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BRIEF COMMUNICATION

IRF8 is a transcriptional determinant for microglial motility

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Abstract Microglia, the resident immune cells of the central nervous system, are constitutively mobile cells that undergo rapid directional movement toward sites of tissue disruption. However, transcriptional regulatory mechanisms of microglial motility remain unknown. In the present study, we show that interferon regulatory factor-8 (IRF8) regulates microglial motility. We found that ATP and complement component, C5a, induced chemotaxis of IRF8 wild-type microglia. However, these responses were markedly suppressed in microglia lacking IRF8 (*Irf8*$^{-/-}$). In a consistent manner, phosphorylation of Akt (which plays a crucial role in ATP-induced chemotaxis) was abolished in *Irf8*$^{-/-}$microglia. Real-time polymerase chain reaction analysis revealed that motility-related microglial genes such as $P2Y_{12}$ receptor were significantly suppressed in *Irf8*$^{-/-}$microglia. Furthermore, *Irf8*$^{-/-}$microglia exhibited a differential expression pattern of nucleotide-degrading enzymes compared with their wild-type counterparts. Overall, our findings suggest that IRF8 may regulate microglial motility via the control of microglial gene expression.

Keywords Microglia · Chemotaxis · IRF8 · Transcription factor · ATP

Introduction

Microglia are highly plastic immune cells of the central nervous system (CNS). They actively move their branched processes to sense pathological alterations or disturbances to ultimately maintain CNS homeostasis [1–4]. Once abnormalities are detected, microglia dramatically transform into a reactive phenotype through a progressive series of cellular and molecular changes, including morphological hypertrophy, proliferation, and the expression of various genes [3, 5]. Following these changes, activated microglia destroy infectious agents, remove cell debris, and promote tissue repair [1, 6, 7], that is, microglia form the first line of defense in the CNS. When acute injuries occur in the brain or spinal cord, microglia densely extend their processes toward the lesion site within several minutes [8, 9], or migrate to the site[10], by which they prevent the spread of the lesion. These early responses are mediated by metabotropic purinergic $P2Y_{12}$ receptors ($P2Y_{12}$Rs), stimulated by extracellular adenosine triphosphate (ATP)/adenosine diphosphate (ADP), which are leaked or released from dying or injured neurons [8, 9]. In addition, in vitro studies have shown that ionotropic P2X4 receptors (P2X4Rs) and adenosine A_3 receptors (A3Rs) are involved in ATP-induced chemotaxis of microglia [11, 12]. Adenosine is released from cells or generated from extracellular ATP by ectonucleoside triphosphate diphosphohydrolases (ENTPDases) and ecto-5′-nucleotidase enzymes (NT5e; also known as CD73), both of which are expressed on the cell surface [13, 14]. Therefore, the expression pattern of these

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purine receptors and even ATP-degrading enzymes may be a critical component for the ability of microglial motility. Furthermore, a variety of extracellular (such as complement component C5a [15]) and intracellular signaling molecules [11, 16] has been shown to participate in the control of microglial motility. These observations led us to speculate that microglial motility may be rigorously controlled at a transcriptional level by transcription factors.

Interferon regulatory factor-8 (IRF8) is a member of the IRF family of transcription factors. We have previously reported that IRF8 is expressed specifically in microglia in the CNS and plays a central role in regulating microglial gene expression [5]. Furthermore, recent studies have also shown that IRF8 is required for the full maturation of microglia [17] in addition to its physiological functions, including phagocytosis and cytokine production [18, 19]. These observations led us to speculate that IRF8 may regulate microglial motility by controlling an expression pattern of microglial genes. In the present study, we explored this hypothesis and found that IRF8 regulates the motility of microglia.

Materials and methods

All experimental procedures were performed under the guidelines of Kyushu University, and this study was approved by the animal care and use committee of Kyushu University

Microglial culture

Mouse primary cultured microglia were prepared as described previously [5, 35]. Briefly, a mixed glial culture was prepared from neonatal IRF8 knockout, hetero-knockout, and wild-type mice and maintained for 10–16 days in DMEM with 10 % fetal bovine serum [5, 36]. Immediately before experiments, microglia were collected by a gentle shake as the floating cells over the mixed glial culture. The microglia were placed in EZ-TAXIScan for migration assay or were transferred to dishes for subsequent experiments. The cultures were of >99 % purity. All animals were treated in accordance with the guidelines issued by the animal care and use committee of Kyushu University.

