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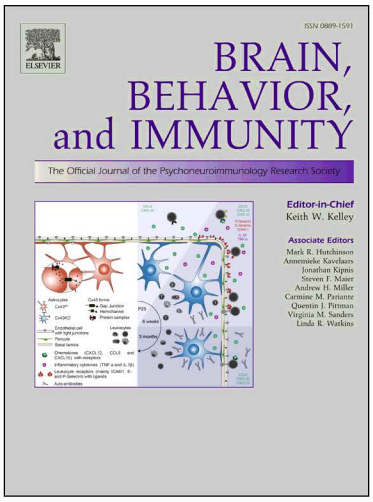

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TLR2 controls random motility, while TLR7 regulates chemotaxis of microglial cells via distinct pathways

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Abstract

Microglial cells are the pathologic sensor of the brain, and any pathologic event triggers microglial activation, which involves migration of these cells to a lesion site. Employing different migration assays, we show that ligands for toll-like receptor (TLR) 2 stimulate random motility, while TLR7 ligands are chemoattractants. The subtype specificity of the TLR ligands was verified by using different TLR-deficient (TLRKO) mouse lines. PI3K and Rac inhibition impairs both TLR2- and TLR7-stimulated microglial migration. In contrast, Akt phosphorylation is only required for the TLR2-, but not for the TLR7-stimulated pathway. Interestingly, P2Y12 receptor signaling is involved in the TLR2 activation-induced microglial migration but not TLR7. Furthermore, TLR7 mRNA expression is down-regulated by TLR2 and TLR7 activation. We conclude that TLRs control the migratory behavior of microglia in a distinct manner.

Introduction

Microglia are considered as the immune cells of the central nervous system (CNS) and are activated in any type of pathologic context (David and Kroner, 2011; Hanisch and Kettenmann, 2007). Various mediators including cytokines, chemokines, and also agonists of toll-like receptors (TLR) trigger microglial activation (Farber and Kettenmann, 2006; Rosenberger et al., 2014). Microglia express all known members of the TLR family identified to date (Hanke and Kielian, 2011). They constitutively express TLR2 (Kielian et al., 2002; Laflamme et al., 2003; Olson and Miller, 2004; Rasley et al., 2002; Zekki et al., 2002) and are capable of recognizing numerous TLR2 ligands, including lipoproteins and the synthetic lipoprotein analogue Pam3CSK4 (synthetic triacylated lipoprotein), as well as Pam2CSK4 (synthetic diacylated lipoprotein) and LTA (lipoteichoic acid) (Chien et al., 2005; Ebert et al., 2005; Jung et al., 2005; Kielian et al., 2002; Olson and Miller, 2004; Omueti et al., 2005). We have recently found that TLR2 plays an important role for mediating interaction between microglia and glioma cells. Versican is an endogenous ligand released from glioma, which activates TLR2 on microglia and converts these cells into a pro-tumorigenic phenotype (Shin et al., 2009). Recent studies demonstrated that TLR7, which recognizes viral single-stranded RNA, can play an important role in both activation of innate

immune responses and pathogenesis of autoimmune diseases (Butchi et al., 2010; Desire et al., 2005; Lewis et al., 2008; Town et al., 2009). These immune responses are not only elicited by GU-rich viral single-stranded RNA but also by synthetic chemicals that include imidazoquinoline compounds such as imiquimod and resiquimod as well as guanosine analogues such as loxoribine (Diebold et al., 2004; Heil et al., 2004; Hemmi et al., 2002). TLR signaling does not only lead to the release of neurotoxic molecules such as inflammatory cytokines and reactive oxygen species (Boje and Arora, 1992; Lehnardt et al., 2008), but also influences other cellular functions such as proliferation and cell migration. In pancreatic cells, activation of TLR7 has been reported to inhibit proliferation and migration (Zou et al., 2015), while TLR2 agonists stimulate human neutrophil migration via activation of mitogen-activated protein kinases (Aomatsu et al., 2008). In microglia, motility is controlled by a variety of substances such as ATP (Davalos et al., 2005), which is also a chemoattractant for microglial cells (Honda et al., 2001), bradykinin (Ifuku et al., 2007), galanin (Ifuku et al., 2011), or morphine (Horvath and DeLeo, 2009; Takayama and Ueda, 2005). The direct effect of TLR agonists on microglial migration has not been investigated so far.

In the present study, we investigated the TLR agonist-induced increase in microglial migration and its underlying signaling cascade. We show that primary cultured

microglial cells display enhanced random migration in response to the TLR2 agonists Pam3CSK4 and Pam2CSK4, whereas the TLR7 agonists imiquimod and loxoribine induce microglial chemotaxis but not random migration. We also found that activation of TLR2 and TLR7 trigger distinct intracellular pathways. Our results suggest that each TLR ligand may have a distinct role under various pathological and physiological conditions.

Material and Methods

Cell culture

Primary microglia cultures were prepared from the cerebral cortex and midbrain of newborn male and female C57BL/6 and TLRKO mice (P0-P3), as described previously (Prinz et al., 1999). In brief, the forebrain was carefully freed of blood vessels and meninges. Cortical tissue was trypsinized for 2 min, dissociated with a fire-polished pipette and washed twice. Mixed glial cells were cultured for 9-12 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum and antibiotics, with medium changes every third day. Microglial cells were then separated from the underlying astrocytic monolayer by gentle shaking of the flasks for 1 h at 37 °C in a shaker-incubator (100 r.p.m.). Cultures usually contained >95% microglia as detected by staining with isolectin B4 (*Griffonia simplicifolia*). Cells were maintained at 37 °C in a 5 % CO_2 humidified atmosphere.

Agarose spot assay

The agarose spot assay was performed as described before in (Wiggins and Rappoport, 2010). 0.1 g of low-melting point agarose (Promega Corporation, Madison, Wl, USA) was placed into a 100-mL beaker and diluted into 20 mL sterile PBS to make a 0.5 %

agarose solution. This was heated in microwave until boiling, swirled to facilitate complete dissolution, and then taken off the microwave. When the temperature cooled to 40 °C, 90 µl of agarose solution was pipetted into a 1.5 mL Eppendorf tube containing 10 µl of PBS with or without the TLR agonist. Ten-microliter spots of agarose were pipetted onto a 35 mm diameter glass-bottomed cell culture dish (MatTek Corporation, MA, USA) and allowed to cool for 10 min at 4 °C. Four spots per dish were pipetted, three containing the TLR agonist and one containing only PBS. Microglial cells were plated into the dish (3-5 × 10^5 cells in 2 ml DMEM) and then incubated at 37 °C in 5 % CO_2 to allow the cells to adhere and migrate. After 6 h, microglial cells under the spot were counted. The values are the average obtained from six dishes. When inhibitors (LY294002; 25 and 50 µM, Wortmannin; 0.1 and 1 µM, ET1864; 50 µM, Akt inhibitor IV; 1 and 10 µM) were added, cells were pre-treated for 30 min before plated into the dish.

Microchemotaxis assay

TLRs agonist-induced chemotaxis was tested using a 48-well microchemotaxis Boyden chamber (Neuroprobe, Bethesda, MD, USA). Upper and lower wells were separated by a polycarbonate filter (8 µm pore size; Poretics, Livermore, CA, USA). Microglial cells

(2-4 × 10^4 cells) in 50 µl of serum-free DMEM medium were added to the upper compartment, while the lower wells contained the TLRs agonist in serum-free DMEM medium. Serum-free DMEM medium was used as a control. Cells were pre-treated with the PI3K (LY294002; 25 µM) and Rac inhibitors (EHT1864; 1-100 µM) for 30 min before added to the upper compartment. The chamber was incubated at 37 °C and 5 % CO_2 for 6 h. Cells remaining on the upper surface of the membrane were removed by wiping, and cells in the lower compartment were fixed in methanol for 10 min and subjected to Diff-Quik stain (Medion Grifols Diagnostics AG, Düdingen, Switzerland). The rate of microglial migration was calculated by counting cells in four random fields of each well using a 20 × bright-field objective. For each condition 2-4 fields in 4-8 wells were analyzed. Under control conditions, there were ~40-80 cells per field, and the number of cells in each field was normalized to the average in control condition (100%).

Wound-healing scratch assay

The scratch assay was performed as described previously (Jeon et al., 2012; Karlstetter et al., 2014). Briefly, microglial cells were seeded at a density of 5 × 10^4 cells/dish in the glass-bottomed cell culture dish, and incubated at 37 °C, 5 % CO_2 for 48 h. A

scratch wound was created with a 10 µl pipette tip on the cell monolayer. Thereafter, the cells were stimulated with TLRs agonist or PBS as a control for 6 h, and preincubated with Rac inhibitor EHT1864 (50 µM) for 30 minutes. The number of migrating cells was quantified by counting all cells in a cell-free zone area. The number of migrated cells was normalized to the average in control condition (100%). A minimum of five individual cultures was used to calculate the mean migratory capacity at each condition.

