Mas-related G protein-coupled receptor D exacerbates inflammatory hyperalgesia by promoting NF-κB activation in dorsal root ganglia

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Abstract

Background

Mas-related G protein–coupled receptor D (MrgprD) is mainly expressed in small-diameter sensory neurons of the dorsal root ganglion (DRG). Results from previous studies suggest that MrgprD participates in mechanical allodynia and nerve injury-induced neuropathic pain. However, it remains elusive whether and how MrgprD is involved in inflammatory pain.

Methods

we used a mouse model of inflammatory hyperalgesia established by intraperitoneal administration of lipopolysaccharide (LPS). The generation of neuroinflammation and inflammatory hyperalgesia were determined using animal behavioral tests, Western blotting, quantitative real-time PCR and ELISA assay. The expression levels of MrgprD were assessed by Western blotting. The involvements of MrgprD in the LPS-triggered neuroinflammation and inflammation-related signaling pathways were assessed by animal behavioral tests, Western blotting, immunohistochemistry, ELISA, and co-immunoprecipitation assays.

Results

The LPS injection induced an evident peripheral neuroinflammation and mechanical allodynia in the mice and increased MrgprD expression in the DRG. The LPS administration also augmented the proportion of MrgprD-expressing neurons in the lumbar 4 DRG. The LPS-induced hypersensitivities to mechanical and cold stimuli, but not to a heat stimulus, were substantially attenuated in Mrgprd- knockout mice compared with wildtype littermates. Mrgprd deletion suppressed the LPS-triggered activation of the NF-κB signaling pathway and attenuated LPS-induced up-regulation of proinflammatory cytokines. Moreover, ectopic overexpression of MrgprD in HEK293 cells stably expressing mouse toll-like receptor 4 (TLR4) markedly promoted the LPS-induced NF-κB activation and enhanced NF-κB's DNA-binding activity. Furthermore, MrgprD physically interacted with TGF-β-activated kinase 1 (TAK1) and I-kappa-B-kinase (IKK) complexes in DRGs and macrophage-like RAW 264.7 cells, and MrgprD agonist treatment was sufficient to elicit the activation of NF-κB signaling, but not of mitogen-activated protein kinases (MAPKs) in RAW 264.7 cells.

Conclusions

Our findings demonstrate that MrgprD exacerbates LPS-induced inflammatory hyperalgesia by promoting canonical NF-κB activation, indicating that MrgprD could be a potential therapeutic target for managing NF-κB-mediated inflammation and inflammatory pain.

Background

Chronic or persistent pain is a major public and clinical burden affecting up to 30% of adults in the world, of which a large percentage cannot be treated effectively [1]. Unlike acute pain that warns us of noxious

stimuli or tissue injury, chronic pain becomes a pathological condition as a consequence of abnormal pain signaling and is often manifested in numerous diseases, such as diabetes, multiple sclerosis, and cancer [2]. Recent progress indicates that the development of neuroinflammation, the inflammation of nervous system, is responsible for generating and sustaining the sensitization of nociceptive neurons in DRG and spinal cord that leads to chronic pain [3–5]. Therefore, targeting the molecules that are involved in neuroinflammation might present promising strategy for the development of drugs to ameliorate chronic pain.

Systemic inflammation during bacterial infection or pathogenic lipopolysaccharide (LPS) administration causes neuroinflammation that contributes to chronic pain [6, 7]. LPS-induced neuroinflammation may also stimulate the pathological cascade in central nervous system leading to cognitive impairment and amyloidogenesis, an underlying cause of Alzheimer's disease [8]. It is generally believed that excessive pro-inflammatory cytokines and chemokines are produced during immune response to infection and activate nociceptors, and eventually lead to the peripheral and the central sensitization, resulting in hyperalgesia and allodynia [5, 9]. Accumulating evidence supports that immune cells and tissue-resident glial cells are critical to chronic pain sensitization during neuroinflammation [5, 7, 10–12]. These non-neuronal cells can communicate with nociceptive neurons and modify their activities by secreting neuroactive signaling molecules to alter pain states during inflammation [13]. However, the impact of sensory neurons on neuroinflammation has been underappreciated.

