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Short communication

Transcription factor IRF1 is responsible for IRF8-mediated IL-1β expression in reactive microglia

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ABSTRACT

Interferon regulatory factor-8 (IRF8) plays a crucial role in the transformation of microglia to a reactive state by regulating the expression of various genes. In the present study, we show that IRF1 is required for IRF8-induced gene expression in microglia. Peripheral nerve injury induced IRF1 gene upregulation in the spinal microglia in an IRF8-dependent manner. IRF8 transduction in cultured microglia induced *de novo* gene expression of IRF1. Importantly, knockdown of the IRF1 gene in IRF8-transduced microglia prevented upregulation of interleukin-1β (IL-1β). Therefore, our findings suggest that expression of IL-1β is dependent on IRF1 in IRF8-expressing reactive microglia.

Microglia are the principal immune cells and, therefore, the major inflammatory mediators of the central nervous system (CNS) (1,2). They play diverse roles in numerous CNS pathologies, such as neuronal injury, trauma, ischemia, infection, and several neurological diseases. Under such pathological conditions, microglia undergo a dramatic change in their gene expression pattern and phenotypically transform into a reactive state, and thus become the first line of defense in the CNS (3,4). Reactive microglia produce various proinflammatory cytokines such as interleukin-1β (IL-1β), which has been shown to cause tissue inflammation (5,6) and abnormal neuronal transmission (7). Therefore, chronic excessive activation of microglia is considered to disrupt CNS homeostasis, which has been implicated in the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease and multiple sclerosis, and in neuropathic pain (2,8,9). However, the molecular mechanisms for the control of such inflammatory reactive state of microglia remain elusive.

Transcription factors are induced under pathological conditions and may control the reactive state of microglia (9). We have previously shown that the transcription factor, interferon regulatory factor-8 (IRF8), plays a crucial role in microglial activation as demonstrated by the dramatic changes in the expression of a variety of genes involved in microglial innate responses [toll-like receptor 2 (*Tlr2*)], chemotaxis [purinergic receptor P2Y12R (*P2ry12*) and CX3 chemokine receptor CX3CR1 (*Cx3cr1*)], inflammatory components [IL-1β (*Il1b*), cathepsin S (*Ctss*), and ATP-gated ionotropic receptor P2X4R (*P2rx4*)] (4). Notably, IRF8-expressing microglia are involved in the pathogenesis of several neurological diseases, including neuropathic pain (4) and multiple sclerosis (10). Furthermore, we have also shown that IRF5 is upregulated in spinal microglia after PNI in an IRF8-dependent manner, and is responsible for direct transcriptional control of P2X4 receptor (11). Therefore, the IRF8-mediated transcriptional network plays an

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important role in determining the reactive state of microglia; however, its mechanism of action is not fully understood.

IRF1 plays an important role in the inflammatory response (12) and forms a complex with IRF8 that subsequently activates the transcription of target genes (12). However, little is known about the nature of IRF1 in microglia. Therefore, in the present study, we explored the roles of IRF1 and its relationship with IRF8 in reactive microglia.

Male IRF8-deficient mice (*Irf8*$^{-/-}$) and their wild-type littermates, and C57BL/6 mice (Clea Japan, Japan) were used. All mice used were aged 9–12 weeks at the start of each experiment, and were housed in groups of two or three per cage at 22 ± 1 °C under a 12-h light–dark cycle, and had access to food and water ad libitum. All experimental procedures were performed under the guidelines of Kyushu University.

We performed spinal nerve injury as previously described (4). Extraction of total RNA was performed as previously described (4). For reverse transcription, 150 ng of total RNA was transferred to the reaction with Prime Script reverse transcriptase (Takara, Japan). qRT-PCR was performed using the 7500 real-time PCR system (Applied Biosystems, Foster City, CA) with the Premix Ex Taq (Takara, Japan), and the LightCycler 96 (Roche, Switzerland) with the FastStart Essential DNA Probes Master (Roche, Switzerland). Expression levels were normalized to the values for 18s ribosomal RNA.

