microglial Cells

Thus, PGN used in this study is free of LPS. These results indicate that activation of TLR2 in mouse microglial cells enhanced the expression of functional mFPR2.

Increased Uptake of Aβ1-42 by PGN-activated Microglial Cells—We then investigated whether mFPR2 expressed by PGN-activated microglial cells mediates the internalization of Aβ1-42. Fig. 4A shows that N9 cells treated with PGN for 24 h increased their capacity to endocytose Aβ1-42 peptides as demonstrated by markedly increased Aβ1-42 fluorescence measured with confocal microscopy. This process was dependent on mFPR2 because PTX, an inhibitor of Gαi protein-coupled receptors and W peptide, another mFPR2 agonist, each abrogated the uptake of Aβ1-42 by PGN-activated microglial cells. In contrast, cholera toxin, a Gαs protein inhibitor, failed to show any inhibition of Aβ1-42 uptake by PGN-activated microglial cells (Fig. 4B), suggesting that activation of Gαs protein coupled to mFPR2 is essential for PGN-activated microglial cells to ingest Aβ1-42.

Requirement of p38 and ERK1/2 MAPK Pathways for Induction of mFPR2 by PGN in Microglial Cells—Because the MAPK pathway has been implicated in the induction of mFPR2 in microglial cells via activation of TLR4 by LPS (16) and also because PGN has been reported to increase IL-1Ra gene expression in macrophages via p38 MAPK activation (21), we assessed the role of MAPKs in PGN induction of mFPR2 in microglial cells. As shown in Fig. 5A, PGN induced a rapid and potent phosphorylation of p38 MAPK in N9 cells. However, in cells pretreated with SB202190, a p38 MAPK inhibitor, the level of mFpr2 mRNA induced by PGN was significantly reduced (Fig. 5B), and the cells failed to migrate in response to mFPR2 agonists (Fig. 5C). PGN additionally induced increased phosphorylation of ERK1/2 in N9 cells (Fig. 6A), and the MAPK/extracellular signal-regulated kinase inhibitor PD98059 reduced the expression of mFpr2 mRNA induced by PGN with decreased cell chemotaxis to mFPR2 agonists (Fig. 6, B and C). These results indicate that p38 and ERK1/2 MAPKs play an essential role in PGN induction of the mFpr2 gene and function in mouse microglial cells.

Requirement of NF-κB Activation in PGN Induction of mFPR2 in Microglial Cells—We further determined the activation of IκB, a regulatory molecule of NF-κB in microglial cells induced by PGN. Stimulation of N9 cells with PGN elicited a biphasic increase in IκBα phosphorylation, which peaked at 5 min followed by an apparent degradation and then a marked phosphorylation at 30 min (Fig. 7A), suggesting de novo synthesis of IκB (22). To study whether NF-κB activation was essential for the up-regulation of mFPR2 by PGN, N9 cells were treated with PGN in the presence...
FIGURE 8. The role of TLR2 in microglial activation by PGN. A, N9 cells transfected with TLR2-siRNA cultured in the absence or presence of PGN (20 μg/ml) for 24 h were examined for surface expression of TLR2 by flow cytometry. The results are presented as percentage of positive cells and MFI. B, N9 cells transfected with TLR2-siRNA were incubated in the presence of PGN (20 μg/ml) or LPS (300 ng/ml) for 24 h. Total RNA was extracted and examined for mFPR2 and β-actin mRNA expression by RT-PCR. ImageJ (NIH software) was used to calculate relative units to indicate the fold difference between PGN-stimulated and nonstimulated cells after normalization with β-actin transcripts. C, N9 cells transfected with TLR2-siRNA were stimulated with PGN (20 μg/ml) or LPS (300 ng/ml) for 24 h and were then examined for migration in response to fMLF (10^{-5} M). * indicates statistically significant (p < 0.05) decrease in cell migration compared with cells treated with PGN only.

FIGURE 7. The role of IκB in PGN induction of mFPR2. A, N9 cells were cultured in the presence of PGN (20 μg/ml) at 37 °C. Total proteins were examined for phospho-IκB and total IκB. B, N9 cells were cultured in the presence of different concentrations of BAY117082 (BAY) for 1 h at 37 °C before stimulation with PGN (20 μg/ml) for an additional 24 h. Total RNA was examined for mRNA expression of mFPR2 and β-actin by RT-PCR. C, N9 cells were cultured with BAY117082 (BAY) for 1 h at 37 °C before stimulation with PGN (20 μg/ml) for an additional 24 h at 37 °C, and the cells were examined for migration in response to fMLF (10^{-5} M). * indicates a statistically significant (p < 0.05) decrease in cell migration compared with cells treated with PGN only.
of a highly selective IκBε phosphorylation inhibitor BAY117082. As shown in Fig. 7, B and C, PGN-induced mFpr2 gene expression in N9 cells and cell chemotaxis in response to mFPR2 agonists were attenuated by BAY117082. Because the phosphorylation of IκBε implies release of NF-κB for nuclear translocation, our results suggest a key role for NF-κB in PGN-mediated induction of mFPR2 in mouse microglial cells. In mouse microglial cells, PGN did not increase the phosphorylation of JNK, in contrast to p38 and ERK1/2 MAPKs (data not shown).