Western blot analysis

Western blotting was performed using the whole cell lysates. Before collection, cells were washed with cold PBS. RIPA buffer (radioimmunoprecipitation assay buffer) was used as lysis buffer that contained 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS dissolved in 1% TBS buffer. Protease inhibitor cocktail (Roche, Grenzach-Wyhlen, Germany) and phosphatase inhibitor mixture (Sigma-Aldrich, Darmstadt, Germany) was added to the RIPA buffer before cell lysis. Cells were homogenized by a syringe needle, followed by centrifugation at 15,000 rpm for 10 minutes to remove insoluble cell debris. Protein concentration was measured by the BCA protein assay kit (Thermo Fisher Scientific, Pittsburgh, PA, USA), and a total of 20 µg protein was separated by 10% SDS-PAGE and electrophoretically transferred onto PVDF membranes (Bio-Rad

Laboratories, Hercules, CA, USA). After protein transfer, PVDF membrane were blocked with Tris-buffered saline (TBS) containing 5% BSA for 1 h at room temperature. Membranes were then incubated at 4 °C overnight with the antibodies against phospho-AKT (S-473) and Akt purchased from Cell Signaling (Danvers, MA, USA). Membranes were then washed after the treatments with primary antibodies and incubated with anti-rabbit IgG secondary antibody (Cell Signaling) at room temperature for 2 h. Signals from the transferred protein onto the PVDF membrane were then visualized by ECL Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) using ChemiDoc XRS system. Quantification of the images was performed by the Image J software.

qPCR for TLRs mRNA expression in microglia

Primary neonatal microglia were seeded at 10^6 cells per dish in a 6 well plate. After 24h, the cells were stimulated with Pam2CSK4 (300ng/ml), Pam3CSK4 (300ng/ml), imiquimod (5µg/ml), loxoribine (1mM) or LPS (100ng/ml) for 24h. Subsequently cell lysates were collected and RNA extracted, followed by cDNA synthesis and quantitative PCR expression. The TLR2 and TLR7 primers were purchased from

QIAGEN (Hilden, Germany). For the qPCR cycle used see *Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix* product information.

Drugs and reagents

Pam3CSK4, Pam2CSK4, imiquimod, and loxoribine were purchased from InvivoGen (San Diego, CA, USA). LY294002 was obtained from Cell Signaling. Wortmannin was purchased from Sigma-Aldrich. EHT1863 was provided by Tocris Bioscience (Bristol, UK). Akt inhibitor IV was purchased from Calbiochem (San Diego, CA, USA). Ticagrelor was purchased from Cayman Chemical (Hamburg, Germany).

Statistics

Results are expressed as the mean ± SEM. Statistical analyses of the results were evaluated using Dunnett's or Tukey's multiple comparison test after one-way ANOVA. Value of $p<0.05$ were considered statistically significant.

Results

TLR2 and TLR7 activation increase microglial migratory activity

To test the effects of the TLR2 agonists Pam3CSK4 and Pam2CSK4 and the TLR7 agonists imiquimod and loxoribine on microglial migratory activity, we analyzed the accumulation of cells in an agarose spot containing either PBS (as control) or a TLR agonist. The spots were placed on glass Petri dishes, and microglial cells in suspension were subsequently added. After 6 h incubation time, microglial cells that had migrated into the agarose spot were quantified (Fig. 1A). Pam3CSK4 did not significantly increase the number of accumulated microglial cells at 10 ng/ml, but at 100 and 300 ng/ml, while Pam2CSK4 increased cell numbers already at 10 and also at 100 and 300 ng/ml (Fig. 1B and Table 1). Likewise, imiquimod increased numbers of accumulated microglial cells at 1, 3, and 5 µg/ml. loxoribine increased accumulation of microglial cells at 0.1, 0.5, and 1 mM (Fig. 1C, Table 1).

To determine the specificity of the subtype-specific ligands, we cultured microglia from TLR1 knock out (KO), TLR6KO, TLR2KO, and TLR7KO mice and tested the impact of Pam3CSK4, Pam2CSK4, imiquimod, and loxoribine on microglial migration. Pam3CSK4 augmented the migration of microglia derived from TLR1KO and TLR6KO mice, but did not significantly increase the migration of TLR2KO microglia (Fig. 2A, B

and C, Table 1). Similarly, imiquimod and loxoribine did not induce migration of microglia from TLR7KO mice (Fig. 2D, Table 1).

As a second approach to evaluate microglial migratory activity induced by TLR2 and TLR7 agonists, we used the Boyden Chamber assay. We obtained similar results as observed with the agarose spot assay. As shown in Fig. 3, TLR2 and TLR7 agonists significantly increased migration of microglia in a concentration-dependent manner (Table 1). Neither Pam3CSK4 nor Pam2CSK4 stimulated migration of microglia from TLR2KO mice, and neither imiquimod nor loxoribine increased migration of cells from TLR7KO mice, substantiating the findings obtained with the agarose spot assay (Fig. 3C and Table 1).

TLR7 agonists induce microglial chemotaxis, while TLR2 agonists increase random motility

To distinguish between chemotaxis, i. e. a directed migration, and non-directed motility, we added the TLR agonists named above either with or without gradient to the agarose spot assay or the Boyden chamber. In the agarose spot assay, the respective ligand was present either only in the spot or in spot and supernatant, while in the Boyden chamber it was present in the upper, in the lower, or in both compartments. In these and the

subsequent studies we used 300 ng/ml of Pam3CSK4 and Pam2CSK4, 5 μg/ml of imiquimod, and 1 mM of loxoribine. Pam3CSK4 and Pam2CSK4 increased microglial motility in the agarose spot assay in the absence and presence of a gradient at a similar magnitude (Fig. 4A) as it was observed in the Boyden chamber (Fig. 4B). In contrast, imiquimod and loxoribine did only increase motility when a gradient was present, but not in the absence of a gradient.

To confirm these data we employed another test, namely the wound-healing scratch assay, which detects changes in motility, but not chemotaxis. Microglial cultures were scratched to generate a cell-free zone and were subsequently treated with either TLR2 or TLR7 agonists for 6 h. Treatment with 300 ng/ml Pam3CSK4 and Pam2CSK4 resulted in a larger accumulation of cells in the previously cell-free zone, leading to a 373.7 ± 31.3% and 568.4 ± 45.7% increase, respectively. Treatment with imiquimod and loxoribine did not affect the number of cells in the scratched zone (Fig. 5). These findings substantiate that both Pam3CSK4 and Pam2CSK4 increase random migration, while imiquimod and loxoribine stimulate migration along a gradient.

PI3K inhibition impairs microglial migration stimulated via TLR2 and TLR7

In this series of experiments, we assessed the role of PI3K in microglial migration

induced by TLR2 and TLR7 agonists through the use of two PI3K inhibitors, namely LY294002 and Wortmannin. LY294002 is a synthetic selective PI3K inhibitor that binds to the catalytic domain of the kinase. Wortmannin serves as a bacteria-derived semiselective PI3K inhibitor that binds to the ATP-binding domain of the kinase (Nakanishi et al., 1992; Vlahos et al., 1994). As shown in Fig. 6A, the Pam3CSK4- and Pam2CSK4-induced migration was strongly suppressed by the pretreatment of microglial cells with LY294002 or Wortmannin in the agarose spot assay. Similar results were obtained with imiquimod and loxoribine. Pretreatment with LY294002 or Wortmannin impaired the motility increase induced by the two TLR7 agonists (Fig. 6B). These results were confirmed by using the Boyden chamber. Pam3CSK4, Pam2CSK4, imiquimod, and loxoribine increased migration of microglia by 195.6 ± 17.2%, 253.4 ± 10%, 428.6 ± 31.1% and 247.3 ± 17.7%, respectively. These increases in migration were completely blocked by LY294002 and Wortmannin (Fig. 6C), suggesting that PI3K was involved in the increase in microglial migration induced by TLR2 and TLR7 activation.

Rac activation is required for microglial migration induced by TLR2 and TLR7 agonists

To determine whether Rac activation downstream of the respective TLR is also involved in migration control, we investigated the effects of a Rac inhibitor on TLR agonist-induced migration using the agarose spot assay. Microglial cells were incubated with the Rac inhibitor EHT1864 (50 µM) for 30 min before treatment with the respective TLR ligand. Pam3CSK-, Pam2CSK4-, imiquimod-, and loxoribine-induced microglial motility was inhibited by EHT1864 (Fig. 7A). We confirmed these results for the TLR7 agonists using the Boyden chamber and in addition tested different concentrations of EHT1864. Imiquimod- and loxoribine-induced microglial chemotaxis was blocked by 50 and 100 µM EHT1864, while 1 µM had no effect, and 10 µM led to a partial blockade (Fig. 7B). The effects of the Rac inhibitor on microglial motility induced by TLR2 activation were also tested in the wound-healing scratch assay. As shown by representative images in Fig. 7C and by quantification from three independent experiments in Fig. 7D, EHT1864 strongly suppressed the increase in Pam3CSK4- and Pam2CSK4-induced microglial motility from 352.2 ± 41.2% and 377.8 ± 29.4% of control to 88.8 ± 10.3% and 111.1 ± 21.4% in the presence of EHT1864. These results suggest that a functional Rac signaling is a prerequisite for TLR2 and TLR7-induced microglial migration.