Peripheral sensory neurons are adapted to recognize danger to the organism by virtue of their sensitivity to intense mechanical, thermal and irritant chemical stimuli [14]. Of note, these sensory neurons also express cytokine receptors and pattern recognition receptors (PRRs), which execute crucial functions in immune responses [14]. In particular, toll-like receptor 4 (TLR4) among the TLR family, a classic LPS receptor, has been detected in primary dorsal root ganglia (DRG) neurons and illustrated to be involved in both acute and chronic pain in rodent models [15, 16]. Upon engagement of the receptor by LPS, TLR4 recruits an array of adaptor proteins to initiate a signaling cascade that leads to activate several important signaling pathways, of which nuclear factor kappa B (NF-κB) signaling pathway plays a crucial role in inflammatory response [12, 17]. In the nervous system, NF-κB activity is widely modulated under physiological and pathological conditions, ranging from developmental cell death to acute or chronic neurodegenerative disorders [18]. Moreover, an increasing number of studies demonstrate the importance of NF-κB in pathological pain. Specifically, inflammation was recently shown to augment the activation of NF-κB in DRG neurons [19], and NF-κB activation was documented to contribute to the development of both neuropathic and inflammatory pain [20]. Furthermore, inhibition of NF-κB in DRG neurons using NF-κB decoy significantly suppresses Complete Freund's Adjuvant (CFA)-induced inflammatory pain [21], and intrathecal treatment with NF-κB inhibitors substantially reduces thermal and mechanical hyperalgesia after peripheral inflammation induced by CFA [22]. Blockade of NF-κB activation by intrathecal injection of PTDC, an NF-κB inhibitor, completely blocks the mechanical allodynia triggered by nerve injury [23], and genetic deletion of NF-κB p50 subunit reduces nociceptive responses to inflammatory stimuli in mice [24]. These studies underscore the essential role of NF-κB in neuron health and disorders; however, the

detailed molecular mechanisms on NF-κB signal transduction in the neuron in pathophysiological settings, particularly during systemic inflammation, have remained elusive.

One family of signaling molecules, the Mas-related G protein-coupled receptor (Mrgpr) family is predominantly expressed in small-diameter sensory neurons of the trigeminal ganglia and DRGs [25–27]. This G protein-coupled receptor family consists of MrgprA-MrgprH and a primate-specific MrgprX subfamily [28]. A growing body of evidence suggests the important roles of Mrgpr family in mediating pain and itch sensation [28, 29]. In the DRG, one member of this family, MrgprD, also termed as MrgD or TGR7, is expressed in unmyelinated sensory neurons that bind isolectin-B4 (IB4) and express the ATP-gated ion channel P2 × 3, with a proposed role in pain sensation and/or modulation [25, 28, 30]. The MrgprD receptor innervates the outer layers of mammalian skin and responds to mechanical and heat stimuli, as well as to cold stimuli in some cases [31]. Deletion of Mrgprd gene results in a significantly reduced excitability in these sensory afferents, which consists of a decreased sensitivity to thermal and mechanical stimuli [31]. In addition, MrgprD has been supposed to mediate itch behavior evoked by β-alanine [32], which has been demonstrated as a specific ligand for MrgprD [30]. Moreover, the expression of MrgprD has been documented in aortic endothelia cells, smooth muscle cells [33, 34], neutrophils [35] and macrophages [34], indicating versatile functions of MrgprD in a variety of cellular procedures. Recently, we reported that MrgprD is upregulated in the sciatic nerve chronic constriction injury (CCI)-operated mouse DRGs and functionally coupled to Transient receptor potential ankyrin 1 (TRPA1) to promote CCI-induced neuropathic pain [36]. The pathophysiology of CCI-induced neuropathic pain involves peripheral nerve injury and the following inflammatory responses [4]. Therefore, it is possible that MrgprD is involved in modulation of inflammation. However, the potential role of neuronal-expressing MrgprD in the inflammatory response, especially the development of inflammatory pain, has not been examined yet. In this study, we assessed the impact of MrgprD on the LPS-induced inflammatory pain caused by neuroinflammation. Our results reveal that MrgprD executes an indispensable function in inflammatory pain, via linking to the activation of NF-κB during the inflammatory process.