Migration assay

Microglial migration was examined using the EZ-TAXIScan device (GE Healthcare UK Ltd., Buckinghamshire, UK) [21]. Cultured microglial cells were injected into one of two compartments through a hole connected to the compartment. Two microliters of chemoattractant solution was injected into the other compartment to initiate chemotaxis, and cell migration at 37 °C was recorded every 2 min for 30 min. All the cells that moved during the 30-min test period were manually tracked by clicking each cell body on a display in each chemotaxis experiment. The mean migratory velocity, directionality were calculated using the values obtained every 2 min, and the velocity was expressed as micrometers per second using software (Taxiscan Analyzer 2; GE Healthcare UK Ltd.). The travel distance each cell migrated was measured by plotting the positions of each cell nucleus.

Quantitative real-time PCR

Extraction of total RNA from primary cultured microglia was performed using TRIsure (Bioline) according to the protocol of the manufacturer and purified with RNeasy mini plus kit (Qiagen, Valencia, CA). The amount of total RNA was quantified by measuring OD_{260} using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE). For reverse transcription, 100 ng of total RNA was transferred to the reaction with Prime Script reverse transcriptase (Takara). Quantitative polymerase chain reaction (PCR) was performed with Premix Ex *Taq* (Takara) using a 7,500 real-time PCR system (Applied Biosystems), and the data were analyzed using 7500 System SDS Software 1.3.1 (Applied Biosystems). Expression levels were normalized to the values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), then results were presented relative to those of control microglia cells treated with GFP-coding viral particles (Fig. 2). The sequences of TaqMan primer pairs and probe are described below.

P2X4R (*P2rx4*): 5′-ACAACGTGTCTCCTGGCTACAAT-3′ (forward), 5′-GTCAAACTTGCCAGCCTTTCC-3′ (reverse), 5′-FAM-CAATGAGCAACGCACACTCACCAAGG-TAMRA-3′ (probe)

P2Y12R (*P2ry12*): 5′-TGAAGACCACCAGGCCATTT-3′ (forward), 5′-AGGCCCAGATGACAACAGAAA-3′ (reverse), 5′-FAM-AAACGTCCAGCCCCAGCAATCTCTTG-TAMRA-3′ (probe)

A3R (*Adora3*): 5′-TGACAGTCAGATATAGAACGGTTACCA-3′ (forward), 5′-CGCTAAGGTTGCTTTTCTATTCCA-3′ (reverse), 5′-FAM-CTATTCTTGGGCCTTTGCTGGCTAGTTTCC-TAMRA-3′ (probe)

ENTPDases1 (*Entpd1*): 5′-GGTGCCTATGGGTGGATTACTATT-3′ (forward), 5′-GGTTTCCTGTTTCTGACTGTCTGA-3′ (reverse), 5′-FAM-TTCACTCAGGAACAGAGTTGGCTAAGCCTC-TAMRA-3′ (probe)

Ecto-5′-nucleptidase (*Nt5e*): 5′-TGCAACATGGGAAACCTGATC-3′ (forward), 5′-GGACACGTGGTTCCAAAACAT-3′ (reverse), 5′-FAM-TGCCATGATTAACAACAACCTCAGACACCC-TAMRA-3′ (probe)

C5aR (*C5ar1*): 5′-CACTCTAAACATCTGCTACACCTTCCT-3′ (forward), 5′-CACCACTTTGAGCGTCTTGGT-3′ (reverse), 5′-FAM-CCTGGAGTCGCAAGGCCACGC-TAMRA-3′ (probe)

Cannabinoid receptor-2 (*Cnr2*): 5′-GGATGCCGGGAGACAGAA-3′ (forward), 5′-TGCTCAGGATCATGTACTCC

TTCA-3′ (reverse), 5′-FAM-CCAACGGCTCCAACGGTG GCTT-TAMRA-3′ (probe)