The Akt pathway is involved in TLR-2, but not in TLR7-triggered migration

Pretreatment with Akt inhibitor IV significantly inhibited the TLR2-induced migration of microglia (Fig. 7E). However, imiquimod- and loxoribine-induced microglial motility was not affected by Akt inhibitor IV in the agarose spot assay (Fig. 7F), indicating that Akt activation is required for TLR2-, but not TLR7-stimulated migration. The results outlined above indicate that Akt phosphorylation controls Pam3CSK4- and Pam2CSK4-induced microglial migration. We therefore investigated whether TLR2 ligands trigger Akt phosphorylation by Western blot analysis of microglial lysates using a phosphor-specific Akt antibody following Pam3CSK4 and Pam2CSK4 stimulation. For these experiments, we exposed microglia to 300 ng/ml Pam3CSK4 or Pam2CSK4 for 0, 5, 15, 30, and 60 min, and subsequently assessed pAkt and total Akt expression. Pam3CSK4 and Pam2CSK4 induced a significant increase in Akt phosphorylation after 15 min, and the phosphorylation levels peaked at 30 min (Fig. 8A). Total Akt levels remained unchanged during the entire 60 min period. To determine whether Pam3CSK4- and Pam2CSK4-mediated Akt phosphorylation is dependent on PI3K, we again used the PI3K inhibitor LY294002. Both 25 and 50 μM of LY294002 significantly inhibited Akt phosphorylation triggered by Pam3CSK4 and Pam2CSK4 (Fig. 8B). To confirm that Pam3CSK- and Pam2CSK4-mediated Akt phosphorylation is

TLR2-dependent, we used microglial cells from TLR2KO mice. Pam3CSK4 and Pam2CSK4 had no effect on Akt phosphorylation in microglia lacking TLR2 (Fig. 8C). As a positive control, 100 μM ATP significantly increased phosphorylation of Akt. These results indicate that Pam3CSK4 and Pam2CSK4 increase phosphorylation of Akt in a PI3K- and TLR2-dependent manner.

Involvement of the P2Y12 pathway in TLR2, but not TLR7 agonists-induced microglial migration

It is well established that ATP-induced microglial membrane ruffling and chemotaxis are mediated by Gi/o-protein coupled P2Y12 receptor (Honda et al., 2001; Sasaki et al., 2003) and P2Y12 receptor stimulation results in activation of the PI3K pathway and subsequent increase in microglial migration (Irino et al., 2008; Ohsawa et al., 2007; Wu et al., 2007). To determine whether P2Y12 activation is required for microglial migration by induced by TLR2 and TLR7 agonists, we investigated the effects of the P2Y12 receptor inhibitor ticagrelor in the agarose spot assay. As shown in Figure 9A, ticagrelor inhibited the increase in Pam3CSK4- and Pam2CSK4-induced microglial migration. In contrast, imiquimod- and loxoribine-induced microglial chemotaxis was

not affected by ticagrelor (Fig. 9B). These results indicate that P2Y12 receptor are involved in the TLR2, but not TLR7 mediated increase in microglial migration.

TLR7 but not TLR2 expression is regulated by TLR2 and TLR7 activation

To determine the effects of the TLR activation on the expression of TLR2 and TLR7 in microglia, mouse primary microglial cell cultures were treated with normal medium (as control) or TLR agonists (Pam3CSK4, Pam2CSK3, imiquimod, loxoribine and LPS) for 24 hours. We subsequently performed quantitative PCR (qPCR) and found that the TLRs agonists did not significantly alter the TLR2 mRNA expression (Fig. 10). The TLR7 mRNA expression, however, was significantly downregulated by TLR2 and TLR7 agonists, indicating that TLR2 and TLR7 activation results in downregulation of microglial TLR7, but not TLR2 (Fig. 10).

Discussion

Parenchymal microglia are the main cell type that contributes to CNS innate immune signaling and express the complete repertoire of identified TLRs as well as several inflammasome-related molecules, which together endow the cell with potent inflammatory capacity. As the resident immune cells of the brain, microglia serve as sensors of events occurring within their environment and provide the first line of defense against invading microbes. Upon recognition of pathogens, activated microglia migrate rapidly to the sites of tissue damage and express genes related to inflammation such as pro-inflammatory cytokines and radicals (Hua et al., 2007).

We used three different methods to demonstrate the effect on TLR2 and TLR7 agonists on microglial migratory activity. Moreover, we distinguished between a general increase in motility by using the agarose spot assay and the Boyden chamber without gradient and the wound-healing scratch assay and directed chemotaxis by using the agarose spot assay and the Boyden chamber with gradient. We found that the TLR2 agonists Pam3CSK4 or Pam2CSK4 increased microglial motile activity, but not chemotaxis. This finding is similar to the behavior of human neutrophils in which TLR2 activation induces random migration (Aomatsu et al., 2008). In contrast, the TLR7 agonists imiquimod and loxoribine stimulated directed migration towards their respective

gradients. Thus, the two receptor systems control distinct migratory behavior of microglia, namely directed migration, i. e. chemotaxis by TLR7, and a general motility increase induced by TLR2, independently of a gradient.

Another difference between TLR2 and TLR7 induced migration is the involvement of the purinergic signaling. We found that Pam3CSK4- and Pam2CSK4-induced microglial migration was significantly inhibited by ticagrelor, a P2Y12 antagonist (Fig. 9A). These findings indicated that TLR2 activation enhance microglial migration through activation of P2Y12 receptor signaling. It is well established that P2Y12 receptors control microglial migration (Honda et al., 2001; Ohsawa et al., 2007). How TLR2 activation induces the activation of P2Y12 remains speculative. We did also not distinguish whether the intracellular pathways which we inhibited were directly down-stream of TLR2 or P2Y12. In contrast, ticagrelor had no effect on imiquimod- and loxoribine-induced microglial chemotaxis (Fig. 9B), suggesting that the microglial chemotaxis by TLR7 activation is not dependent on P2Y12 receptor signaling pathway.

Typically, TLR2 forms heterodimers with either TLR1 or TLR6 (Shioi et al., 2000). Since we found no influence of deleting either TLR1 or TLR6 on Pam3CSK4- and Pam2CSK4-induced microglial migration, we assume that these agonist can bind to both TLR1/2 and TLR2/6. Previous studies have shown that Pam3CSK4 is a strong

TLR1/2 agonist but at higher concentrations (>100 ng/ml) exhibited some activity toward TLR2/6 and Pam2CSK4 exhibited comparable activities toward both the TLR1/2 and TLR2/6 pairs (Omueti et al., 2005).

Our experiments show that microglial migratory activity induced by activation of both TLR2/P2Y12 and TLR7 were completely inhibited by LY294002 and Wortmannin, suggesting that activation of PI3K is involved in both responses. PI3Ka are lipid kinases that phosphorylate phosphoinositides, generating second messengers further involved in the regulation of a variety of cellular processes such as proliferation, survival, and cell migration (Cao et al., 2013; Ha et al., 2008; Kanno et al., 2015). The mechanism for TLR-mediated activation of the PI3K pathway has been explored and a direct linkage of TLRs mediated by phosphorylated tyrosine residues activates PI3K. P2Y12 receptor is known to be coupled to activation of PI3K (Czajkowski et al., 2004). Phosphorylation of tyrosine residues at the C terminus of TLR2 was required for recruitment of PI3K-p85 subunit and subsequent activation by Rac1 (Karlstetter et al., 2014). Recent evidence suggests that there is a cross talk between TLR signaling and the PI3K/Akt pathway (Irino et al., 2008; Li et al., 2000b). The stimulation of TLRs activates the PI3K/Akt pathway, which has been shown to prevent cardiac myocyte apoptosis and protect the myocardium from ischemia-reperfusion injury (Brazil et al., 2004; Ohsawa

et al., 2007).

Many studies have reported a cross-talk between Rho-GTPases and PI3K (Fuhler et al., 2008; Li et al., 2005; Servant et al., 2000). Rac is a member of the Rho family small GTP-binding proteins, which regulate the assembly of actin cytoskeletal structures associated with cell migration (Small et al., 1999), and is known to be a downstream signal of PI3K (Hall, 1998; Li et al., 2000a). Previous studies suggested that EHT1864 is a Rac-specific inhibitor that can inhibit association of Rac with its effector Pak (p21-activated kinase) as well as a variety of downstream Rac signaling pathways (Desire et al., 2005). We now show that microglial migratory activity induced by TLR2 and TLR7 was abolished by pretreatment with the Rac inhibitor EHT1864. This is consistent with the recent report by Shin et al. (2009) showing that downstream signaling of MyD88-mediated phagocytosis is dependent on PI3K and Rac activation during *Borrelia* infection. It has also been reported that TLR2 activates NF-kB via a Rac-regulated pathway (Karlstetter et al., 2014).