Methods

Animals

Animal experiments and care were performed in strict compliance with the guidelines outlined within the Guide to Animal Use and Care from the Nanjing Normal University. Male C57BL/6 mice were housed in groups of five per cage in our animal facility, with free access to food and water. The Mrgprd knockout ($Mrgprd^{-/-}$) mice, with the entire open reading frame of MrgprD replaced with an in-frame fusion of EGFP (also termed as $Mrgprd\Delta^{EGFP}$), were kindly shared by Dr. Wenqin Luo (Department of Neuroscience, University of Pennsylvania) [37, 38]. $Mrgprd^{-/-}$ mice were backcrossed 7 times in the C57BL/6 background. Offspring $Mrgprd^{-/-}$ and $Mrgprd^{+/+}$ littermate controls were generated by breeding $Mrgprd^{+/-}$ heterozygotes. Before the experiments, the knockout of Mrgprd gene was verified by genomic DNA extraction and PCR amplification (see Additional file 1: Figure S1).

LPS administration

The TLR4 ligand LPS (Escherichia coli 055:B5, Catalog no. L4524) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Systemic inflammation in mice was induced by a single intraperitoneal injection of LPS (2.5 mg/kg, dissolved in saline), as previously described [6]. Saline-injected mice served as controls.

Mechanical allodynia

All animal behavior was observed and recorded by investigators who were blind to the treatment. Animals were placed in the testing environment for 30 minutes before the beginning of behavior testes. Mechanical allodynia was assessed by measuring the paw withdraw threshold (PWT) with a set of Von Frey filaments (0.04–0.2 g; Ugo Basile, Gemonio, Italy). The filament was applied to the plantar surface of the left hind paw at a vertical angle for up to 3 s from the bottom. Fifty percent mechanical withdrawal threshold (MWT) values were determined using the up-down method [39].

Cold allodynia

Cold allodynia was evaluated by the cold plate testing. Each animal was placed on a cold Plexiglas plate maintained at 4 ± 1 °C and covered with a transparent plastic box. Brisk lifting of the left hind paw was counted as a nociceptive response, and the number of paw lifts over a 5 min period was recorded and scored by an observer who was blind to the treatment condition. Walking steps and slow paw lifting related to locomotion were not counted.

Heat hyperalgesia

Heat hyperalgesia was assessed by measuring the paw withdraw latency to radiant heat stimuli. Each animal was placed in elevated chambers on a Plexiglas floor and was acclimated to the testing environment for 30 min before the experiments. The radiant heat source (plantar test, 37370; Ugo Basile, SRL, Gemonio, Italy) was applied to the center of the plantar surface of the left hind paw with at least 3-minute interval. The average withdrawal latency of the trials was recorded as the response latency.

Cell culture and transfection

The HEK293 cells stably expressing mouse TLR4 was purchased from InvivoGen (Catalog No. hkb-mtlr4; San Diego, CA, USA). Cells were cultured in DMEM (Wisent Corporation, Nanjing, China) supplement with 10% fetal bovine serum (Wisent Corporation), 100 U/ml penicillin and 100 μg/ml streptomycin (Wisent Corporation, Nanjing, China) at 37 °C in an atmosphere of 5% CO_2. Cells were transfected with PEI (Catalog no. 408727; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. In all cases, the total amount of transfected DNA was normalized by empty control plasmid.