Western blotting

The protein extracts from whole-cell lysates of cultured microglial cells were prepared in accordance with methods described previously [5]. Aliquots (0.5–10 μg) were subjected to a 10 % polyacrylamide gel electrophoresis, and proteins were transferred electrophoretically to PVDF membranes. After blocking with blocking one (Nacalai tesque), the membranes were incubated with anti-P2X4R rabbit polyclonal antibody (1:1,000, Alomone), anti-Akt rabbit polyclonal antibody (1:1,000, Cell Signaling), anti-phospho-Akt rabbit monoclonal antibody (1:1,000, Cell Signaling), anti-β-actin mouse monoclonal antibody (1:2,000, Sigma), and then incubated with HRP-conjugated secondary antibody (1:1,000). The blots were detected using a chemiluminescence method (Chemi-Lumi One; Nacalai tesque) and exposed to films.

Statistical analysis

Statistical analyses were performed using the Student's *t* test (Figs. 1f–h and 3a–d) or one-way ANOVA with a post hoc Dunnett's test (Figs. 1c–e and 2b, d) using GraphPad Prism 5.04 software. Differences were considered significant at $P<0.05$.

Results

IRF8 deficiency affects nucleotide-induced chemotactic activities of cultured microglia

To address whether IRF8 is involved in microglial cell motility, we initially placed primary cultured microglial cells from wild-type ($Irf8^{+/+}$) or IRF8-deficient ($Irf8^{-/-}$) mice in an EZ-Taxiscan chamber, which generated a concentration gradient of chemoattractants (e.g., ATP and ADP) and assessed chemotactic activity [20, 21]. As previously reported [12, 22], $Irf8^{+/+}$microglia showed clear and robust chemotactic activity and moved toward the top edge of the chamber where higher concentrations of ATP were located (Fig. 1a). Likewise, $Irf8^{-/-}$microglia migrated in response to ATP (Fig. 1b). However, calculating the distances migrated at the single cell level revealed that the mean travelling distance of $Irf8^{-/-}$microglia was significantly ($P<0.001$) shorter than that of their $Irf8^{+/+}$counterparts (Fig. 1d). In addition, $Irf8^{-/-}$cells showed lower magnitude of the migration mean velocity and directionality during the 30-min test (Fig. 1c, e). Such attenuated responses to ATP were also observed when assessed with IRF8-heterozygous knockout ($Irf8^{+/-}$) microglia (Fig. 1c–e). Furthermore, $Irf8^{-/-}$microglia migrated toward the higher concentration of ADP but showed less travelling distance (Fig. 1g), which may have been due to abnormal directionality (Fig. 1h). However, the mean velocity of $Irf8^{-/-}$microglia was comparable with that of $Irf8^{+/+}$microglia (Fig. 1f). These results suggest that IRF8 influences ATP/ADP-induced chemotactic responses of microglia.

IRF8 regulates the expression of cell surface receptors and activation of its downstream molecule involved in microglial motility

ATP- and ADP-induced chemotaxis of microglia have been reported to be induced predominantly through $P2Y_{12}R$ signals [12, 22]. Furthermore, P2X4R and A3R participate cooperatively in these responses [11, 12]. Therefore, we hypothesized that the abnormal chemotactic responses in $Irf8^{-/-}$microglia were attributed to the lack of signals mediated by these receptors. Akt is a downstream molecule of both $P2Y_{12}R$ and P2X4R, and its activation has recently been implicated in the regulation of both cell migration and motility [16, 23]. We therefore determined the level of Akt phosphorylation in $Irf8^{-/-}$microglia after ATP treatment by western immunoblotting analysis. Consistent with previous reports [12, 16], the amount of phosphorylated Akt after ATP treatment was enhanced in $Irf8^{+/+}$microglia (Fig. 2a). By contrast, such activation was absent in $Irf8^{-/-}$microglia (Fig. 2a), indicating that ATP-mediated signaling is suppressed in $Irf8^{-/-}$microglia.