We also provide evidence that the TLR2/P2Y12 and TLR7 signaling pathways diverge in the context of migratory activity. While the TLR2 signaling pathway depends on Akt phosphorylation, the TLR7 pathway does not. The Akt pathway has been implicated in cellular signaling processes that control cell migration, growth, proliferation, and

apoptosis (Brazil et al., 2004). There is also evidence that Akt, a serine/threonine kinase, is activated downstream of PI3K in microglia (Irino et al., 2008). The results of the present study show that microglial migration induced by Pam3CSK4 and Pam2CSK4 is suppressed by the Akt inhibitor. PI3K inhibition also reduces ATP-induced migration and Akt phosphorylation, implicating that the PI3K/Akt pathway could in addition act down-stream of P2Y12 receptor activation (Irino et al., 2008; Ohsawa et al., 2007). We also show that Pam3CSK4- and Pam2CSK4-induced Akt phosphorylation is dependent on TLR2 and PI3K, similar to the results shown for ADP (Irino et al., 2008). Thus TLR2/P2Y12 and TLR7 signaling in microglia diverge in their intracellular pathways which results in the control of different microglial functions, namely chemotaxis and undirected motility.

While our study used cultured microglial cells as a model, there is sufficient evidence that TLRs play an important role as a signaling system in different brain diseases. TLR7, TLR8 and TLR9 can enhance microglial Aβ uptake in the early stage of Alzheimer´s disease (Gambuzza et al., 2014). In particular TLR2 has been identified to play a pivotal role in glial activation and neuroinflammation in neurodegenerative diseases (Hayward and Lee, 2014). In glioma, TLR2 and TLR4 mediate the communication between glioma cells and microglial cells. Indeed versican activating TLR2 converts microglia

into a pro-tumorigenic phenotype (Hambardzumyan et al., 2016). Several TLRs are involved in spinal cord injury, amyotrophic lateral sclerosis and neuropathic pain (Heiman et al., 2014). All these data indicate that TLRs play important roles in many brain diseases.

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References

Aomatsu, K., Kato, T., Fujita, H., Hato, F., Oshitani, N., Kamata, N., Tamura, T., Arakawa, T., Kitagawa, S., 2008. Toll-like receptor agonists stimulate human neutrophil migration via activation of mitogen-activated protein kinases. Immunology 123, 171-180.

Boje, K.M., Arora, P.K., 1992. Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death. Brain research 587, 250-256.

Brazil, D.P., Yang, Z.Z., Hemmings, B.A., 2004. Advances in protein kinase B signalling: AKTion on multiple fronts. Trends in biochemical sciences 29, 233-242.

Butchi, N.B., Du, M., Peterson, K.E., 2010. Interactions between TLR7 and TLR9 agonists and receptors regulate innate immune responses by astrocytes and microglia. Glia 58, 650-664.

Cao, Z., Ren, D., Ha, T., Liu, L., Wang, X., Kalbfleisch, J., Gao, X., Kao, R., Williams, D., Li, C., 2013. CpG-ODN, the TLR9 agonist, attenuates myocardial ischemia/reperfusion injury: involving activation of PI3K/Akt signaling. Biochimica et biophysica acta 1832, 96-104.

Chien, H.F., Yeh, K.Y., Jiang-Shieh, Y.F., Wei, I.H., Chang, C.Y., Chang, M.L., Wu, C.H., 2005. Signal transduction pathways of nitric oxide release in primary microglial culture challenged with gram-positive bacterial constituent, lipoteichoic acid. Neuroscience 133, 423-436.

Czajkowski, R., Banachewicz, W., Ilnytska, O., Drobot, L.B., Baranska, J., 2004. Differential effects of P2Y1 and P2Y12 nucleotide receptors on ERK1/ERK2 and phosphatidylinositol 3-kinase signalling and cell proliferation in serum-deprived and nonstarved glioma C6 cells. British journal of pharmacology 141, 497-507.

Davalos, D., Grutzendler, J., Yang, G., Kim, J.V., Zuo, Y., Jung, S., Littman, D.R., Dustin, M.L., Gan, W.B., 2005. ATP mediates rapid microglial response to local brain injury in vivo. Nature neuroscience 8, 752-758.

David, S., Kroner, A., 2011. Repertoire of microglial and macrophage responses after spinal cord injury. Nature reviews. Neuroscience 12, 388-399.

Desire, L., Bourdin, J., Loiseau, N., Peillon, H., Picard, V., De Oliveira, C., Bachelot, F., Leblond, B., Taverne, T., Beausoleil, E., Lacombe, S., Drouin, D., Schweighoffer, F., 2005. RAC1 inhibition targets amyloid precursor protein processing by gamma-secretase and decreases Abeta production in vitro and in vivo. The Journal of biological chemistry 280, 37516-37525.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., Reis e Sousa, C., 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303, 1529-1531.

Ebert, S., Gerber, J., Bader, S., Muhlhauser, F., Brechtel, K., Mitchell, T.J., Nau, R., 2005.

Dose-dependent activation of microglial cells by Toll-like receptor agonists alone and in combination. Journal of neuroimmunology 159, 87-96.

Farber, K., Kettenmann, H., 2006. Purinergic signaling and microglia. Pflugers Archiv : European journal of physiology 452, 615-621.

Fuhler, G.M., Drayer, A.L., Olthof, S.G., Schuringa, J.J., Coffer, P.J., Vellenga, E., 2008. Reduced activation of protein kinase B, Rac, and F-actin polymerization contributes to an impairment of stromal cell derived factor-1 induced migration of CD34+ cells from patients with myelodysplasia. Blood 111, 359-368.

Gambuzza, M.E., Sofo, V., Salmeri, F.M., Soraci, L., Marino, S., Bramanti, P., 2014. Toll-like receptors in Alzheimer's disease: a therapeutic perspective. CNS & neurological disorders drug targets 13, 1542-1558.

Ha, T., Hua, F., Liu, X., Ma, J., McMullen, J.R., Shioi, T., Izumo, S., Kelley, J., Gao, X., Browder, W., Williams, D.L., Kao, R.L., Li, C., 2008. Lipopolysaccharide-induced myocardial protection against ischaemia/reperfusion injury is mediated through a PI3K/Akt-dependent mechanism. Cardiovascular research 78, 546-553.

Hall, A., 1998. Rho GTPases and the actin cytoskeleton. Science 279, 509-514.

Hambardzumyan, D., Gutmann, D.H., Kettenmann, H., 2016. The role of microglia and macrophages in glioma maintenance and progression. Nature neuroscience 19, 20-27.

Hanisch, U.K., Kettenmann, H., 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nature neuroscience 10, 1387-1394.

Hanke, M.L., Kielian, T., 2011. Toll-like receptors in health and disease in the brain: mechanisms and therapeutic potential. Clin Sci (Lond) 121, 367-387.

Hayward, J.H., Lee, S.J., 2014. A Decade of Research on TLR2 Discovering Its Pivotal Role in Glial Activation and Neuroinflammation in Neurodegenerative Diseases. Experimental neurobiology 23, 138-147.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., Bauer, S., 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303, 1526-1529.

Heiman, A., Pallottie, A., Heary, R.F., Elkabes, S., 2014. Toll-like receptors in central nervous system injury and disease: a focus on the spinal cord. Brain, behavior, and immunity 42, 232-245.

Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., Akira, S., 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nature immunology 3, 196-200.

Honda, S., Sasaki, Y., Ohsawa, K., Imai, Y., Nakamura, Y., Inoue, K., Kohsaka, S., 2001. Extracellular ATP or ADP induce chemotaxis of cultured microglia through Gi/o-coupled

P2Y receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience 21, 1975-1982.

Horvath, R.J., DeLeo, J.A., 2009. Morphine enhances microglial migration through modulation of P2X4 receptor signaling. The Journal of neuroscience : the official journal of the Society for Neuroscience 29, 998-1005.

Hua, F., Ha, T.Z., Ma, J., Li, Y., Kelley, J., Gao, X., Browder, I.W., Kao, R.L., Williams, D.L., Li, C.F., 2007. Protection against myocardial ischemia/reperfusion injury in TLR4-deficient mice is mediated through a phosphoinositide 3-kinase-dependent mechanism. Journal of Immunology 178, 7317-7324.

Ifuku, M., Farber, K., Okuno, Y., Yamakawa, Y., Miyamoto, T., Nolte, C., Merrino, V.F., Kita, S., Iwamoto, T., Komuro, I., Wang, B., Cheung, G., Ishikawa, E., Ooboshi, H., Bader, M., Wada, K., Kettenmann, H., Noda, M., 2007. Bradykinin-induced microglial migration mediated by B1-bradykinin receptors depends on Ca2+ influx via reverse-mode activity of the Na+/Ca2+ exchanger. The Journal of neuroscience : the official journal of the Society for Neuroscience 27, 13065-13073.

Ifuku, M., Okuno, Y., Yamakawa, Y., Izumi, K., Seifert, S., Kettenmann, H., Noda, M., 2011. Functional importance of inositol-1,4,5-triphosphate-induced intracellular Ca2+ mobilization in galanin-induced microglial migration. Journal of neurochemistry 117, 61-70.

Irino, Y., Nakamura, Y., Inoue, K., Kohsaka, S., Ohsawa, K., 2008. Akt activation is involved in P2Y12 receptor-mediated chemotaxis of microglia. Journal of neuroscience research 86, 1511-1519.

Jeon, H., Kim, J.H., Lee, W.H., Lee, M.S., Suk, K., 2012. Plasminogen activator inhibitor type 1 regulates microglial motility and phagocytic activity. Journal of neuroinflammation 9, 149.