Mice were deeply anesthetized and perfused transcardially with phosphate-buffered saline (PBS) followed by ice-cold 4% paraformaldehyde/PBS. The L4 segment of the lumbar spinal cord were removed, postfixed in the same fixative, and placed in 30% sucrose solution for 24 h at 4 °C. Transverse L4 spinal cord sections (30 μm) were cut on a Leica CM 1850 cryostat and incubated for 2 h at room temperature in a blocking solution (3% normal goat or donkey serum), and then incubated for 48 h at 4 °C in the primary antibody for IRF1 (1:1000, Cell Signaling), IRF8 (1:500, Santa Cruz). Identification of cell types was performed using the following markers: microglia, CD11b (1:1000, Serotec) and Iba1 (1:2000, Wako); astrocytes, GFAP (glial fibrillary acidic protein, 1:1000, Chemicon); neurons, NeuN (Neuronal Nuclei, 1:2000, Abcam). Spinal sections were incubated with secondary antibodies conjugated to Alexa Fluor™ 405, 488 or 546 (1:1000, Molecular Probes) and mounted in VECTASHIELD with or without DAPI (Vector Laboratories). Three to five sections from the L4 spinal cord segments of each mouse were randomly selected and analyzed using an LSM510 Imaging System (Carl Zeiss Japan).

We used lentiviral CS2 vectors (RIKEN, Japan) encoding mouse IRF8-green fluorescent protein (GFP), IRF8(K79E)-GFP, IRF1 or GFP under the control of the human EF-1α promoter, or vectors encoding *Irf1* or control shRNA sequences expressed under the control of H1 promoter. Each vector with pCAG-HIVgp (packaging plasmid; RIKEN, Japan) and pCMV-VSV-G-RSV-Rev (RIKEN, Japan) was cotransfected into HEK293T cells. After mixing with polyethylene glycol, viral particles and 8 μg/ml polybrene were added to primary cultured microglia (1.2×10^5 cells/well), microglial BV2 cells (1.0×10^4 cells/well), astrocytic A1 cells (1.0×10^4 cells/well) or neuronal Neuro2A cells (1.0×10^4 cells/well) contained in 24-well plates.

All data are expressed as the mean ± SEM and were analyzed by the Student's *t* test, two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test, or one-way ANOVA with the Bonferroni multiple comparison test or the Tukey's multiple comparison test. Statistical analysis was performed using GraphPad Prism 5.04 software. Significance was reached at values of $P < 0.05$, $P < 0.01$, or $P < 0.001$.

To examine the relationship between IRF1 and IRF8 in microglial cells under *in vivo* condition, we investigated expressional pattern of *Irf1* in spinal cord of the PNI model mice in which the expression of IRF8 is known to be increased in spinal microglia (4). We found that *Irf1* expression was markedly increased in the ipsilateral spinal cord of wild-type (WT) PNI mice (Fig. 1A and B), whereas the other IRFs (*Irf2* and *Irf3*) were not altered (Fig. 1A and B). In these mice, the *Irf1* expression increased from 2 days, peaking at 7 days and was maintained for more than 2 weeks after PNI (Fig. 1A), with a time course matching that of IRF8 (4) (Fig. 1A). In contrast, no detectable change was found in the contralateral spinal cord (Fig. 1A). Notably, the PNI-induced upregulation of *Irf1* was almost completely suppressed in IRF8-deficient (*Irf8*$^{-/-}$) mice (Fig. 1B). These results suggest that in the spinal cord after PNI, gene expression of IRF1 is regulated by IRF8.

Although IRF8 expression is highly restricted in microglia in the spinal cord (4), IRF1 has been shown to be ubiquitously expressed in CNS cells including microglia, astrocytes, and neurons (13,14). To determine the cell types showing increased IRF1 expression in the spinal cord after PNI, we examined the localization of IRF1 protein using an immunohistochemical approach. There were detectable but weak signals of IRF1 immunofluorescence in the contralateral sides of the spinal cord (Fig. 1C). However, following PNI, IRF1 immunofluorescence was markedly increased in the ipsilateral spinal dorsal horn of WT mice (Fig. 1C). The increase in IRF1 immunofluorescence was not observed in *Irf8*$^{-/-}$ mice (Fig. 1C). We found that immunofluorescence of IRF1 was observed in cells labeled by CD11b, a microglia marker (Fig. 1C). In addition, some IRF1 immunofluorescence-positive cells were also labeled by GFAP (an astrocyte marker) (Fig. 1D). However, NeuN (a neuronal marker) was not double-labeled with IRF1 (Fig. 1E). Furthermore, distinct enhancement of IRF1 expression also occurred in the ipsilateral ventral horn after PNI (Fig. 1F–H). Interestingly, in this region, about half of cells positive to IRF1 immunofluorescence were observed in CD11b-positive microglia (Fig. 1F), and the others were CD11b-negative cells including astrocytes (Fig. 1G). GFAP-positive astrocytes did not express IRF8 (Fig. 1H). These results indicate that, in the spinal cord after PNI, IRF1 expression is increased in microglia as well as other cells including astrocytes, and is dependent on IRF8-mediated signals.