As IRF8 regulates the expression of various microglial genes [5, 18, 19], we hypothesized that the lack of Akt activation was due to a loss of functional purinoceptors in $Irf8^{-/-}$microglia. As expected, we found that mRNA levels of *P2ry12* and *P2rx4* were markedly lower in $Irf8^{-/-}$microglia compared with $Irf8^{+/+}$microglia (Fig. 2b). Western immunoblot analysis confirmed that the P2X4R protein was markedly suppressed in $Irf8^{-/-}$microglia (Fig. 2c). In addition, the expression of *Adora3* was also attenuated in this group (Fig. 2b). Taken together, these results suggest that IRF8 regulates the expression of purinoceptors, which are involved in microglial motility.

IRF8 regulates the expression of nucleotide-degrading enzymes

To further confirm that low expression of purinoceptor-induced abnormal chemotactic responses of microglia, we also examined the gene transcripts in $Irf8^{+/-}$microglia, which showed the attenuated responses to ATP as did $Irf8^{-/-}$microglia (Fig. 1c, d). Unexpectedly, $Irf8^{+/-}$microglia exhibited a comparable expression of *P2ry12* and *P2rx4* with $Irf8^{+/+}$microglia (Fig. 2b), although *Adora3* mRNA tended to decline (Fig. 2b). These results suggest that the attenuated chemotactic activity of $Irf8^{-/-}$microglia in response to ATP is