Jung, D.Y., Lee, H., Jung, B.Y., Ock, J., Lee, M.S., Lee, W.H., Suk, K., 2005. TLR4, but not TLR2, signals autoregulatory apoptosis of cultured microglia: a critical role of IFN-beta as a decision maker. J Immunol 174, 6467-6476.

Kanno, A., Tanimura, N., Ishizaki, M., Ohko, K., Motoi, Y., Onji, M., Fukui, R., Shimozato, T., Yamamoto, K., Shibata, T., Sano, S., Sugahara-Tobinai, A., Takai, T., Ohto, U., Shimizu, T., Saitoh, S., Miyake, K., 2015. Targeting cell surface TLR7 for therapeutic intervention in autoimmune diseases. Nature communications 6, 6119.

Karlstetter, M., Nothdurfter, C., Aslanidis, A., Moeller, K., Horn, F., Scholz, R., Neumann, H., Weber, B.H., Rupprecht, R., Langmann, T., 2014. Translocator protein (18 kDa) (TSPO) is expressed in reactive retinal microglia and modulates microglial inflammation and phagocytosis. Journal of neuroinflammation 11, 3.

Kielian, T., Mayes, P., Kielian, M., 2002. Characterization of microglial responses to

Staphylococcus aureus: effects on cytokine, costimulatory molecule, and Toll-like receptor expression. Journal of neuroimmunology 130, 86-99.

Laflamme, N., Echchannaoui, H., Landmann, R., Rivest, S., 2003. Cooperation between toll-like receptor 2 and 4 in the brain of mice challenged with cell wall components derived from gram-negative and gram-positive bacteria. European journal of immunology 33, 1127-1138.

Lehnardt, S., Schott, E., Trimbuch, T., Laubisch, D., Krueger, C., Wulczyn, G., Nitsch, R., Weber, J.R., 2008. A vicious cycle involving release of heat shock protein 60 from injured cells and activation of toll-like receptor 4 mediates neurodegeneration in the CNS. The Journal of neuroscience : the official journal of the Society for Neuroscience 28, 2320-2331.

Lewis, S.D., Butchi, N.B., Khaleduzzaman, M., Morgan, T.W., Du, M., Pourciau, S., Baker, D.G., Akira, S., Peterson, K.E., 2008. Toll-like receptor 7 is not necessary for retroviral neuropathogenesis but does contribute to virus-induced neuroinflammation. Journal of neurovirology 14, 492-502.

Li, C., Hu, Y., Sturm, G., Wick, G., Xu, Q., 2000a. Ras/Rac-Dependent activation of p38 mitogen-activated protein kinases in smooth muscle cells stimulated by cyclic strain stress. Arteriosclerosis, thrombosis, and vascular biology 20, E1-9.

Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A.V., Wu, D., 2000b. Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. Science 287, 1046-1049.

Li, Z., Paik, J.H., Wang, Z., Hla, T., Wu, D., 2005. Role of guanine nucleotide exchange factor P-Rex-2b in sphingosine 1-phosphate-induced Rac1 activation and cell migration in endothelial cells. Prostaglandins & other lipid mediators 76, 95-104.

Nakanishi, S., Kakita, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H., Matsuda, Y., et al., 1992. Wortmannin, a microbial product inhibitor of myosin light chain kinase. The Journal of biological chemistry 267, 2157-2163.

Ohsawa, K., Irino, Y., Nakamura, Y., Akazawa, C., Inoue, K., Kohsaka, S., 2007. Involvement of P2X4 and P2Y12 receptors in ATP-induced microglial chemotaxis. Glia 55, 604-616.

Olson, J.K., Miller, S.D., 2004. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J Immunol 173, 3916-3924.

Omueti, K.O., Beyer, J.M., Johnson, C.M., Lyle, E.A., Tapping, R.I., 2005. Domain exchange between human toll-like receptors 1 and 6 reveals a region required for lipopeptide discrimination. The Journal of biological chemistry 280, 36616-36625.

Prinz, M., Kann, O., Draheim, H.J., Schumann, R.R., Kettenmann, H., Weber, J.R., Hanisch, U.K., 1999. Microglial activation by components of gram-positive and -negative bacteria:

distinct and common routes to the induction of ion channels and cytokines. Journal of neuropathology and experimental neurology 58, 1078-1089.

Rasley, A., Anguita, J., Marriott, I., 2002. Borrelia burgdorferi induces inflammatory mediator production by murine microglia. Journal of neuroimmunology 130, 22-31.

Rosenberger, K., Derkow, K., Dembny, P., Kruger, C., Schott, E., Lehnardt, S., 2014. The impact of single and pairwise Toll-like receptor activation on neuroinflammation and neurodegeneration. Journal of neuroinflammation 11, 166.

Sasaki, Y., Hoshi, M., Akazawa, C., Nakamura, Y., Tsuzuki, H., Inoue, K., Kohsaka, S., 2003. Selective expression of Gi/o-coupled ATP receptor P2Y12 in microglia in rat brain. Glia 44, 242-250.

Servant, G., Weiner, O.D., Herzmark, P., Balla, T., Sedat, J.W., Bourne, H.R., 2000. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. Science 287, 1037-1040.

Shin, O.S., Miller, L.S., Modlin, R.L., Akira, S., Uematsu, S., Hu, L.T., 2009. Downstream signals for MyD88-mediated phagocytosis of Borrelia burgdorferi can be initiated by TRIF and are dependent on PI3K. J Immunol 183, 491-498.

Shioi, T., Kang, P.M., Douglas, P.S., Hampe, J., Yballe, C.M., Lawitts, J., Cantley, L.C., Izumo, S., 2000. The conserved phosphoinositide 3-kinase pathway determines heart size in mice. Embo J 19, 2537-2548.

Small, J.V., Rottner, K., Kaverina, I., 1999. Functional design in the actin cytoskeleton. Current opinion in cell biology 11, 54-60.

Takayama, N., Ueda, H., 2005. Morphine-induced chemotaxis and brain-derived neurotrophic factor expression in microglia. The Journal of neuroscience : the official journal of the Society for Neuroscience 25, 430-435.

Town, T., Bai, F., Wang, T., Kaplan, A.T., Qian, F., Montgomery, R.R., Anderson, J.F., Flavell, R.A., Fikrig, E., 2009. Toll-like receptor 7 mitigates lethal West Nile encephalitis via interleukin 23-dependent immune cell infiltration and homing. Immunity 30, 242-253.

Vlahos, C.J., Matter, W.F., Hui, K.Y., Brown, R.F., 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). The Journal of biological chemistry 269, 5241-5248.

Wiggins, H., Rappoport, J., 2010. An agarose spot assay for chemotactic invasion. BioTechniques 48, 121-124.

Wu, L.J., Vadakkan, K.I., Zhuo, M., 2007. ATP-induced chemotaxis of microglial processes requires P2Y receptor-activated initiation of outward potassium currents. Glia 55, 810-821.

Zekki, H., Feinstein, D.L., Rivest, S., 2002. The clinical course of experimental autoimmune encephalomyelitis is associated with a profound and sustained transcriptional activation of

the genes encoding toll-like receptor 2 and CD14 in the mouse CNS. Brain Pathol 12, 308-319.

Zou, B.B., Wang, F., Li, L., Cheng, F.W., Jin, R., Luo, X., Zhu, L.X., Geng, X., Zhang, S.Q., 2015. Activation of Toll-like receptor 7 inhibits the proliferation and migration, and induces the apoptosis of pancreatic cancer cells. Molecular medicine reports 12, 6079-6085.

Figure Legends

Fig. 1 Effect of TLR2 and TLR7 agonists on microglial migration in the agarose spot assay.

The TLR2 agonists Pam3CSK4 and Pam2CSK4 and the TLR7 agonists imiquimod and loxoribine increase migratory activity in microglia. ***(A)***, Microglia was plated on 35-mm cover slips with spots containing PBS or a TLR agonist. Agarose spot assay without TLR agonists shows low microglial invasion after 6 h incubation. An agarose spot containing 300 ng/ml Pam3CSK4 and 300 ng/ml Pam2CSK4, 5 µg/ml imiquimod and 1 mM loxoribine showed significant invasion after 6 h. Scale bar: 100 µm. ***(B) (C)***, Cells migrated into the spots were counted, and the increase in migratory activity is displayed in relation to the PBS control. Bars represent the mean ± SEM from three independent experiments. (*$p<0.05$ and **$p<0.01$ vs. PBS, Dunnett's test, each group, n=8).

Fig. 2 TLR2- and TLR7-induced migratory activity of microglia deficient of the respective TLR.

Microglial migration was determined after 6 h incubation in the agarose spot assay as described in the legend to Fig. 1. Pam3CSK3- or Pam2CSK4-induced migration of TLR1KO ***(A)*** and TLR6KO ***(B)*** microglia was not affected, but impaired in TLR2KO cells ***(C)***. imiquimod and loxoribine did not induce migratory activity in TLR7KO microglia ***(D)*** (**p<0.01 vs. PBS, Dunnett's test, each group, n=6).

Fig. 3 TLR2 and TLR7 agonist-induced migratory activity analyzed in the Boyden Chamber.