To examine further mechanistic detail of the IRF8-mediated IRF1 expression in microglia, IRF8-GFP gene was transduced into primary cultured microglial cells. A GFP-encoding vector was used as a control. We found that forced expression of IRF8 significantly increased the expression of *Irf1* (Fig. 1I). The mutant IRF8 that is formed by replacing lysine at amino acid position 79 with Glu [IRF8(K79E)] resulting in a lack of DNA binding activity (4) failed to increase *Irf1* (Fig. 1I). In contrast, ectopic IRF8 expression in astrocytic A1 cells or neuronal Neuro2A cells failed to increase *Irf1* transcripts (Fig. 1J and K), suggesting a cell-type-specific mechanism. These results together suggest that gene expression of microglial IRF1 is controlled by IRF8 and is dependent on its DNA binding ability.

To determine the role of IRF1 in microglia, we employed IRF8-transduced BV2 cells that have been shown to exhibit increased expression of various microglial genes (4,11). We then investigated the involvement of IRF1 in IRF8-mediated expression of microglial genes by incorporating in BV2 cells a lentiviral vector that encoded shRNA for *Irf1* to knock down this gene expression (Fig. 2A). IRF8 transduction in BV2 cells caused upregulation of *Irf1* expression (Fig. 2A), as observed in primary microglial cells (Fig. 1I). In addition, IRF8-transduced cells without *Irf1* shRNA increased the expression of *Tlr2*, *P2ry12*, *P2rx4*, *Cx3cr1*, *Il1b*, *Ctss*, and *Irf5* (Fig. 2B). Expression of *Aif1* that encodes ionized calcium binding adaptor molecule 1 (Iba1, a microglial marker) was also increased (Fig. 2B). The IRF8 transduction in A1 astrocytes or Neuro2A cells did not alter the gene expressions (Fig. 2C), as is the case for *Irf1* (Fig. 1J and K). In contrast, the knockdown of IRF1 in BV2 cells markedly attenuated IRF8-