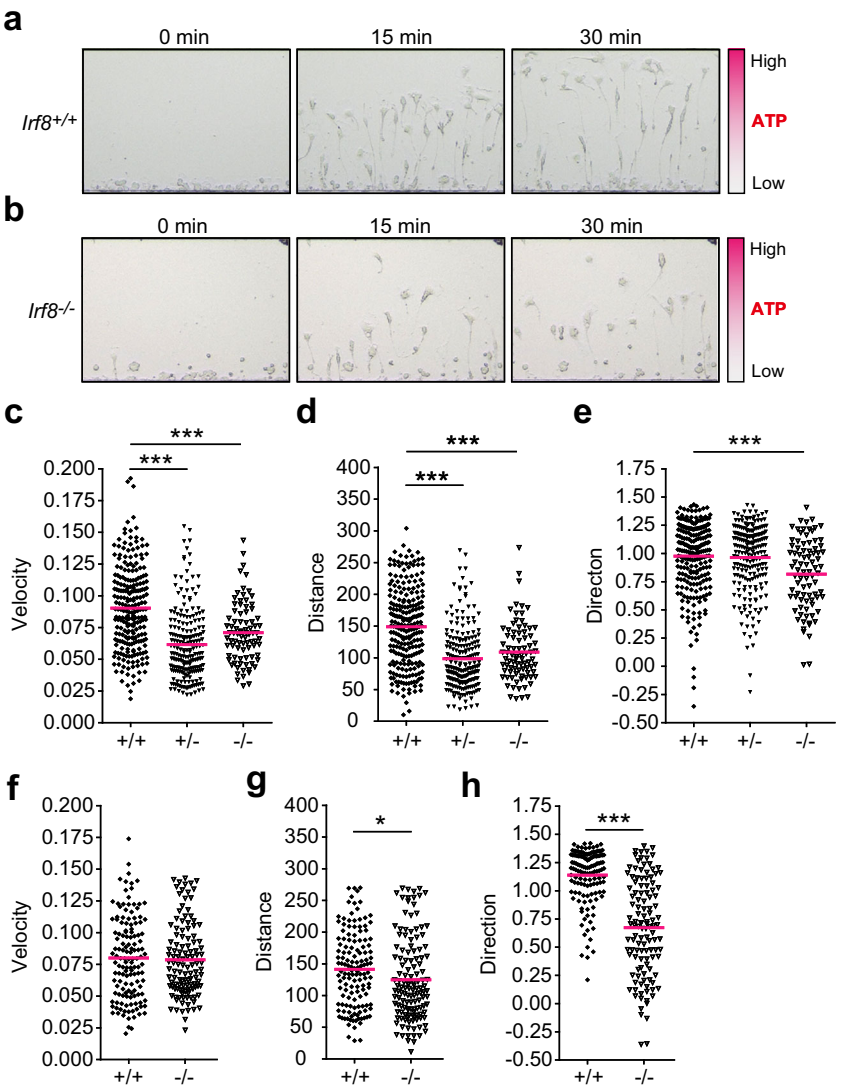

Fig. 1 IRF8 is required for ATP / ADP-induced microglial chemotaxis. Chemotactic responses of **a** wild-type ($Irf8^{+/+}$) and **b** IRF8-deficient ($Irf8^{-/-}$) microglia to ATP (0, 15, and 30 min) in the well chamber, using the EZ-TAXIScan system. Mean velocity (**c**), travel distance (**d**), and directionality (**e**) of $Irf8^{+/+}$, IRF8-heterozygous knockout ($Irf8^{+/-}$), and $Irf8^{-/-}$microglial cells during the 30-min test period after ATP injection (n=83–237). Mean velocity (**f**), travel distance (**g**), and directionality (**h**) of $Irf8^{+/+}$and $Irf8^{-/-}$microglial cells during the 30-min test period after ADP injection (n=120–138). *P<0.05; ***P<0.001

not merely due to the loss of purinoceptors. Extracellular ATP is hydrolyzed by ENTPDases (e.g., ENTPdase1, which is the dominant subtype in microglia [24]) to ADP and adenosine monophosphate (AMP) [14]. ADP activates $P2Y_{12}$Rs, which is crucial for ATP-induced microglial chemotaxis [13]. Importantly, ATP fails to induce chemotaxis in ENTPdase1-deficient microglia but rather inhibits spontaneous migratory activity [13]. However, the addition of a soluble ectonucleotidase restores the ability of ATP to be a chemoattractant [13]. AMP is further hydrolyzed by NT5e to adenosine [14], resulting in the activation of microglial A3Rs and subsequently further enhancing ATP-induced microglial chemotaxis [11]. Therefore, the balance of the expression of these enzymes may be critical for exhibiting chemotactic responses properly. Interestingly, the lack of microglial IRF8 caused a significant reduction in the mRNA expression of *Entpd1* (Fig. 2d). By contrast, expression of *Nt5e* was markedly increased with a deficiency of IRF8 (Fig. 2d), suggesting that IRF8 may control microglial migratory responses by altering the balance of nucleotide-degrading enzymes. Taken together, these findings indicate that in addition to its role as a transcriptional activator, IRF8 suppressed the transcription of certain genes, thus affecting the responsiveness of microglia toward ATP.

IRF8 controls C5a-mediated microglial chemotaxis

To investigate whether IRF8 also controls the responsiveness of microglia to chemoattractants other than nucleotides, we assessed the chemotactic behavior of microglia using another well-established chemoattractant, complement component C5a [15]. We found that $Irf8^{+/+}$microglia showed a robust chemotactic activity toward the high concentration of C5a (Fig. 3a–c). By contrast, these responses were markedly suppressed in $Irf8^{-/-}$microglia (Fig. 3a–c). In line with these