(A) Cells penetrating the filter into the Pam3CSK4-, Pam2CSK4-, imiquimod-, and loxoribine-filled lower chamber were stained by Diff-quick and were displayed in light microscopic images. Scale bar: 100 μm. ***(B)*** The relative increase in cell numbers compared to the PBS control is shown in a dose-dependent relationship for Pam3CSK4, Pam2CSK4, imiquimod and loxoribine at the indicated concentrations. *p<0.05, **p<0.01 vs. control (Dunnett's test, each group, n=10). ***(C)*** In TLR2KO microglia, Pam3CSK4 and Pam2CSK4 did not induce an increase in migratory activity, while

imiquimod- and loxoribine were ineffective in TLR7KO microglia (Dunnett's test, each group, n=8).

Fig. 4 Analysis of migratory activity with or without gradient in the agarose spot assay and the Boyden chamber.

(A) Migration assay with the agarose spot assay. Pam3CSK4 (300 ng/ml), Pam2CSK4 (300 ng/ml), imiquimod (5 µg/ml), and loxoribine (1 mM) were either added to the spot alone (gradient) or in the spot and the surrounding medium at the same concentration (no gradient). imiquimod and loxoribine only increased migratory activity in the presence of a gradient, in contrast to the TLR2 agonists. The data represent mean ± SEM (n=4) **p<0.01 vs control, ##p<0.01 (one-way ANOVA followed by Tukey's test). ***(B)*** Likewise, migratory activity was tested in the Boyden chamber with the agonist either only in the upper or lower chamber or in equal concentrations in both chambers. While Pam3CSK4 (300 ng/ml) and Pam2CSK4 (300 ng/ml) consistently stimulated microglial migration, imiquimod (5 µg/ml) and loxoribine (1 mM) were only effective when being exclusively present in the lower chamber. The microglial suspension was added to the upper well. After incubation for 6 h, the number of cells migrating to the

lower well was determined. Data are expressed as mean ± SEM (n=8) of three independent experiments. **p<0.01 vs control, ##p<0.01 (one-way ANOVA followed by Tukey's test).

Fig. 5 Only TLR2 agonists, but not the TLR7 agonists, stimulate microglial invasion in the scratch assay.

(A) A scratch was implemented with a needle in a layer of microglial cells in the culture dish. Cell cultures were analyzed after incubation for 6 hr. Representative images at 6 h after scratching and treatment with Pam3CSK4 (300 ng/ml), Pam2CSK4 (300 ng/ml), imiquimod (5 µg/ml), or loxoribine (1 mM). Scale bar: 100 µm. ***(B)*** Number of cells in the scratched zone relative to the control. Data show mean ± SEM (n=8/group) **p<0.01 vs control. one-way ANOVA followed by Tukey's test)

Fig. 6 The increase in microglial migration induced by TLR2 and TLR7 agonists is mediated by activation of PI3K.

(A) Using the agarose spot assay, migration was stimulated with Pam3CSK4 (300 ng/ml) and Pam2CSK4 (300 ng/ml). The agonist-induced increase was inhibited by

pretreatment with the PI3K inhibitors LY294002 (25 and 50 μM) or Wortmannin (0.1 and 1 μM). Results are expressed as mean ± SEM from three independent experiments. *p<0.05, **p<0.01 vs PBS (Dunnett's test, each group, n=8). ***(B)*** The impact of these PI3K inhibitors was tested on the microglial migration with the agarose spot assay induced by imiquimod (5 μg/ml) or loxoribine (1 mM). Results are expressed as mean ± SEM from three independent experiments. *p<0.05, **p<0.01 vs PBS (Dunnett's test, each group, n=8) ***(C)*** Similar approach as described in A, B was performed using the Boyden chamber assay. TLR2 and TLR7 agonist-induced microglial chemotaxis was inhibited by pretreatment of LY294002 (25 μM) or Wortmannin (1 μM). Data are expressed as mean ± SEM of three independent experiments. **p<0.01 vs PBS (Dunnett's test, each group, n=10).

Fig. 7 Rac signaling is crucial for TLR2- and TLR7- induced migration, while Akt is required for TLR2-induced migration of microglia

(A) Analysis of the effect of the Rac inhibitor EHT1864 (50 μM, pretreatment for 30 min) on microglial migration induced by Pam3CSK4 (300 ng/ml) and Pam2CSK4 (300 ng/ml), and by imiquimod (5 μg/ml) and loxoribine (1 mM) determined in the spot assay **p<0.01 vs PBS. Data are expressed as mean ± SEM of three independent

experiments. (Dunnett's test, each group, n=8). ***(B)*** A similar set of experiments as described above was performed on imiquimod- (5 µg/ml) and loxoribine-induced migration (1 mM) using different concentrations of EHT1864 (1, 10, 50 and 100 µM). **p<0.01 vs PBS. Data are expressed as mean ± SEM of three independent experiments. (Dunnett's test, each group, n=10). ***(C)*** Impact of 50 µM EHT1864 on Pam3CSK4- (300 ng/ml) and Pam2CSK4-induced migration (300 ng/ml) in the scratch assay. Microglia were preincubated for 30 min with Rac inhibitor, and cell cultures were analyzed after 6 hr. Scale bar: 100 µm. ***(D)*** Images from the scratched areas were quantified 6 hr after treatment. Data are expressed as mean ± SEM of three independent experiments. **p<0.01 vs DMSO, ##p<0.01. (Tukey's test, each group, n=8). ***(E)*** Pam3CSK4- (300 ng/ml) and Pam2CSK4-inducded migration (300 ng/ml) of microglia was inhibited by the Akt inhibitor IV (1 and 10 µM, pretreatment for 30 min) in the spot assay. Data are expressed as mean ± SEM of three independent experiments. **p<0.01 vs PBS (Dunnett's test, each group, n=5). ***(F)*** Imiquimod- (5 µg/ml) and loxoribine-induced microglial chemotaxis (1 mM) was not affected by Akt inhibitor IV (1 and 10 µM, pretreatment for 30 min). Data are expressed as mean ± SEM of three independent experiments. **p<0.01 vs PBS (Dunnett's test, each group, n=5).

Fig. 8 Pam3CSK4 and Pam2CSK4 enhance Akt phosphorylation in a PI3K- and TLR2-dependent manner.

(A) Western blot analysis of microglial lysates with anti-p-Akt and total Akt antibody after treating microglial cells with 300 ng/ml Pam3CSK4 or 300 ng/ml Pam2CSK4 for 0, 5, 15, 30 and 60 min. Examples of the blots are shown on the left. The graphs on the right present the average intensity ratio of the bands corresponding to p-Akt (ser473) and total Akt in each group relative to the value at 0 min. *$p<0.05$, **$p<0.01$ vs 0 min (control). Data are expressed as mean ± SEM of three independent experiments. (Dunnett's test, each group, n=4). ***(B)*** Western blot analysis of microglial lysate after treatment of microglial cells for 30 min with 300 ng/ml Pam3CSK4 or 300 ng/ml Pam2CSK4 in the presence or absence of LY294002 (pretreated for 30 min, 25 or 50 μM). **$p<0.01$ vs 0 min (control). Data are expressed as mean ± SEM of three independent experiments. (Dunnett's test, each group, n=4). ***(C)*** Western blot of microglial lysate after treatment of TLR2KO microglia for 30 min with 300 ng/ml Pam3CSK4 or Pam2CSK or with 100 μM ATP (positive control) for15 min. The quantification is shown on the left. $p<0.01$ vs PBS (control). Data are expressed as mean

± SEM of three independent experiments. (Tukey's test, each group, n=3)..

Fig.9 TLR2, but not TLR7 induced microglial migration depend on P2Y12 receptor activity

Using the agarose spot assay microglial migration was measured and compared to control values in PBS. ***(A)*** Pam3CSK4 (300 ng/ml, left graph) and Pam2CSK4 (300 ng/ml, right graph) was applied alone and in the presence of ticacrelor (Tic, 10 µM). As a control, ticacrelor was also applied without the agonist.

(B) Similarly the impact of ticacrelor was tested on the TLR7 agonists imiquimod (5 µg/ml, left graph) and loxoribine (1 mM, right graph).

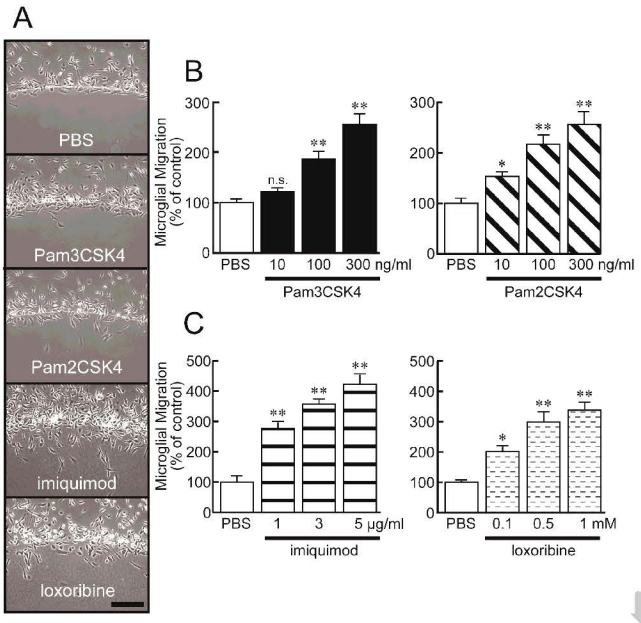
Bars represent the mean ± SEM from five independent experiments, **p<0.01 vs PBS, ##p<0.01, Tukey's test, each group, n=14).

