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Synergy of TRIF-dependent TLR3 and MyD88-dependent TLR7 in up-regulating expression of mouse FPR2, a promiscuous G-protein-coupled receptor, in microglial cells

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Abstract

Human G-protein-coupled formyl peptide receptor-like 1 and its mouse homologue formyl peptide receptor 2 mediate the chemotactic activity of a variety of pathogen and host-derived peptides, including amyloid β_{42} , a key causative factor in Alzheimer's disease (AD). Here, we found that Polyinosine-polycytidylic acid (Poly(I:C)), which is a specific TLR3 ligand, and Imiquimod (R837), which is a specific TLR7 ligand, when used alone, each increased MAPK-dependent functional mFPR2 expression in microglial cells, and the combination of Poly(I:C) and R837 exhibited additive effect by enhancing the level of I κ B- α phosphorylation. Our results indicated that RNA virus infection may actively participate in the pathogenic processes of brain inflammation and neurodegenerative diseases by TLR3- and TLR7-mediated TRIF-dependent and MyD88-dependent signaling pathways.

Keywords

TLR3; TLR7; Cooperation; Microglia; mFPR2

Introduction

Microglia are cells of the myeloid origin populating the CNS during embryogenesis where they become resident innate immune cells. As a type of tissue macrophages, microglia form the first line of defense against invading pathogens and provide a key link between the CNS and the immune system (1,2). In the normal brain, microglia exist in a quiescent state, but they are

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rapidly activated in response to injury and infection by bacteria or viruses. In the event of encephalitis caused by bacterial or viral invasion, microglia secrete acute-phase reactants and proinflammatory cytokines such as TNF- α , IL-6, and IL-12, which may aid in the clearance of the pathogen, but potentially at the cost of inflammation-associated damage to brain tissues (3). In addition, activation of microglia is a key component seen in the pathogenesis of degenerative CNS diseases, including Alzheimer's disease (AD), Parkinson's disease (3,4) and prion disease (5).

Detection and response to microbial infection by the immune system depends to a considerable extent on the family of Toll-like receptors (TLRs), evolutionarily conserved to recognize pathogen-associated molecular patterns (PAMPs) including Gram-positive and -negative bacteria, DNA and RNA viruses, fungi and protozoa, as well as some host derived "endogenous agonists" produced in inflammatory responses (6). Human microglia express almost all TLRs identified so far, including TLR2 and TLR3 at high levels, TLR1, TLR4, TLR5, TLR6, TLR7 and TLR8 at moderate levels and TLR9 at low but detectable levels (7). Mouse microglia express TLRs 1, 2, 3, 4, 6, 7, 8 and 9, but not TLR5 (8). Although most TLRs signal through the adaptor protein MyD88, TLR3 has been shown to trigger its signaling cascade through TRIF, independent of MyD88 (6).

Activation of TLRs converts microglial cells into potent mediators of CNS inflammation and anti-microbial responses by enhancing the transcription of a number of genes coding for cytokines and receptors crucial for host defense. Among such molecules up-regulated by several TLR agonists is a G-protein coupled formyl peptide receptor mFPR2, a homologue of human FPRL1, which recognizes bacterial and host-derived chemotactic agonist peptides, including Gram-bacterial formyl peptides (9), peptides released by damaged cells (10) and the 42 amino acid form of β -amyloid peptide ($A\beta_{42}$) associated with AD (4). Mouse microglia stimulated with ligands for TLR4 (11), TLR2 (12,13), or TLR9 (14) not only exhibited increased chemotactic responses to $A\beta_{42}$, but also markedly enhanced mFPR2-mediated uptake of $A\beta_{42}$. Thus, TLR-activated microglia may actively participate in the proinflammatory responses of the AD brain and in the processing of $A\beta_{42}$ (15), which is implicated for both direct neurotoxicity and for stimulating microglia to release neurotoxic mediators (16).

Studies have shown that in infection and inflammation, multiple TLR pathways in immune cells are likely to be activated by different components of microorganisms, and that the interplay between TLR signaling pathways may have a major impact on host responses and the outcome of the pathological processes (17–21). It is therefore important to understand the mechanistic basis of the interplay of such TLR signaling and its pathophysiological significance. In this study, we examined the effect on mFPR2 expression in mouse microglia of both TRIF-dependent TLR3 and the MyD88-dependent TLR7. Here, we report a potent synergistic up-regulation of mFPR2 in microglia by combination of suboptimal concentrations of TLR3 and TLR7 agonists through an enhanced NF- κ B activation.

Materials and methods

Reagents and cells

Polyinosine-polycytidylic acid (Poly(I:C)) and Imiquimod (R837) were purchased from InvivoGen (San Diego, CA). mFPR2 agonist peptides MMK-1 and W-peptide (WKYVMm, W pep) were synthesized and purified at the Department of Biochemistry, Colorado State University (Fort Collins, Co.), in accordance with the published sequences (22). fMLF, LPS and Resveratrol were purchased from Sigma (Saint Louis, MO). $A\beta_{42}$ peptide was from California Peptide Research (Napa, CA). Monoclonal antibody (T3.7C3) that recognizes the extracellular domain of mouse TLR3 was purchased from eBioscience (San Diego, CA). Rabbit

polyclonal anti-TLR3 antibody was purchased from ProSci (Poway, CA). Antibodies specific for total ERK1/2, ERK1/2 phosphorylated at Tyr-204, phosphor (P)-p38 MAPK, total p38 MAPK, phosphorylated (P)-I κ B- α , and total I κ B- α were purchased from Cell Signaling Technology (Beverly, MA). SB202190, PD98059, BAY117082 and LY294002 were obtained from Calbiochem (San Diego, CA). The murine microglial cell line N9 (23,24) was a kind gift from Dr. P. Ricciardi-Castagnoli (Universita 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 μ g/ml streptomycin, and 50 μ M 2-mercaptoethanol. Primary murine microglial cells were isolated from 1-day-old newborn C57BL/6 mice and were grown in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1 M HEPES, 2.5 μ g/ml Fungizone, 100 μ M nonessential amino acids, and 5 μ g/ml insulin. The purity of neonatal microglial cell population was determined by the expression of CD11b using flow cytometry to be more than 95% (11).