Co-immunoprecipitation and Western blot analysis

Freshly isolated DRGs or cultured cells were homogenized with cell lysis buffer (P0013J; Beyotime, Shanghai, China) containing proteinase inhibitor cocktail (ab201119; Abcam, Shanghai, China) and incubated on ice for 30 min. Cell lysates were centrifuged (12500 rpm) at 4 °C for 15 min. Equal amount

of the proteins was immunoprecipitated overnight with indicated antibodies at 4 °C. The protein A/G plus-agarose beads (Santa Cruz Biotechnology, Dallas, TX, USA) were incubated with immunocomplexes for additional 2 h and washed four times with the prechilled lysis buffer. The immunoprecipitates were resolved on SDS-PAGE followed by blotting onto nitrocellulose membranes (Whatman, GE Healthcare, NJ, USA). The membrane was blocked with 5% non-fat dry milk in TBS-Tween-20 (0.1%, v/v) for 2 h at room temperature. After briefly washing with cold TBS, the membrane was incubated with indicated primary antibody (the dilution used according to the manufacturer's instructions) overnight at 4 °C, followed by three times washing. The antibody-antigen complexes were visualized by the LI-COR Odyssey Infrared Imaging System according to the manufacturer's instruction using IRDye800 flurophore-conjugated antibody (LI-COR Biosciences, Lincoln, NE, USA). Quantification was directly performed on the blot using the LI-COR Odyssey Analysis software. Aliquots of whole cell lysates were subjected to immunoblotting to confirm appropriate expression of proteins.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from freshly isolated DRGs using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, MA, USA) and the contaminated DNA was digested using DNase I (Roche, Basel, Switzerland). Complementary DNA (cDNA) was carried out with Prime Script RT reagent kit (TaKaRa, Dalian, China). Real-time PCR was performed using SYBR Premix ExTaq (TaKaRa, Dalian, China). The reaction was run in a StepOnePlus Real-Time PCR system using 2 μL of the cDNA in a 20 μL reaction according to the manufacturer's instructions. The sequences of the mouse Mrgprd primers were as follows: forward, 5'-TTTTCAGTGACATTCCTCGCC-3'; and reverse, 5'- GCACATAGACACAGAAGGGAGA-3'. The sequences of the mouse IL-1β primers were as follows: forward, 5'-TGGTGTGTGACGTTCCCATTA-3', and reverse, 5'-CAGCACGAGGCTTTTTTGTTG-3'. The sequences of the mouse TNF-α primers were as follows: forward, 5'-GACCCTCACATCAGATCATCTTT-3', and reverse, 5'-CCTCCACTTGGTGGTTTGCT-3'. The sequences of the mouse MCP-1 primers were as follows: forward, 5'- GCCTGCTGTTCACACAGTTGC-3', and reverse, 5'-CAGGTGAGTGGGGCGTTA-3'. The sequences of mouse β-Actin primers were as follows: forward, 5'-TGAGAGGGAAATCGTGCGTGAC-3', and reverse, 5'- AAGAAGGAAGGCTGGAAAAGAG-3'. β-Actin was used as housekeeping gene. The relative transcription level was calculated using the ΔΔCt method. All samples for each gene were run at least in triplicate.

Immunohistochemistry on DRG

DRGs were collected from spinal level L4, fixed in 4% paraformaldehyde in 1 x PBS for 20 min, cryoprotected with 20% sucrose in 1 x PBS, and embedded into OCT. Tissues were cut into sections at the thickness of 10 μm, air dried and stored at -80 °C. In Mrgprd$^{-/-}$ mice, the MrgprD positive neurons were visualized by EGFP fluorescence in DRG [37]. For immunostaining of TLR4, sections were incubated in blocking solution (containing 10% donkey serum, 0.3% Triton X-100 in PBS) for 1 h at room temperature, and then incubated with rabbit anti-TLR4 (1:100, ab62053; Abcam, Shanghai, China) at 4℃ overnight. Next, the sections were washed and incubated with Alexa Fluor-conjugated donkey anti-rabbit IgG secondary antibody (1:200, Invitrogen) at 37℃ for 1 h. Lastly the cell nuclei were counterstained with

DAPI for 5 min at room temperature. The pictures were captured using Axio Zoom. V16 microscope (ZEISS, Oberkochen, Germany). At least three mice from each group were analyzed.