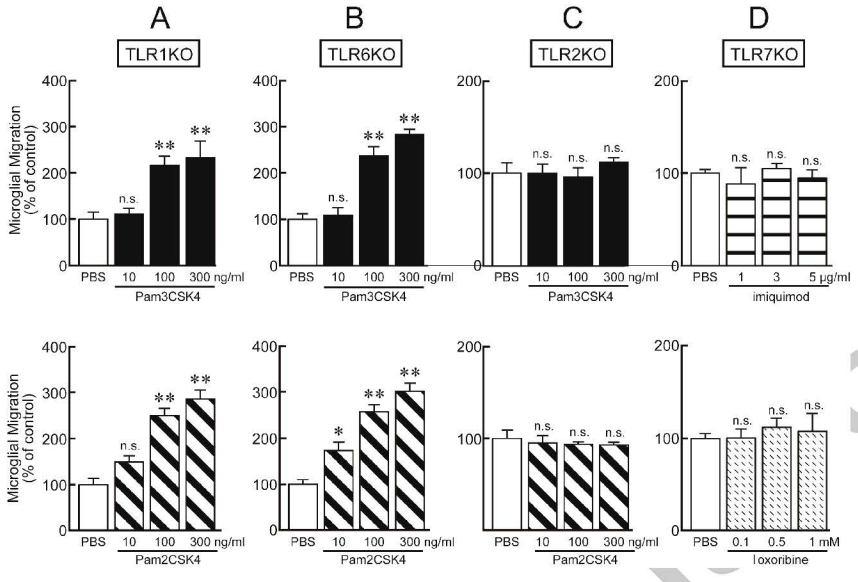
Fig. 10 TLR7 mRNA is downregulated by 24h stimulation with TLR2 and TLR7 agonists.

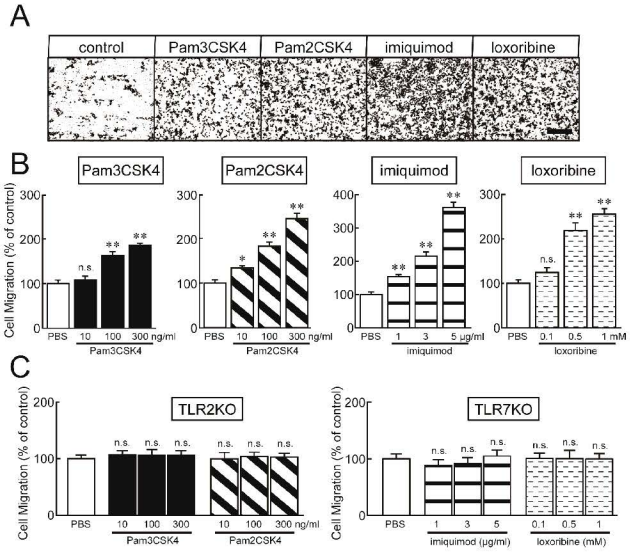
Primary microglia was stimulated with the TLR2 agonist Pam3CSK4 (300 ng/ml), and Pam2CSK4 (300 ng/ml), with the TLR7 agonists imiquimod (5 µg/ml) and loxoribine (1 mM,) and TLR4 ligand LPS (100 ng/ml) for 24 h. RNA was extracted from cell

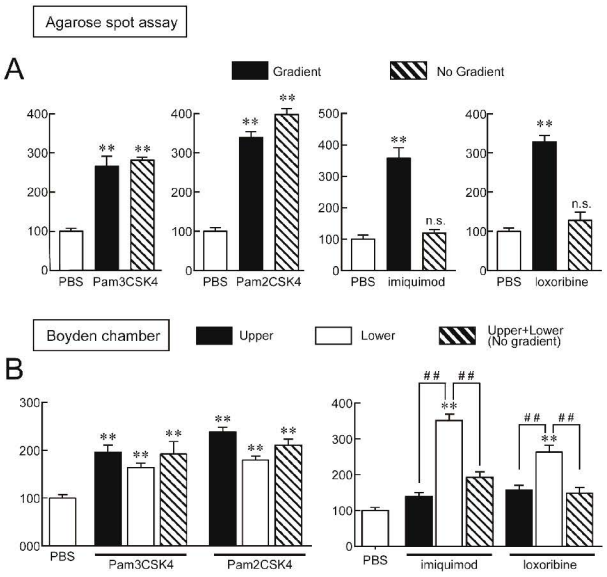
lysates and TLR2 (left graph) and TLR7 mRNA levels (right graph) were evaluated by quantitative PCR (qPCR). Results are expressed as the mean ± SEM from seven independent experiments. **p<0.01 vs control (naive), Tukey's test, each group, n=7)

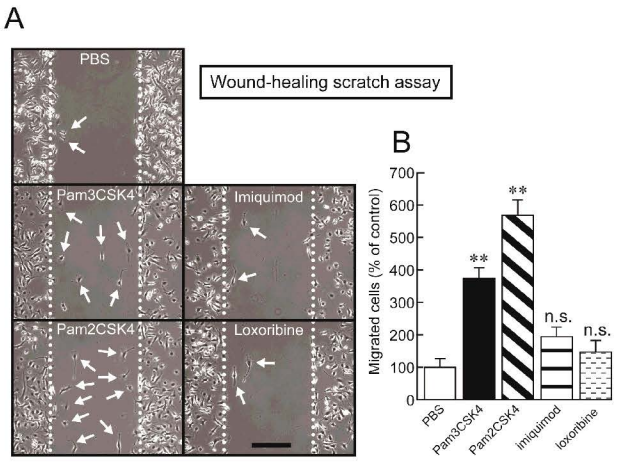
Table 1 Agarose spot assay and Boyden Chamber. Summary of change (%) of microglial migration and chemotaxis responsive to the TLR2 and TLR7 agonist-treated cells when compared with controls.