Chemotaxis Assays

Chemotaxis assays for microglial cells were performed with 48-well chemotaxis chambers and polycarbonate filters (8- μ m pore size) (NeuroProbe, Cabin John, MD) as described previously (12). The results are expressed as the mean $\pm$ S.D. of the chemotaxis index (CI), which represents the fold increase in the number of migrated cells, counted in three high powered fields ($\times$ 400), in response to chemoattractants over spontaneous cell migration (to control medium).

RT-PCR

Total RNA was extracted from cells with an RNeasy mini kit and depleted of contaminating DNA with RNase-free DNase (Qiagen, Valencia, CA). For amplification of mouse TLR3 gene, primers 5'-TTGCGTTGCGAAGTGAAG-3' (sense) and 5'-TAAAAAGAGCGAGGGGACAG-3' (antisense) and for mouse TLR7 gene, primers 5'-GCTGTGTGGTTTGTCTGGTG-3' (sense) and 5'-CCCCTTTATCTTTGCTTTCC-3' (antisense) were designed to yield a 405-bp and 269-bp product, respectively. For amplification of mFPR2 gene, primers 5'-TCTACCATCTCCAGAGTTCTGTGG-3' (sense) and 5'-TTACATCTACCACAATGTGAAC-3' (antisense) were designed to yield a 268-bp product. Mouse β -actin primers were used as a control with primers 5'-TGTGATGGTGGGAATGGGTCAG-3' (sense) and 5'-TTTGATGTACACGACGATTTCC-3' (antisense) that yielded a product of 514-bp. RT-PCR was performed with 0.1 μ g of total RNA for each sample (High Fidelity ProSTAR HF System, Stratagene, La Jolla, CA), consisting of a 15-min reverse transcription at 42°C, a 1-min inactivation at 95°C, 40 cycles of denaturation (30 cycles for TLR3) at 95°C (30 s), annealing at 60°C (30 s), and extension at 68°C (2 min), with a final extension for 5 min at 68°C. All PCR products were resolved by 1.5% agarose gel electrophoresis and visualized with ethidium bromide staining. For quantitation, gels were scanned, and the pixel intensity for each band was determined using the ImageJ program (NIH Image, Bethesda, MD) and normalized against the levels of β -actin.

Flow Cytometry

Microglial cells were stimulated with R837 for 24 h, harvested, fixed and permeabilized with Fixation/Permeabilization Kit (BD Biosciences, San Diego, CA). Intracellular TLR7 expression was examined by staining the cells with a polyclonal antibody to TLR7 (IMGENEX, San Diego, CA), followed by Alexa Fluor® 488 F(ab')₂ fragment of goat anti-rabbit IgG (H +L) (Invitrogen, Carlsbad, CA). All staining procedures were completed at 4°C in Dulbecco's phosphate-buffered saline (DPBS) containing 5 mM EDTA and 1% FCS. After extensive

washing, the cells were analyzed using a FACScan flow cytometer (BD Biosciences). The results are presented as percentage of positive cells and mean fluorescence intensity (MFI).

Western Immunoblotting

N9 cells grown in 60-mm dishes to subconfluency were cultured overnight in FCS-free medium. After treatment with Poly(I:C) or R837 or in combination, 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, Rockford, IL). Western blotting of TLR3 protein, phosphorylated p38, ERK1/2, IκB-α was performed using phospho-specific antibodies according to the manufacturer's instructions. Briefly, proteins were electrophoresed on 10% SDS-PAGE precast gels (Invitrogen) under reducing conditions and transferred onto ImmunoBlot polyvinylidene membrane (Bio-Rad). The membranes were blocked with 5% 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. Rochester, NY). For detection of total p38, ERK1/2, IκB-α and β-actin, the membranes were stripped with Restore Western blot Stripping Buffer (Pierce) followed by incubation with specific antibodies.

Fluorescence Confocal Microscopy

N9 cells were seeded at 2.0×10^4 cells/well on eight-well chamber slides (Nalge Nunc International) for 24 h. The cells were then treated at 37°C with Poly(I:C) or R837 or in combination for 12 h. Activated N9 cells were further treated in the presence or absence of G protein receptor deactivator pertussis toxin (PTX), cholera toxin (CTX) or W-peptide (W-pep) for 1 h followed by Aβ₄₂ (50 μg/ml) for 30 min. The cells were fixed in 2% paraformaldehyde for 20 min at room temperature, washed with PBS, and incubated with 5% normal goat serum (Sigma-Aldrich) in PBS plus 0.05% Tween 20 for 1 h to reduce nonspecific binding of antibodies to the cell surface and for cell permeabilization. An anti-Aβ₄₂ antibody (Sigma-Aldrich) was applied to the slides, which were further incubated for 1 h at room temperature. After three rinses with PBS, the slides were incubated with FITC-conjugated goat anti-mouse IgG (BD Pharmingen) in TBS buffer containing 1% BSA for 60 min. After washing with PBS, the slides were stained with propidium iodide (PI) for 20 min at room temperature. The slides were then mounted with an anti-fade, water-based mounting medium and analyzed under a laser-scanning confocal fluorescence microscope (Zeiss LSM510 NLO Meta). Excitation wavelengths of 488 nm (for FITC) and 561 nm (for PI) were used to generate fluorescence emission in green (for Aβ₄₂) and red (for nuclei), respectively. The intensity of green fluorescence detected for Aβ₄₂ was analyzed with ImageJ (NIH software).