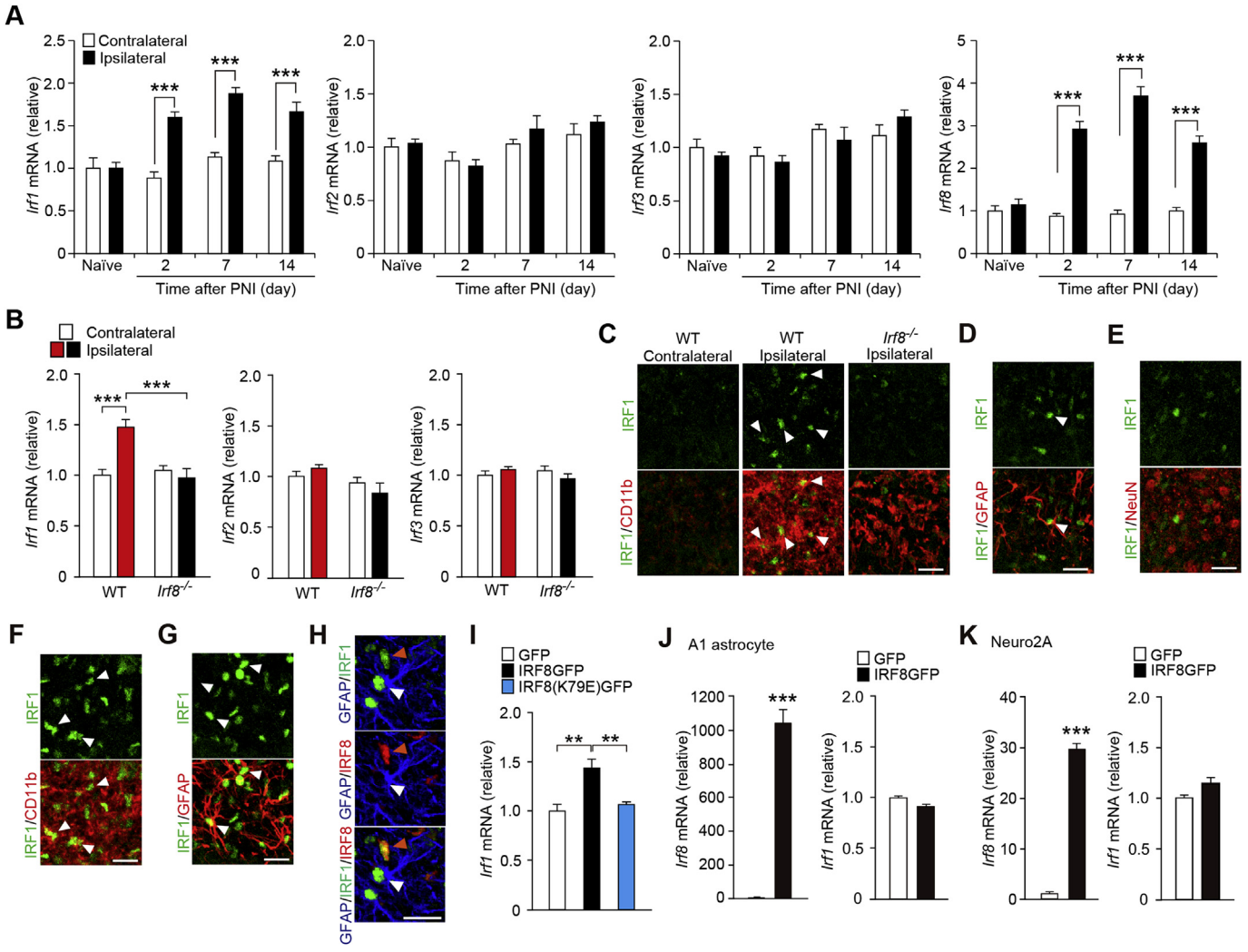

Fig. 1. Interferon regulatory factor-8 (IRF8) regulates IRF1 expression in spinal cord and microglial cells (**A**) Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of *Irf1*, *Irf2*, *IRF3* and *Irf8* in total RNA extracted from spinal cord ipsilateral and contralateral to PNI before (naive) and after PNI. Values represent the relative ratio of genes [normalized to the value for 18s ribosomal RNA (*18s*)] to the contralateral side of naive mice (n = 6, $^{***}P < 0.001$). (**B**) qRT-PCR of *Irf1*, *Irf2*, and *Irf3* in the spinal cords of wild-type (WT) and IRF8-deficient ($Irf8^{-/-}$) mice 7 days after PNI. Values represent the relative ratio of genes (normalized to the value for *18s*) to the contralateral side of WT mice (n = 6, $^{***}P < 0.001$). (**C**–**E**) Double immunolabeling of IRF1 with CD11b (**C**), GFAP (**D**) and NeuN (**E**) in the dorsal spinal cord 7 days after PNI. Arrowheads indicate co-localization of IRF1 immunofluorescence with that of CD11b (**C**) and GFAP (**D**). (**F**, **G**) Double immunolabeling of IRF1 with CD11b (**F**) and GFAP (**G**) in the ventral spinal cord 7 days after PNI. Arrowheads indicate co-localization of IRF1 immunofluorescence with that of CD11b (**F**) and GFAP (**G**). (**H**) Triple immunolabeling of IRF1 with IRF8 and GFAP in the ventral spinal cord 7 days after PNI. White arrowhead indicates localization of IRF1 immunofluorescence in an IRF8-negative cell labeled with GFAP. Orange arrowhead indicates co-localization of IRF1 immunofluorescence with that of IRF8, but not GFAP. (**I**) qRT-PCR analysis of *Irf1* in primary cultured microglial cells transduced with IRF8-green fluorescent protein (GFP) (IRF8GFP), IRF8(K79E)-GFP [IRF8(K79E)], or GFP alone (GFP). Values represent the relative ratio of *Irf1* (normalized to the value for *18s*) to the GFP-transduced cells (n = 8, $^{**}P < 0.01$). (**J**, **K**) qRT-PCR analysis of *Irf8* and *Irf1* in (**J**) astrocytic A1 cells and (**K**) neuronal Neuro2A cells, transduced with IRF8-GFP or GFP. Values represent the relative ratio of genes (normalized to the value for *18s*) to the GFP-transduced cells (n = 6, $^{***}P < 0.001$). Scale bars: 20 μm (**C**–**H**).