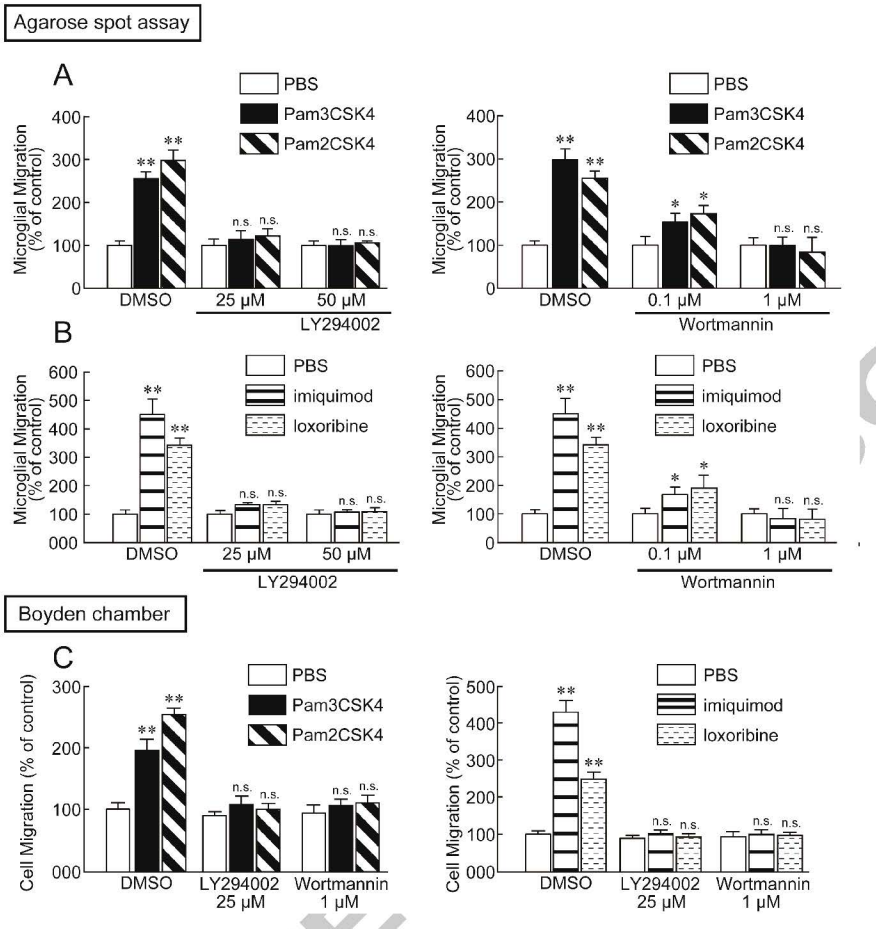


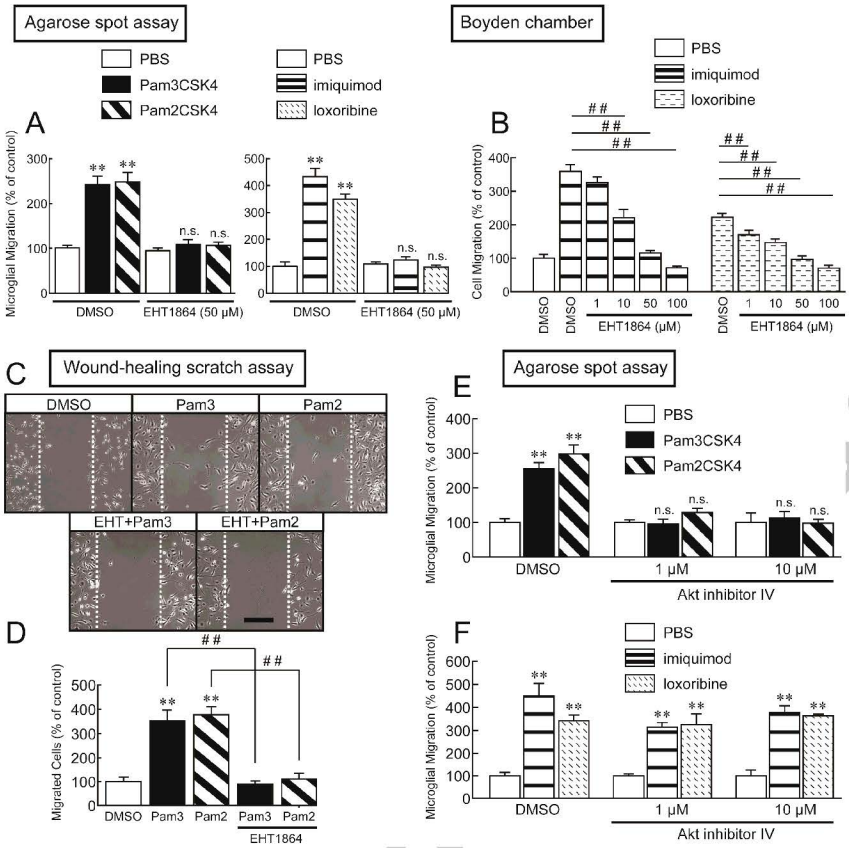


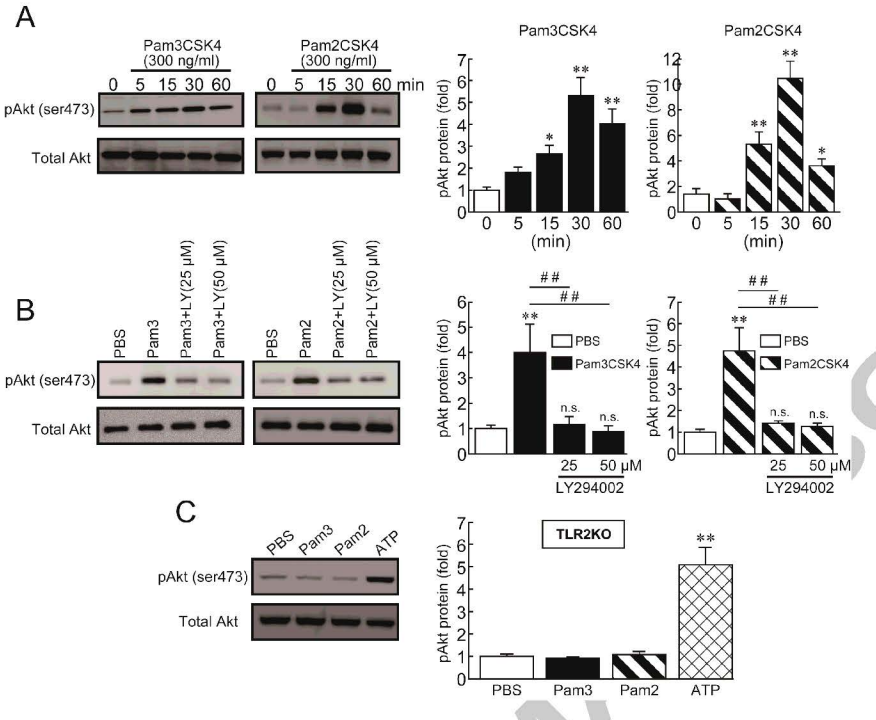


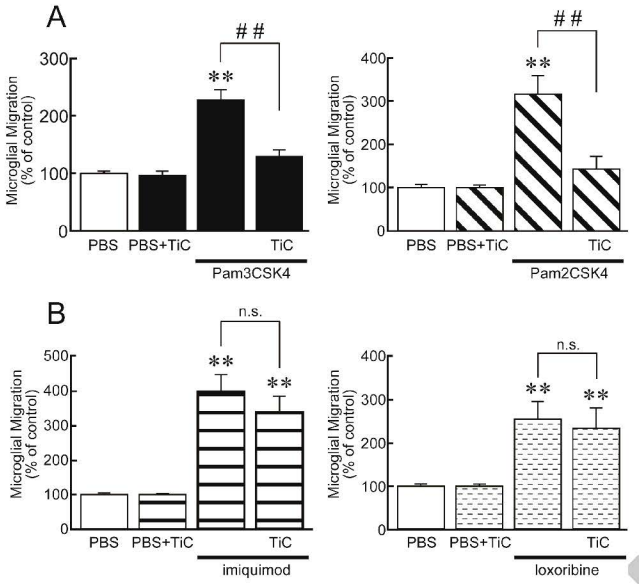


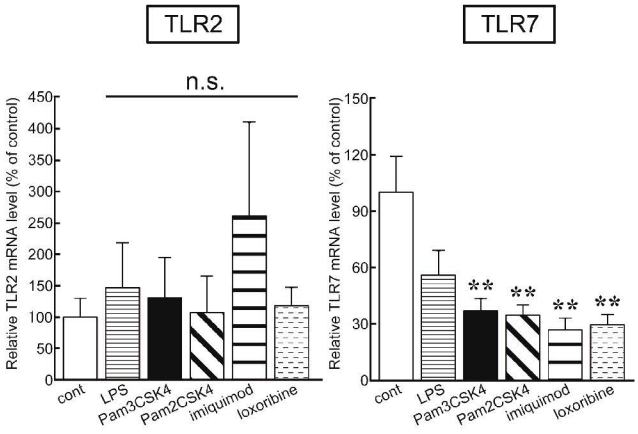












Agarose spot assay **Table 1**

Conc	*10 ng/ml*		*100 ng/ml*		*300 ng/ml*	
Strain	WT	TLR2-KO	WT	TLR2-KO	WT	TLR2-KO
Pam3CSK4	122.2±4.8	96.6±9.5	186.8±13.5	95.6±9.1	254.3±20.6	111.6±4.1
Pam2CSK4	143.2±10.4	95.1±6.9	217.5±16.7	93.8±0.8	256.4±23.7	93.1±1.8

Conc	*10 ng/ml*		*100 ng/ml*		*300 ng/ml*	
Strain	TLR1-KO	TLR6-KO	TLR1-KO	TLR6-KO	TLR1-KO	TLR6-KO
Pam3CSK4	110.9±11.6	108.7±14.8	216.1±18.1	236.9±18.1	232.9±34.2	283±9.4
Pam2CSK4	149.4±11.6	173.3±17.8	249.7±13.2	257.7±12.7	285.1±18.9	301.6±15.6

Conc	*1 µg/ml*		*3 µg/ml*		*5 µg/ml*	
Strain	WT	TLR7-KO	WT	TLR7-KO	WT	TLR7-KO
imiquimod	277.9±19.1	88.1±16.7	356.9±13.9	104.8±4.8	422.4±31.6	94.4±7.8

Conc	*0.1 mM*		*0.5 mM*		*1 mM*	
Strain	WT	TLR7-KO	WT	TLR7-KO	WT	TLR7-KO
loxoribine	201.1±18.1	100.8±8.9	298.9±32.6	112.2±8.5	338.1±24.9	107.8±18.2

Boyden chamber

Conc	*10 ng/ml*		*100 ng/ml*		*300 ng/ml*	
Strain	WT	TLR2-KO	WT	TLR2-KO	WT	TLR2-KO
Pam3CSK4	107.9±47.9	106.4±7.0	162.7±8.6	105.8±9.4	185.2±5.1	105.8±7.4
Pam2CSK4	133.8±4.3	99.5±11.2	182.6±8.9	103.9±6.8	244.3±11.5	102.9±5.7

Conc	*1 µg/ml*		*3 µg/ml*		*5 µg/ml*	
Strain	WT	TLR7-KO	WT	TLR7-KO	WT	TLR7-KO
imiquimod	153.6±5.8	88.5±9.5	214.8±11.7	91.8±9.4	360.5±15.4	104.6±10.2

Conc	*0.1 mM*		*0.5 mM*		*1 mM*	
Strain	WT	TLR7-KO	WT	TLR7-KO	WT	TLR7-KO
loxoribine	124.2±9.8	100.5±8.7	218.7±16.2	100.3±13.9	255.2±11.8	99.7±8.8

Highlights

TLR2 signaling controls random motility in cultured mouse microglia

TLR7 signaling controls chemotaxis

Both pathways rely on PI3K and Rac activation

Akt phosphorylation is only required for the TLR2 pathway