Statistical Analysis

All experiments were performed at least three times and representative results were shown. For cell migration, the significance of the difference between test and control groups was analyzed with Student's *t* test aided by the Prism software (Prism software corporation, Irvine, CA), and *p* values equal to, or less than, 0.05 were considered statistically significant.

Results

Mouse microglia express TLR7 and TLR3

The mouse microglial cell line N9 and primary microglia isolated from the brains of newborn mice constitutively expressed TLR7 mRNA and protein (Fig. 1A, B, C) and TLR3 mRNA and

proteins (Fig. 1D, E, F). TLR3 and TLR7 mRNA and protein were enhanced by treatment of the cells with the TLR7 ligand R837, or TLR3 ligand Poly(I:C) in both N9 cell line and primary microglia (Fig. 1).

Induction of functional mFPR2 by TLR7 and TLR3 agonists in microglia

We next examined the capacity of activated TLR7 or TLR3 in microglial cells to promote the expression of the G-protein coupled receptor mFPR2. N9 cells treated with R837 or Poly(I:C) increased the expression of mFPR2 mRNA (Fig. 2A, B), which was associated with the development of chemotactic responses to the mFPR2 agonist peptide, W-peptide (W-pep) (Fig. 2B) (9). Similarly, primary murine microglial cells treated with R837 or Poly(I:C) showed markedly increased mFPR2 mRNA expression and chemotactic responses to MMK-1, another mFPR2 agonist peptide (22) (Fig. 2C, D). Microglial cells treated with R837 or Poly(I:C) also migrated in response to A β ₄₂, a key causative factor of Alzheimer's disease (AD) (Fig. 2B).

Requirement of p38, ERK1/2 MAPKs and NF- κ B for induction of mFPR2 by R837 or Poly(I:C)

Because the activation of p38, ERK1/2 and NF- κ B has been implicated in the induction of mFPR2 in microglial cells when activated by ligands for TLR2, TLR4 and TLR9 (11,12,14), we assessed the role of MAPKs and NF- κ B in TLR3 and 7 induction of mFPR2. As shown in Fig. 3A, a rapid phosphorylation of p38 MAPK at 5 min and ERK1/2 at 15 min was elicited by TLR7 agonist R837 in N9 cells. In contrast, the TLR3 agonist Poly(I:C) induced a slower phosphorylation of p38 and ERK1/2 at 30 min in N9 cells. We further determined the activation of I κ B- α , a regulator of NF- κ B and found R837 elicited a biphasic increase in I κ B- α phosphorylation, which appeared at 5 min followed by a reduction presumably due to degradation, and then again by a marked phosphorylation at 60 min, suggesting *de novo* synthesis of I κ B- α (25). In contrast, Poly(I:C) induced only a monophasic increase in I κ B- α phosphorylation at 60 min, suggesting the signals induced by Poly(I:C) in microglia are relatively weaker than those of R837.

The effect of TLR3 and TLR7 agonists on functional expression of mFPR2 in microglia was dependent on MAPKs p38 and ERK, as well as I κ B- α , since chemical inhibitors of these molecules suppressed the chemotaxis of microglia to fMLF, a Gram⁻ bacterial product and a low affinity mFPR2 agonist peptide (Fig. 3B). Both TLR3 and TLR7 agonists failed to induce phosphorylation of JNK (Thr183/Tyr185) (Data not shown), suggesting this MAPK subtype is not involved in the TLR3 and TLR7 signaling that results in mFPR2 expression.

Increased cell capacity to endocytosis A β ₄₂ in microglial Cells activated with R837 or Poly(I:C)

We investigated whether mFPR2 expressed by R837 or Poly(I:C)-activated microglial cells mediates the internalization of A β ₄₂, a key causative factor in Alzheimer's disease. As Fig. 4A shown, N9 cells treated with R837 or Poly(I:C) for 24 h increased their capacity to endocytose A β ₄₂ peptides as demonstrated by markedly increased A β ₄₂ fluorescence measured with confocal microscopy. This process was dependent on mFPR2 because PTX, an inhibitor of G α_i protein-coupled receptors abrogated the uptake of A β ₄₂ by R837 or Poly(I:C)-activated microglial cells, suggesting that activation of G α_i protein coupled to mFPR2 is essential for R837 or Poly(I:C)-activated microglial cells to internalization A β ₄₂.

Cooperation between TLR3 and TLR7 in promoting the functional expression of mFPR2 by microglia

We also found that the low level of R837 combined with the low level of Poly(I:C) increased microglial capacity to endocytose A β ₄₂ peptide as compared with them used alone, respectively (Fig. 4B). The ingestion of A β ₄₂ by microglial cells stimulated with R837 in combination with

Poly(I:C) was significantly inhibited by PTX, and inhibitor of $G\alpha_i$ protein-coupled receptors, or by another mFPR2 agonist, W-peptide. In contrast, cholera toxin, a $G\alpha_s$ protein inhibitor, failed to show any inhibition of $A\beta_{42}$ uptake by microglia activated with R837 combined with Poly(I:C) (Fig. 4B).

In order to further elucidate that R837 and Poly(I:C) synergistically promote to endocytose $A\beta_{42}$ peptide by microglial cells was dependent on up-regulation of mFPR2, we examined whether R837 combined with Poly (I:C) synergistically induces functional expression of mFPR2 in microglia. Microglial cells stimulated simultaneously with low concentrations of R837 and Poly(I:C) increased the expression of mFPR2 mRNA (Fig. 5A) and associated with much more potent chemotaxis in response to $A\beta_{42}$ as compared with cells stimulated with a single TLR agonist (Fig. 5B). To ascertain if the stimulatory activity of both R837 and Poly (I:C) is independent of LPS contamination, polymyxin B, a LPS inhibitor, was tested. We found that polymyxin B (10 $\mu\text{g/ml}$) did not affect R837- and Poly(I:C)- induced mFPR2 expression (data not shown) and function. In contrast, polymyxin B inhibited LPS-induced mFPR2 mRNA expression and mFPR2-mediated migration of N9 cells to mFPR2 agonist peptide (Fig. 5C).