**Involvement of TLR2 in PGN-mediated Microglial Activation**—To clarify whether PGN indeed utilized TLR2 to promote the expression of functional mFPR2 in microglial cells, we examined the effect of siRNA targeting TLR2. As shown in Fig. 8, N9 cells stably transfected with TLR2-siRNA expressed lower levels of TLR2 in the nonstimulated state as shown by moderately reduced mean fluorescence intensity (MFI) on the cell surface (Fig. 8A). Upon stimulation with PGN, N9 cells containing TLR2-siRNA did not exhibit substantially increased levels of TLR2 MF1. This is in contrast to N9 cells without TLR2-siRNA, which showed a markedly increased MFI for TLR2 on the cell surface. The decreased expression of TLR2 level on PGN-stimulated N9 cells transfected with TLR2-siRNA was associated with substantially reduced expression of mFpr2 mRNA (Fig. 8B) as well as attenuated cell chemotaxis induced by the mFPR2-specific agonist peptide Aβ42 (Fig. 8C). N9 cells containing TLR2-siRNA responded normally to the TLR4 agonist LPS by exhibiting increased mFpr2 mRNA as well as Aβ42-induced cell chemotaxis (Fig. 8, B and C), indicating that TLR2 is essential for the effect of PGN on microglial cells. We also tested another reported TLR2 agonist, PamCAG, and we found that PamCAG induced the expression and function of mFPR2 in both N9 and primary microglial cells (Fig. 8, D and E). Furthermore, in HEK-293 cells transfected with mouse Tlr2 (from InvivoGen), PamCAG as well as PGN induced phosphorylation of p38 MAPK, a kinase well known to be a component of the TLR2 signaling cascade. These results indicate that TLR2 in microglial cells can be activated by PamCAG and PGN and thus plays an important role in the induction of mFPR2 by molecular patterns.

**DISCUSSION**

We have shown that activation of TLR2 promoted the expression of functional mFPR2 in murine microglial cells through a MAPK-dependent signaling pathway in this study. PGN concomitantly down-regulated the chemotactic response of microglia to SDF-1α without significantly affecting the mRNA expression of the receptor CXCR4. The induction of mFPR2 by PGN in microglial cells is associated with a markedly increased Aβ42 internalization, which was inhibited by PTX and another mFPR2 agonist W peptide. To our knowledge, this is the first demonstration of the capacity of TLR2 to potentiate the microglial interaction with Aβ42, a key pathogenic factor in AD, via the induction of the G-protein-coupled receptor mFPR2.

Microglia are resident mononuclear phagocytes in the CNS and are key mediators of innate immune responses and inflammation in the brain (23, 24). In fact, activation of microglia is an essential component in the pathogenesis of many brain diseases, including AD, Parkinson disease (25), multiple sclerosis, AIDS dementia (26), and brain trauma caused by stroke (27). Our previous studies showed that murine microglial cells in the nonstimulated state express low levels of mFPR2 (16), a receptor that recognizes a diverse array of chemotactic agonists, including not only Aβ42 but also the bacterial fMLF, human immunodeficiency virus, type 1, envelope protein-derived peptides, and a neuroprotective peptide humanin (5, 13, 15, 17). When stimulated with LPS, the level of mFPR2 is enhanced, and the cells become responsive to mFPR2-specific agonists (16, 17, 28), suggesting that activation of TLR4 may promote microglial responses in CNS diseases in which agonists for mFPR2 are elevated (29, 30). Activation of mFPR2 and human FPR1 in phagocytic leukocytes by their peptide agonists elicits typical proinflammatory responses, including increased cell chemotaxis, phagocytoses, and release of superoxide (9, 11). Our present study reveals that TLR2 engagement by PGN is also able to induce the expression of mFPR2 by microglia. Thus, TLRs on microglial cells act as sensors for proinflammatory signals and orchestrate the host responses in the CNS.