mediated expression of *Il1b* ($P < 0.001$) and *Aif1* ($P < 0.05$) but not the other genes (Fig. 2B). These results indicate that in microglia, IRF8-induced IRF1 expression is crucial for the expression of IL-1β, and to a lesser extent Iba1. To further investigate the role of IRF1 in IL-1β expression, we lentivirally transduced IRF1 into microglial cells and found that forced expression of IRF1 in WT microglial cells caused a marked increase in *Il1b* expression (Fig. 2D). The *Il1b* upregulation by IRF1 was blunted (but not completely abolished) in $Irf8^{-/-}$ microglial cells (Fig. 2D). Together, these results suggest that IRF1 plays a crucial role in regulating *Il1b* expression in microglia.

In the current study, we show that IRF8 is dependent on IRF1 to activate the transcription of IL-1β in reactive microglia. Furthermore, forced expression of IRF8 in cultured microglia induced *de novo* expression of IRF1, although whether IRF8 directly binds to the promoter loci of *Irf1* to induce its expression remains to be elucidated. Furthermore, the lack of IRF8 totally suppressed the PNI-induced IRF1 expression in the spinal cord. Considering the fact that with in the spinal cord IRF8 expression is highly specific to microglia, it is hypothesized that microglial IRF8 contributes to the PNI-induced IRF1 expression not only in microglia but also in other types of cells including astrocytes, presumably in a non-cell autonomous manner.

Given that expression pattern of *Il1b* in the spinal cord was closely correlated with those of *Irf1* and *Irf8* after PNI (Fig. 2E) and the PNI-induced *Il1b* expression was markedly suppressed in $Irf8^{-/-}$ mice (4), our results suggest that IRF8-induced IRF1 expression may play a role for the expression of IL-1β in spinal microglia. The molecular machineries by which upregulated IRF1 contributes to the expression of IL-1β remain to be elucidated. An early study has shown that IRF8 forms a complex with other transcription factors