Cooperative up-regulation of functional mFPR2 by R837 and Poly(I:C) in microglial cells would be dose dependent. As Fig. 5C shown, the synergistic effect of R837 and Poly(I:C) increasing cell chemotaxis in response to W-peptide disappeared when the level of R837 increased to 2 $\mu\text{g/ml}$.

Enhanced activation of $\text{I}\kappa\text{B}-\alpha$ in microglial cells stimulated by R837 in combination with Poly (I:C)

A monoclonal antibody that recognizes the extracellular domain of human TLR3 was able to down-regulate Poly(I:C)-induced production of cytokines such as IL-6, IL-8, MCP-1, RANTES, and IP-10 in human lung epithelial cells and interfere with the known TLR3-dependent signaling pathways (26,27). In order to ascertain the cooperation of TLR3 and TLR7 in up-regulation of functional mFPR2 in microglia, we found this monoclonal antibody was able to down-regulate the expression of mFPR2 and mFPR2-mediated chemotaxis induced by combination of low concentrations of R837 and Poly(I:C) (Fig. 6A, B).

Since NF- κB is a common downstream signaling component activated by both MyD88-and TRIF-dependent TLR agonists (26), we examined the effects of combination of low concentrations of R837 and Poly(I:C) on the level of phosphorylated $\text{I}\kappa\text{B}-\alpha$. When used alone, R837 at concentrations up to 1 $\mu\text{g/ml}$ induced weak phosphorylation of $\text{I}\kappa\text{B}-\alpha$, and Poly(I:C) up to 10 $\mu\text{g/ml}$ failed to activate $\text{I}\kappa\text{B}-\alpha$ in microglia. However, pretreatment of microglial cells with 10 $\mu\text{g/ml}$ Poly(I:C) for 1 h, followed by 1 $\mu\text{g/ml}$ R837, induced more potent $\text{I}\kappa\text{B}-\alpha$ phosphorylation (Fig. 6C), suggesting that R837 and Poly(I:C) cooperated to promote NF- κB release from $\text{I}\kappa\text{B}-\alpha$ for nuclear translocation (25).

Resveratrol (RES) (3,4',5-trihydroxy-*trans*-stilbene) is a polyphenol that specifically inhibits TRIF but not MyD88 signaling coupled to TLRs by targeting TANK-binding kinase 1 and RIP1 in TRIF complex, accompanied by reduced NF- κB activation (28–29). To examine whether NF- κB activation was essential for the up-regulation of mFPR2 in microglia by R837 combined with Poly(I:C) at low concentration, N9 cells were treated with R837 combined with Poly(I:C) in the absence or presence of a highly selective $\text{I}\kappa\text{B}-\alpha$ phosphorylation inhibitor BAY117082 (RAY) or resveratrol (RES). As shown in Fig. 6D, the chemotaxis induced by R837 combined with Poly(I:C) synergistically in response to W-peptide in microglial cells was significantly attenuated by BAY117082 or reduced by RES. No $\text{I}\kappa\text{B}-\alpha$ phosphorylation can be detected in microglial cells after stimulation with R837 combined with Poly(I:C) in the presence of BAY117082 or the $\text{I}\kappa\text{B}-\alpha$ phosphorylation was attenuated in the presence of RES.

(Fig. 6E). Our results suggested TLR3 and TLR7 agonists utilizes both MyD88- and TRIF-dependent signal pathways to increase phosphorylation of I κ B- α and release of NF- κ B, which play a key role in the induction of mFPR2 in microglial cells by TLR7 and TLR3 synergistically.

4. Discussion

In this study, we have shown that in addition to TLR2, 4 and 9 (11,12,14), activation of TLR3 or TLR7 promoted the expression of functional mFPR2 in murine microglial cells through MAPK and NF- κ B dependent signaling pathways. The induction of mFPR2 enabled microglial cells to migrate in response to various peptide agonists, including A β ₄₂ associated with AD (16,31). Activated microglial cells also increased their capacity to endocytose A β ₄₂ in an mFPR2 dependent manner. More importantly, we showed that suboptimal concentrations of TLR3 and TLR7 agonists synergistically cooperated to activate microglial cells through both MyD88- and TRIF-signaling cascades which converge by activation of the key transcription factor NF- κ B.

Cooperation between TLRs in cellular responses is common in mammalian cells. Combination of Poly(I:C) and CPG DNA synergistically induced TNF, IL-6 and IL-12p40 in mouse macrophages (21), and combination of TLR9 with TLR4 agonists promotes IL-12 release by murine DCs at levels higher than the activity of each agonist used alone (32). Other reports showed that gene expression and protein secretion of TNF, IL-1 β , IL-6, IL-10, IL-12, IL-23 and cyclooxygenase-2 (COX2) were several-fold higher in DCs stimulated with combinations of TLR ligands than in cells stimulated with a single agonist (17,33,34). In general, stimulation of TLRs by agonists activates two downstream MyD88-dependent and -independent signaling pathways. MyD88 is the immediate adaptor molecule that is common to all TLRs, with the exception of TLR3. MyD88 recruits IL-1R-associated kinase and TNFR-associated factor 6 (TRAF6) leading to activation of the canonical I κ B kinase (IKK) $\alpha\beta\gamma$ complex. IKK β phosphorylates I κ B- α resulting in its degradation and the release of NF- κ B for nuclear translocation. LPS and Poly(I:C) also activates Toll/IL-1R domain-containing adaptor (TRIF; TICAM-1). This pathway is independent of MyD88, leading to delayed activation of NF- κ B. TRIF also activates the transcriptional regulator, IFN regulatory factor (IRF)3 and the expression of IFN- β and IFN-inducible genes through the activation of TANK-binding kinase (TBK)1 and IKK ϵ . TLR3 primarily uses the TRIF pathway, whereas LPS interaction with TLR4 activates both MyD88- and TRIF-dependent pathways. It has been demonstrated that all MyD88-dependent TLR agonists synergize with Poly(I:C) in vitro in inducing TNF and IL-6 production by mouse bone marrow-derived macrophages (18). Our previous studies showed the cooperation between TLR2 and NOD2 as well as IFN γ and CD40 ligand in promoting mFPR2 expression by microglial cells (13,35). The present study extended the scope of interaction between proinflammatory stimulants by showing a marked synergistic effect of combination of suboptimal concentrations of TLR3 and TLR7 agonists in the induction of mFPR2 in microglial cells.