Although TLR2 expression on microglia has been documented previously (31–34), its biological significance remains to be fully understood. It is suggested that TLR2 expression by microglia may be involved in the host defense against microbial infection and in the maintenance of homeostasis of the CNS. Despite the fact that the CNS is normally isolated from the systemic circulation by the blood-brain barrier, bacterial infection can cause CNS inflammation. Pathogens may deploy various strategies to compromise the integrity of the blood-brain barrier. For instance, *Staphylococcus aureus* establishes brain abscess through passages connecting the infection sites in the oral and facial-maxillary regions (35). Infection at distant organs such as the heart by *S. aureus* (i.e. endocarditis) also may result in brain abscess because of weakened BBB in bacteremia (35). It has been reported that exposure of
Induction of mFPR2 by TLR2 Activation in Microglial Cells

microglia in vitro to heat-inactivated S. aureus or PGN promotes the release of cytokines and chemokines (19), in which TLR2 plays a pivotal role (18, 36). In our study, activation of TLR2 in microglial cells resulted in up-regulation of mFPR2, which enabled microglial cells to respond to agonists derived from bacteria (fmLF) and AD (Aβ(1-42)), supporting the notion that activation of resident microglia through TLR2 may serve to amplify the CNS responses in inflammation and Alzheimer disease (see Fig. 9 for a schematic diagram). The use of TLR2 by PGN in microglial cells was supported by the failure of N9 microglial cells transfected with TLR2-siRNA to show increased expression of mFPR2 mRNA and chemokinesis to the mFPR2 agonist Aβ(1-42). In addition, another well characterized TLR2 agonist PamCAG also was able to up-regulate the expression of functional mFPR2 in microglial cells (Fig. 8).

Although PGN is a potent and maybe a better known TLR2 agonist derived from bacteria, mammalian cells may also constitute alternative sources of TLR2 agonists, thereby promoting inflammatory response in the absence of bacterial infection. Among the reported mammalian molecules, human heat shock proteins HSP60, HSP70, and GP96 and high mobility group box 1 (HMGB1) have been shown to interact with TLR2 as well as TLR4 to activate cells (37–40). Eosinophil-derived neutrotoxin (EDN) has also been reported to activate human dendritic cells via TLR2 (41). Thus, endogenous host-derived ligands for TLR2 can be recognized by TLR2 in the absence of bacterial infection. Among the reported mammalian ligands, the tri-lauroylated lipopeptide analogue (Lau3CSK4) is recognized efficiently by mouse TLR2 but not human TLR2 (42). In our study, human HSP70 and EDN apparently lack the capacity to stimulate mouse microglia to express functional mFPR2 (data not shown). Thus, TLR2 agonists derived from mouse cells have yet to be tested on microglial cells.

Our findings that mFPR2 expressed by TLR2 ligand-activated microglia mediates an increased cellular uptake of Aβ(1-42) may have important pathophysiological significance. In AD brain, microglia accumulating at plaque lesions contain high levels of Aβ(1-42) (8). The notion that microglia actively phagocytose the Aβ peptide is supported by experiments using in vitro culture (43) and animal AD models (44–47). In culture, microglia isolated from AD brain migrate to aggregated Aβ and are able to remove Aβ deposits over a period of 2–4 weeks (48). Consistent with these findings, cultured rat microglia interact with Aβ peptide, which is localized on the cell surface and in phagosome-like intracellular vesicles (49). In in vivo experiments, Aβ peptides injected into rat striatum are rapidly phagocytosed by microglia (44) followed by degradation and clearance of Aβ peptide (47). In mouse AD models, active immunization with Aβ peptide (50, 51) or passive immunization with antibodies to Aβ (52) resulted in substantial removal of existing Aβ deposits apparently by microglia. These observations suggest that microglial ingestion of Aβ peptide represents a host defense response aimed at removing aberrantly produced noxious agents. However, our previous studies also indicated that endocytosis of Aβ peptide by mononuclear phagocytes resulted in fibrillar deposition, a consequence of prolonged exposure of the cells to Aβ(1-42) (14, 15). It is therefore plausible that the clearance or deposition of Aβ peptide may be determined by the Aβ(1-42) peptide burden and the duration of cell exposure. It should be pointed out that Aβ(1-42) has been reported to bind several cell surface receptors, including type A (SR-A) and type B (CD36) scavenger receptors (53) and the receptor for advanced glycation end products (54). However, our present study suggests that in microglia activated by TLR ligands, the G-protein-coupled receptor mFPR2 plays a major role in mediating Aβ(1-42) internalization, because Aβ(1-42) uptake by activated microglia was aborted by the G-protein inactivator and by the mFPR2 agonist W peptide but not by the ligands for SR or receptor for advanced glycation end products (data not shown). Thus, mFPR2 in microglia not only mediates the pro-inflammatory activity of Aβ(1-42) but also may actively participate in the clearance or deposition of the amyloid β peptide. Further research is merited to delineate the beneficial versus detrimental effects of FPR1/ mFPR2 in the pathogenic process of AD and to develop novel therapeutic agents.

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