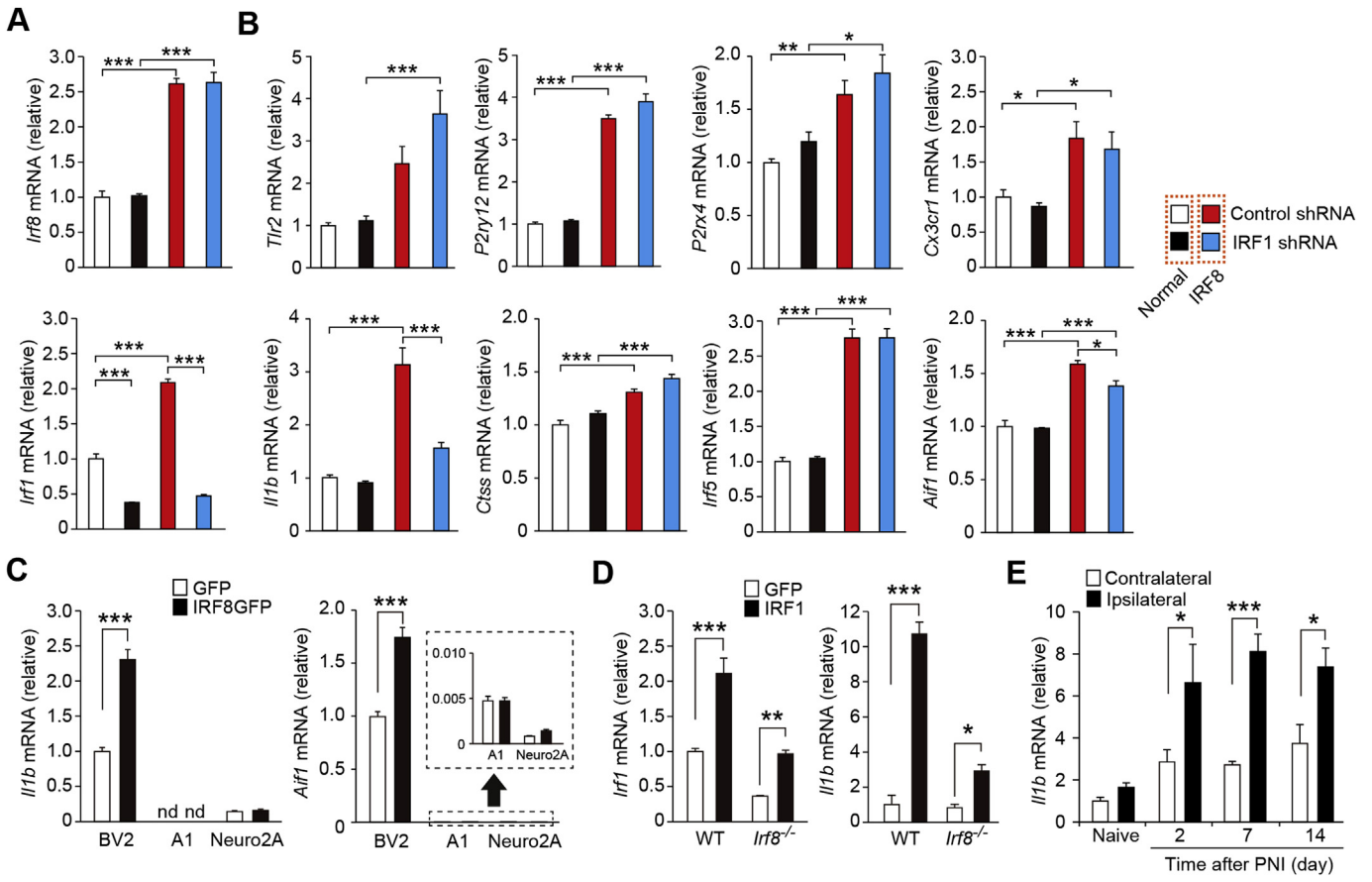

Fig. 2. IRF1 is required for IRF8-mediated expression of interleukin 1β (IL-1β) and ionized calcium binding adaptor molecule 1 (Iba1) in microglia (**A**, **B**) qRT-PCR analysis of (**A**) *Irf8* and *Irf1*, and (**B**) other microglial genes in IRF8-transduced BV2 cells with or without (control) *Irf1* shRNA. Values represent the relative ratio of genes tested (normalized to the value for *18s*) to control shRNA-transduced cells (n = 5–6, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (**C**) qRT-PCR analysis of *Il1b* and *Aif1* in astrocytic A1 cells and neuronal Neuro2A cells, transduced with IRF8-GFP or GFP. Values represent the relative ratio of genes (normalized to the value for *18s*) to the GFP-transduced cells (n = 6, $^{***}P < 0.001$). (**D**) qRT-PCR analysis of *Irf1* and *Il1b* mRNA in total RNA extracted from WT or *Irf8*$^{-/-}$ primary cultured microglia transduced with IRF1 or GFP. Values represent the relative ratio of genes (normalized to the value for *18s*) to the GFP-transduced cells (n = 6, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (**E**) qRT-PCR analysis of *Il1b* mRNA in total RNA extracted from spinal cord ipsilateral and contralateral to PNI before (naive) and after PNI. Values represent the relative ratio of *Il1b* (normalized to the value for *18s*) to the contralateral side of naive mice (n = 6, $^{*}P < 0.05$, $^{***}P < 0.001$).

and binds to the promoter region of *Il1b* (15). IRF8 is known to interact with IRF1, which synergistically activates the expression of target genes (12). Indeed, the upregulation of IL-1β mRNA in cultured microglial cells by forced IRF1 expression was blunted in *Irf8*$^{-/-}$ microglial cells. Therefore, in IRF8-transduced microglial cells, IRF1 might form a complex with IRF8 to increase *Il1b* expression. However, our data showing that the effect of IRF1 on IL-1β expression was not completely abolished in *Irf8*$^{-/-}$ microglial cells raise the possibility that increased IRF1 by itself may also has a potential to increase the expression of IL-1β in microglia.

IL-1β has been shown to play multiple roles in CNS pathologies, such as Alzheimer's disease, Parkinson's disease, and neuropathic pain (5,6). Therefore, our results suggest that IRF8-IRF1 axis may be a possible therapeutic target for the CNS diseases.

Declaration of competing financial interests

The authors have no competing financial interests to declare.

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