The cooperation between TLR7 and TLR3 in activating microglial cells may have important pathophysiological consequences. Many encephalitic retroviruses such as West Nile virus and Japanese encephalitis virus produce dsRNA during replication in the CNS (35–38). Microglia recognize dsRNA through TLR3 and therefore are a key sensor of dsRNA-producing viruses invading the CNS. Murine TLR7 and human TLR8 interact with guanosine (G)- and uridine (U)-rich ssRNA derived from human immunodeficiency virus-1 (HIV-1) (37). As one of the consequences of such interaction, our present study reveals the induction of mFPR2 by TLR3 and TLR7 engagement with dsRNA and ssRNA, bacterial RNA, and endogenous ligands such as mitochondrial RNA and mRNA released from necrotic cells. Thus, TLRs on microglial cells interact with proinflammatory signals and orchestrate host responses in the CNS in the presence

of multiple proinflammatory signals. It should be noted that recently other molecules such as RIG1 have been reported to sense dsRNA and mediate cell activation originally attributed to TLR3 (39). However, structure/function analysis have confirmed the capacity of TLR3 to sense dsRNA that are composed of more than 40 nucleotides. In contrast, RIG1 mainly interacts with dsRNA of shorter sequences (40,41). Thus viral dsRNA was able to interact multiple sensors in the host, which may favor the host anti-viral response.

Upon activation by proinflammatory or injurious stimulants, microglial cells assume a typical macrophage phenotype and secrete a variety of cytokines and chemokines involved in host defense against microbial infection and inflammatory responses. Microglial cells express a plethora of TLRs and are ready to sense PAMPs specific for these receptors. In fact, results from our previous and present studies indicate that PAMPs, including molecules from Gram-positive and -negative bacteria, CPG-containing DNA from bacteria and viruses, as well as viral dsRNA and viral ssRNA, all are capable of inducing functional mFPR2 in microglial cells, suggesting that mFPR2 or the human homologue FPRL1 is one of the major target genes, whose transcription and translation are subject to rapid up-regulation in pathological processes where multiple TLR agonists may be present. A growing body of evidence shows that TLR-activated microglia that express mFPR2/FPRL1 actively participate in the uptake and processing of A β ₄₂, which is a chemotactic agonist for mFPR2 and has direct neurotoxic effects and also stimulates microglia to release neurotoxic mediators in the AD brain (16,31). While microglial endocytosis of A β ₄₂ has been shown to be important for the “lay-down” of fibrillary A β aggregates to form the cores of senile plaques seen in the AD brain, recent studies using TLR gene deletion approach demonstrated that TLR-activation is crucial for mouse microglia to be able to endocytose and degrade A β ₄₂ peptides in a manner depended on the induction of a G protein-coupled receptor (15) and mFPR2 is likely to be the responsible receptor. Thus, based on its promiscuous nature of ligand recognition, mFPR2 in activated microglia may play central roles in innate host defense in the brain and actively participate in the course of AD pathogenesis.

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References

1. Rock RB, Gekker G, Hu S, Sheng WS, Cheeran M, Lokensgard JR, Peterson PK. Role of microglia in central nervous system infections. *Clin Microbiol Rev* 2004;17:942–964. [PubMed: 15489356]
2. Monsonego A, Weiner HL. Immunotherapeutic approaches to Alzheimer's disease. *Science* 2003;302:834–838. [PubMed: 14593170]
3. Nelson PT, Soma LA, Lavi E. Microglia in diseases of the central nervous system. *Ann Med* 2002;34:491–500. [PubMed: 12553488]
4. Le W, Rowe D, Xie W, Ortiz I, He Y, Appel SH. Microglial activation and dopaminergic cell injury: an in vitro model relevant to Parkinson's disease. *J Neurosci* 2001;21:8447–8455. [PubMed: 11606633]
5. Kovacs GG, Budka H. Prion diseases: from protein to cell pathology. *Am J Pathol* 2008;172:555–565. [PubMed: 18245809]
6. Chen K, Huang J, Gong W, Iribarren P, Dunlop NM, Wang JM. Toll-like receptors in inflammation, infection and cancer. *Int Immunopharmacol* 2007;7:1271–1285. [PubMed: 17673142]
7. Bsibsi M, Ravid R, Gveric D, van Noort JM. Broad expression of Toll-like receptors in the human central nervous system. *J Neuropathol Exp Neurol* 2002;61:1013–1021. [PubMed: 12430718]