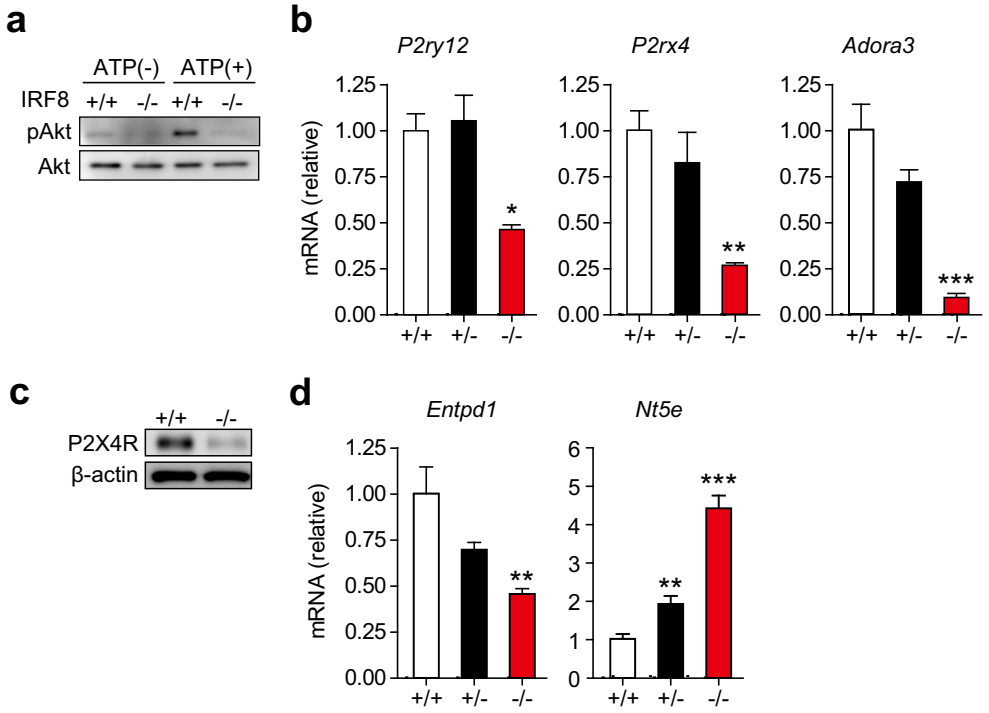

Fig. 2 IRF8 regulates the expression of microglial motility-related genes. **a** Western immunoblot of phosphorylated Akt (*pAkt*) and total Akt in whole cell lysates of *Irf8*$^{+/+}$or *Irf8*$^{-/-}$microglial cells 15 min after ATP treatment. **b** Real-time PCR analysis of purinergic receptors, *P2ry12* and *P2rx4*, and adenosine A_3 receptor, *Adora3*, in *Irf8*$^{+/+}$, *Irf8*$^{+/-}$, and *Irf8*$^{-/-}$microglial cells (n=5–9). **c** Western immunoblot of P2X4R in whole cell lysates of *Irf8*$^{+/+}$and *Irf8*$^{-/-}$microglial cells. **d** Real-time PCR analysis of the ectonucleoside triphosphate diphosphohydrolase (*Entpd1*) and ecto-5′-nucleotidase enzyme (*Nt5e*) in *Irf8*$^{+/+}$, *Irf8*$^{+/-}$, and *Irf8*$^{-/-}$microglial cells (n=5–9). **P*<0.05; ***P*<0.01; ****P*<0.001 vs. *Irf8*$^{+/+}$microglial cells. Values are the mean±SEM

results, expression of C5a receptor (*C5ar1*) was lower in *Irf8*$^{-/-}$microglia compared with *Irf8*$^{+/+}$microglia (Fig. 3d). These data indicate that IRF8 plays a role in C5a-induced chemotaxis of microglia. By contrast, expression of cannabinoid receptor-2 (*Cnr2*), a receptor involved in cannabinoid-induced migration of microglia [25], was comparable between the two genotypes (Fig. 3e). Collectively, these results suggested that IRF8 regulates cell dynamics by regulating an expression repertoire of microglial genes.

Discussion

In the present study, we show a transcriptional regulatory mechanism of IRF8-mediated motility of microglia, thereby tightly controlling the expression pattern of genes responsible for microglial chemotactic responses.

In the migration assay, microglia exhibited dynamic changes in morphology by the extension or retraction of their fine processes, and cell body migration. However, previous in vivo