8. McKimmie CS, Fazakerley JK. In response to pathogens, glial cells dynamically and differentially regulate Toll-like receptor gene expression. *J Neuroimmunol* 2005;169:116–125. [PubMed: 16146656]
9. Le Y, Murphy PM, Wang JM. Formyl-peptide receptors revisited. *Trends Immunol* 2002;23:541–548. [PubMed: 12401407]
10. Gao JL, Guillabert A, Hu J, Le Y, Urizar E, Seligman E, Fang KJ, Yuan X, Imbault V, Communi D, Wang JM, Parmentier M, Murphy PM, Migeotte I. F2L, a peptide derived from heme-binding protein, chemoattracts mouse neutrophils by specifically activating Fpr2, the low-affinity N-formylpeptide receptor. *J Immunol* 2007;178:1450–1456. [PubMed: 17237393]
11. Cui YH, Le Y, Gong W, Proost P, Van Damme J, Murphy WJ, Wang JM. Bacterial lipopolysaccharide selectively up-regulates the function of the chemotactic peptide receptor formyl peptide receptor 2 in murine microglial cells. *J Immunol* 2002;168:434–442. [PubMed: 11751990]
12. Chen K, Iribarren P, Hu J, Chen J, Gong W, Cho EH, Lockett S, Dunlop NM, Wang JM. Activation of Toll-like receptor 2 on microglia promotes cell uptake of Alzheimer disease-associated amyloid beta peptide. *J Biol Chem* 2006;281:3651–3659. [PubMed: 16339765]
13. Chen K, Zhang L, Huang J, Gong W, Dunlop NM, Wang JM. Cooperation between NOD2 and Toll-like receptor 2 ligands in the up-regulation of mouse mFPR2, a G-protein-coupled Abeta42 peptide receptor, in microglial cells. *J Leukoc Biol* 2008;83:1467–1475. [PubMed: 18299458]
14. Iribarren P, Chen K, Hu J, Gong W, Cho EH, Lockett S, Uranchimeg B, Wang JM. CpG-containing oligodeoxynucleotide promotes microglial cell uptake of amyloid beta 1–42 peptide by up-regulating the expression of the G-protein-coupled receptor mFPR2. *FASEB J* 2005;19:2032–2034. [PubMed: 16219804]
15. Tahara K, Kim HD, Jin JJ, Maxwell JA, Li L, Fukuchi K. Role of toll-like receptor signalling in Abeta uptake and clearance. *Brain* 2006;129:3006–3019. [PubMed: 16984903]
16. Cui Y, Le Y, Yazawa H, Gong W, Wang JM. Potential role of the formyl peptide receptor-like 1 (FPRL1) in inflammatory aspects of Alzheimer's disease. *J Leukoc Biol* 2002;72:628–635. [PubMed: 12377930]
17. Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol* 2007;7:179–190. [PubMed: 17318230]
18. Bagchi A, Herrup EA, Warren HS, Trigilio J, Shin HS, Valentine C, Hellman J. MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *J Immunol* 2007;178:1164–1171. [PubMed: 17202381]
19. Roelofs MF, Joosten LA, Abdollahi-Roodsaz S, van Lieshout AW, Sprong T, van den Hoogen FH, van den Berg WB, Radstake TR. The expression of toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. *Arthritis Rheum* 2005;52:2313–2322. [PubMed: 16052591]
20. Ouyang X, Negishi H, Takeda R, Fujita Y, Taniguchi T, Honda K. Cooperation between MyD88 and TRIF pathways in TLR synergy via IRF5 activation. *Biochem Biophys Res Commun* 2007;354:1045–1051. [PubMed: 17275788]
21. Whitmore MM, DeVeer MJ, Edling A, Oates RK, Simons B, Lindner D, Williams BR. Synergistic activation of innate immunity by double-stranded RNA and CpG DNA promotes enhanced antitumor activity. *Cancer Res* 2004;64:5850–5860. [PubMed: 15313929]
22. Hu JY, Le Y, Gong W, Dunlop NM, Gao JL, Murphy PM, Wang JM. Synthetic peptide MMK-1 is a highly specific chemotactic agonist for leukocyte FPRL1. *J Leukoc Biol* 2001;70:155–161. [PubMed: 11435499]
23. Righi M, Mori L, De Libero G, Sironi M, Biondi A, Mantovani A, Donini SD, Ricciardi-Castagnoli P. Monokine production by microglial cell clones. *Eur J Immunol* 1989;19:1443–1448. [PubMed: 2789141]
24. Lockhart BP, Cressey KC, Lepagnol JM. Suppression of nitric oxide formation by tyrosine kinase inhibitors in murine N9 microglia. *Br J Pharmacol* 1998;123:879–889. [PubMed: 9535016]
25. Karin M. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. *Oncogene* 1999;18:6867–6874. [PubMed: 10602462]
26. Duffy KE, Lamb RJ, San Mateo LR, Jordan JL, Canziani G, Brigham-Burke M, Korteweg J, Cunningham M, Beck HS, Carton J, Giles-Komar J, Duchala C, Sarisky RT, Mbow ML. Down