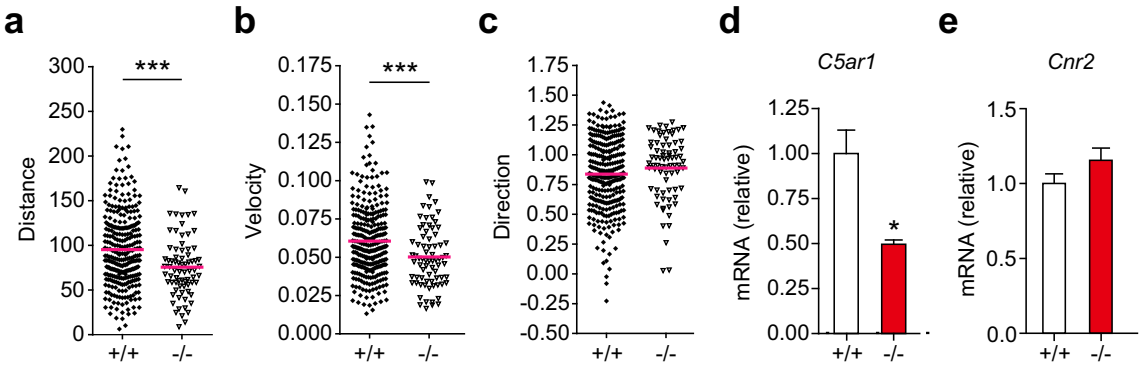

Fig. 3 IRF8 is involved in complement component C5a-induced microglial chemotaxis. Chemotactic responses of *Irf8*$^{+/+}$and *Irf8*$^{-/-}$microglia to C5a (0, 15, and 30 min) in the well chamber using the EZ-TAXIScan system. Mean velocity (**a**), travel distance (**b**), and directionality (**c**) of *Irf8*$^{+/+}$and *Irf8*$^{-/-}$microglial cells during the 30-min test period after C5a injection (n=74–304). Real-time PCR analysis of *C5ar1* (**d**) and *Cnr2* (**e**) in *Irf8*$^{+/+}$and *Irf8*$^{-/-}$microglial cells (n=5–9). **P*<0.05; ****P*<0.001. Values are the mean±SEM (**d**, **e**)

reports have shown that microglia extend their processes to the lesion site without cell body migration in response to laser-induced brain tissue injury [8, 9]. This effect may be explained by tight cell adhesion in the tissue, thus preventing rapid cell body movement within the limited period. By contrast, both process extension and cell body migration of microglia induced by ATP are suppressed by blocking $P2Y_{12}R$ signaling [9, 12, 26], suggesting that these cellular responses may share the same signaling mechanism.

The most striking observation in this study was the reduced expression of P2X4R, $P2Y_{12}R$, and A3R in IRF8-deficient microglia. Moreover, the differential expression pattern of ATP-degrading enzymes (*Entpd1* and *Nt5e*) was observed in IRF8-deficient microglia compared with wild-type microglia. Entpd1 controls the balance of ATP-adenosine and is required for ATP-induced chemotaxis of microglia [13]. In light of the increased expression of *Nt5e* in IRF8-deficient microglia, our results suggest that IRF8 deficiency may disrupt the ATP-ADP-adenosine balance on microglial surface, thereby impairing ATP-induced microglial chemotaxis. In addition, the expression of complement receptor, C5aR1 of the present study, and chemokine receptors (CCR2, CCR5, and CX3CR1) [19], but cannabinoid CB2 receptor, are significantly decreased by IRF8 deficiency. Although the possible involvement of other molecules regulated by IRF8 in microglial chemotaxis was not explored in the present study, our results with previous reports support the idea that IRF8 regulates microglial motility at the transcriptional level. Whether IRF8 directly binds to promoter regions of these genes and induces or represses their expression remains to be elucidated in future studies.

IRF8 is constitutively and predominantly expressed in the nucleus of microglia and activates the transcription of target genes [5, 18, 19]. Under pathological conditions such as following peripheral nerve injury, IRF8 is increased in activated microglia [5]. Ectopic expression of IRF8 causes marked upregulation of P2X4R, $P2Y_{12}R$, and CX3CR1 in cultured microglia [5]. Therefore, elevated expression of IRF8 in microglia may enhance their motility, allowing them to efficiently respond to pathological alterations. In fact, following peripheral nerve injury, reactive microglia engulf myelinated axons with their processes in the spinal dorsal horn in a manner that is dependent on $P2Y_{12}R$ signals [27]. Furthermore, microglial chemotaxis-related genes are upregulated in the spinal cord and are required for the generation of neuropathic pain [28–31]. However, whether microglial motility itself correlates with the degree of pain hypersensitivity remains unclear. Conversely, the treatment with lipopolysaccharide (LPS), a microglial activator, decreased IRF8 expression in microglia (data not shown). These results suggest that activation of microglia by IRF8 may be different from that by LPS and that LPS-treated microglia may respond differently to ATP or C5a.

In conclusion, the present study provides a new insight into the molecular mechanisms regulating the motility of microglia. In addition to their role in maintaining CNS homeostasis, microglia play a central part in CNS pathologies [32–34], such as Alzheimer's disease, multiple sclerosis, and neuropathic pain. Therefore, our results may help to uncover potential targets for developing therapeutic treatments for neurodegenerative diseases.

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Competing financial interests The authors have no competing financial interests to declare.

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