- modulation of human TLR3 function by a monoclonal antibody. *Cell Immunol* 2007;248:103–114. [PubMed: 18048020]
27. Matsumoto M, Kikkawa S, Kohase M, Miyake K, Seya T. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem Biophys Res Commun* 2002;24:293(5):1364–1369. [PubMed: 12054664]
 28. Youn HS, Lee JY, Fitzgerald KA, Young HA, Akira S, Hwang DH. Specific inhibition of MyD88-independent signaling pathways of TLR3 and TLR4 by resveratrol: molecular targets are TBK1 and RIP1 in TRIF complex. *J Immunol* 2005;175:3339–3346. [PubMed: 16116226]
 29. Hain R, Reif HJ, Krause E, Langebartels R, Kindl H, Vornam B, Wiese W, Schmelzer E, Schreier PH, Stocker RH, et al. Disease resistance results from foreign phytoalexin expression in a novel plant. *Nature* 1993;361:153–156. [PubMed: 8421520]
 30. Hain R, Bieseler B, Kindl H, Schroder G, Stocker R. Expression of a stilbene synthase gene in *Nicotiana tabacum* results in synthesis of the phytoalexin resveratrol. *Plant Mol Biol* 1990;15:325–335. [PubMed: 2103451]
 31. Iribarren P, Zhou Y, Hu J, Le Y, Wang JM. Role of formyl peptide receptor-like 1 (FPRL1/FPR2) in mononuclear phagocyte responses in Alzheimer disease. *Immunol Res* 2005;31:165–176. [PubMed: 15888909]
 32. Theiner G, Rossner S, Dalpke A, Bode K, Berger T, Gessner A, Lutz MB. TLR9 cooperates with TLR4 to increase IL-12 release by murine dendritic cells. *Mol Immunol* 2008;45:244–252. [PubMed: 17599410]
 33. Gautier G, Humbert M, Deauevieu F, Scuiller M, Hiscott J, Bates EE, Trinchieri G, Caux C, Garrone P. A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. *J Exp Med* 2005;201:1435–1446. [PubMed: 15851485]
 34. Napolitani G, Rinaldi A, Berton F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* 2005;6:769–776. [PubMed: 15995707]
 35. Chen K, Iribarren P, Huang J, Zhang L, Gong W, Cho EH, Lockett S, Dunlop NM, Wang JM. Induction of the formyl peptide receptor 2 in microglia by IFN-gamma and synergy with CD40 ligand. *J Immunol* 2007;178:1759–1766. [PubMed: 17237425]
 36. Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med* 2004;10:1366–1373. [PubMed: 15558055]
 37. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 2004;303:1526–1529. [PubMed: 14976262]
 38. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001;413:732–738. [PubMed: 11607032]
 39. Saito T, Gale M Jr. Differential recognition of double-stranded RNA by RIG-I-like receptors in antiviral immunity. *J Exp Med* 2008;205:1523–1527. [PubMed: 18591413]
 40. Leonard JN, Ghirlando R, Askins J, Bell JK, Margulies DH, Davies DR, Segal DM. The TLR3 signaling complex forms by cooperative receptor dimerization. *PNAS* 2008;105:258–263. [PubMed: 18172197]
 41. Liu L, Botos I, Wang Y, Leonard JN, Shiloach J, Segal DM, Davies DR. Structural basis of toll-like receptor 3 signaling with double-stranded RNA. *Science* 2008;18, 320(5874):379–81. [PubMed: 18420935]

Fig. 1. Mouse(m)TLR7 and TLR3 expression in microglial cells

N9 cell line (**A, D**) or primary microglial cells (**B, E**) isolated from newborn mice were incubated with different concentrations of R837 or Poly(I:C) at 37°C for 24 h. Total RNA was extracted and examined for TLR7 or TLR3 mRNA expression by RT-PCR. RT-PCR products were electrophoresed on agarose gels and visualized with ethidium bromide staining. The expression of β-actin mRNA was used as a control. The density of product bands was measured by ImageJ (NIH software) with normalization against β-actin bands. **C.** N9 cells were cultured in the absence (medium) or presence of R837 (5 μg/ml) for 24 h, then examined for intracellular expression of TLR7 by flow cytometry. The results are presented as percentage of positive cells and mean fluorescence intensity (MFI). **D.** N9 cells grown in 60-mm dishes to subconfluency were stimulated with Poly(I:C) (20 μg/ml) for 16 hours and then lysed. Equal amounts of total proteins were electrophoresed and blotted, and protein bands were detected with Rabbit polyclonal anti-TLR3 antibody and anti-mouse β-actin antibodies, respectively.

Fig. 2. Induction of mFPR2 mRNA expression and cell chemotaxis to mFPR2 ligands in microglial cells

N9 cell line (A) or primary microglial cells isolated from newborn mice (B) were incubated with different concentrations of R837 or Poly(I:C) at 37 °C, 5% CO₂ for 24 hours. Total RNA extracted and examined for mFPR2 mRNA expression by RT-PCR. RT-PCR products were electrophoresed on agarose gel and visualized with ethidium Bromide staining. LPS was as positive control and the expression of β -actin mRNA was used as a control. The density of product bands was measured by ImageJ (NIH software) with normalization against β -actin bands. C. N9 cells were incubated with indicated concentrations of R837 or Poly(I:C) for 24 hours and then examined for migration in response to mFPR2 ligands W-peptide (10^{-6} M) or A β 42 (75 μ g/ml), LPS was as positive control. D. Primary microglial cells isolated from newborn mice were incubated with R837 or different concentrations of or Poly(I:C) at 37 °C, 5% CO₂ for 24 hours and then examined for migration in response to another mFPR2 ligand MMK-1 (10^{-5} M). The results were expressed as the CI, representing the fold increase in cell migration in response to chemoattractants vs medium control. *, Significantly increased cell migration ($P < 0.05$) as compared with medium control.

Fig. 3. Activation of MAPK and IκB-α in microglial cells by R837 or Poly(I:C) in up-regulation of functional mFPR2 in microglial cells

A. N9 cells were cultured in the absence or presence of R837 (10 μg/ml) or Poly(I:C) (25 μg/ml) at 37 °C and lysed at the indicated time points. Equal amounts of total proteins were electrophoresed and blotted, and protein bands were detected with anti-phospho-p38 and total p38 antibodies, anti-phospho-ERK1/2 and total ERK1/2 antibodies and anti-phospho-IκB-α and total IκB-α antibodies, respectively. B. Murine primary microglial cells were cultured in the absence or presence of different concentrations of SB202190, PD98059, or BAY117082 for 1 hour at 37 °C before stimulation with R837 (5 μg/ml) or Poly(I:C) (25 μg/ml) for additional 24 hours at 37 °C. The cells were examined for migration in response to mFPR2 ligand fMLF (10^{-5} M). The results were expressed as CI representing fold increase in cell migration in response to fMLF over base-line migration (to medium). * indicates statistically significant ($P < 0.05$) decrease in cell migration compared with cells treated with R837 or Poly(I:C) alone.

Fig. 4. Increased cell endocytosis to A β ₄₂ in microglial cells activated with R837 or Poly(I:C) alone or R837 combined with Poly(I:C)

A. N9 cells placed on chamber slides were cultured in the absence or presence of R837 (5 μ g/ml) or Poly(I:C) (25 μ g/ml) at 37 °C for 24 h. Two wells of cells were incubated with PTX (500 ng/ml) for 1 hour before further incubated with A β ₄₂ (50 μ g/ml) at 37 °C for 30 min. The slides were stained by using a monoclonal antibody against A β ₄₂ (green) and propidium iodide for nuclei (red) and evaluated by confocal microscopy. B. N9 cells on chamber slides were cultured in the presence of 10 μ g/ml Poly(I:C), 1 μ g/ml R837, or in combination at 37 °C for 24 hours. The cells were then incubated with PTX (500 ng/ml), cholera toxin (CTX) (500 ng/ml), or W peptide (10⁻⁶M) for 1 h before further incubation with A β ₄₂ (50 μ g/ml) for 30 min. The slides were stained by anti-A β ₄₂ antibody (green) and propidium iodide for nuclei (red). The intensity of green fluorescence detected for A β ₄₂ was analyzed with ImageJ (NIH software) and the results from individual panels were summarized in far right panels.

Fig. 5. Combination of suboptimal concentrations of R837 and Poly(I:C) synergistically enhanced mFPR2 function in microglia

A. N9 cells were cultured in the absence or presence of Poly(I:C) (10 μg/ml), R837 (1 μg/ml) or in combination at 37°C, 5% CO₂, for 24 hours. Total RNA was extracted and examined for mFPR2 mRNA expression by RT-PCR. RT-PCR products were electrophoresed on agarose gel and visualized with ethidium bromide staining. LPS was used as a positive control and the expression of β-actin mRNA was used as a loading control. The density of RT-PCR bands was measured by ImageJ (NIH software) with normalization against β-actin bands. B. Murine primary microglial cells were cultured in the absence or presence of Poly(I:C) (10 μg/ml), R837 (1 μg/ml) or in combination and then examined for migration in response to mFPR2 ligands Aβ₄₂ (75 μg/ml). The results were expressed as the CI, representing the fold increase in cell migration in response to chemoattractants vs medium control. *, Significantly increased cell migration ($p < 0.05$) as compared with cells treated with R837 or Poly(I:C) alone. C. R837 (1 μg/ml) and Poly(I:C) (10 μg/ml) or LPS (100 ng/ml) were pre-incubated with polymyxin B (Poly B) (10 μg/ml) for 1 hour at 37°C. The mixture was then used to stimulate N9 cells for 24 hours at 37°C. The cells were examined for migration in response to W-peptide (10⁻⁶M). * indicates Statistically significant ($p < 0.05$) decrease in cell migration compared with cells treated with LPS only. D. N9 cells were stimulated with Poly(I:C), R837, or in combination at indicated concentrations at 37 °C for 24 hours and then examined for migration in response to W-peptide (10⁻⁶ M). The results were expressed as the CI, representing the fold increase in cell migration in response to chemoattractants vs medium control. *indicate significantly increased cell migration ($p < 0.05$) as compared with cells treated with R837 alone.

Fig. 6. Enhanced activation of IκB-α in microglial cells stimulated by R837 in combination with Poly(I:C)

N9 cells stimulated with 10 μg/ml Poly(I:C), 1 μg/ml R837, or in combination in the presence of mAb against mouse TLR3 for 24 hours at 37 °C. An isotype-matched rat IgG was used as a control. Total RNA extracted and examined for mFPR2 mRNA expression by RT-PCR and the RT-PCR products were electrophoresed on agarose gel and visualized with ethidium Bromide staining. LPS was as positive control and the expression of β-actin mRNA was used as a control. The density of product bands was measured by ImageJ (NIH software) with normalization against β-actin bands (A). Or examine for migration in response to mFPR2 ligands W-peptide (10⁻⁶M) (B). C. N9 cells were stimulated with 10 μg/ml Poly(I:C), 1 μg/ml R837, or in combination at 37 °C and lysed at the indicated time points. Equal amounts of total proteins were electrophoresed and blotted, and protein bands were detected with anti-phospho-IκB-α antibody, the total ERK1/2 bands were used as loading controls. D. N9 cells were incubated with 10 μg/ml Poly(I:C), 1 μg/ml R837, or in combination at 37 °C for 24 hours, or with different concentrations of BAY117082 or Resveratrol for 1 hour at 37 °C before stimulation with R837 (1 μg/ml) combined with Poly(I:C) (10 μg/ml) for additional 24 hours, and then examined for migration in response to W-peptide (10⁻⁶ M). The results were expressed as the CI, representing the fold increase in cell migration in response to chemoattractants vs medium control. *, Significantly increased cell migration (p<0.05) as compared with cells treated with R837 combined with Poly(I:C) alone. E. N9 cells were incubated with 10 μg/ml Poly(I:C), 1 μg/ml R837, or in combination at 37 °C for 30 min or with different concentrations of BAY117082 or Resveratrol for 1 hour at 37 °C before stimulation with R837 (1 μg/ml) combined with Poly(I:C) (10 μg/ml) for additional 30 min. Then the cells were lysed and Equal amounts of total proteins were electrophoresed and blotted, and protein bands were detected with anti-phospho-IκB-α antibody, the total ERK1/2 bands were used as loading controls.