Data analysis

Data were expressed as the mean ± SD. Statistical analysis was carried out using SPSS software (version 17.0). Two-tailed unpaired Student's t test was used to determine the significance of the difference between two groups. A P value of < 0.05 was considered statistically significant.

Results

LPS injection induces hyperalgesia and peripheral neuroinflammation in mice

To generate systemic inflammation, male C57BL/6 mice were intraperitoneally injected with LPS (2.5 mg/kg) on the first day of experiment (designated as day 0). We monitored weight loss and sensitivities to mechanical stimulation in animals after injection with either vehicle control (saline) or LPS within 8 days. Intraperitoneal injection with LPS caused significant weight loss from day 1 to day 7, compared to saline-injected group (Fig. 1a). In the von Frey tests, no differences in the mechanical withdrawal threshold (MWT) of hind paws were observed between saline- and LPS-injected mice at day 0. From day 1 to day 8, the MWT in LPS-injected group was significantly decreased compared to saline-injected control, verifying that a single intraperitoneal injection of LPS elicited mechanical hyperalgesia in mice (Fig. 1b) [6].

Systemic administration of LPS is known to cause immune responses [40]. To ascertain LPS induced inflammatory response in DRGs, we examined the changes of signaling pathways involved in LPS-triggered inflammatory response. The activations of ERK, p38-MAPK, and NF-κB were more profound in LPS-treated DRGs compared to saline-treated controls (day 7, Fig. 1c). Moreover, the protein levels of certain known molecules involved in inflammation, including TLR4, Hsp70 and COX2, were increased in LPS-treated DRGs compared to saline-treated controls (Fig. 1c, d, and Additional file 1: Figure S2). Furthermore, we analyzed the expressions of inflammatory cytokines in DRGs dissected from the mice injected with either saline or LPS using quantitative real-time PCR (qRT-PCR) an ELISA assays to exclude the serum cytokines induced by systemic LPS administration. Indeed, the expressions of IL-1β and TNF-α were markedly increased both at mRNA levels (Fig. 1e, f) and protein levels (Fig. 1g, h) after LPS injection for 7 days. These results demonstrate that LPS administration induces neuroinflammation in mice.

Mrgprd knockout attenuates LPS-induced inflammatory hyperalgesia

As a member of Mrgpr family, MrgprD is specifically expressed in small-diameter sensory neurons of DRG and modulates pain/itch sensation. To investigate the potential involvement of MrgprD in

neuroinflammation, we first examined the MrgprD expression levels after LPS challenge. As illustrated by Western blot analyses, MrgprD protein levels were upregulated from day 5 to day 7 in LPS-treated DRGs compared with saline-treated controls (Fig. 2a, b). Moreover, MrgprD-expressing neurons were enumerated on the sections of lumbar 4 (L4) DRGs derived from Mrgprd$^{-/-}$ mice where the coding region of Mrgprd was replaced with an EGFP gene (MrgprdΔ^{EGFP}) as previously described [37]. Taking advantage of EGFP, the DRG neurons specifically expressing MrgprD can be visualized by imaging GFP fluorescence as shown in Fig. 2c. The percentages of EGFP-positive neurons were significantly increased in LPS-challenged DRGs in comparison with saline-treated DRGs (Fig. 2d, 34 ± 0.81% vs. 28 ± 0.95%, at least 1500 neurons were counted on sections of L4 DRGs using Image J, n = 3 mice/group). These observations strongly suggest an involvement of MrgprD in the LPS-triggered neuroinflammation.

In order to explore the impact of MrgprD on the LPS-induced inflammatory pain, we compared the behaviors of nociceptive responses between wild type (WT) and Mrgprd$^{-/-}$ mice by animal behavioral tests. 7 days after saline injection, Mrgprd$^{-/-}$ mice did not exhibit any differences in the nociceptive responses to mechanical and thermal stimulations compared with their WT littermates (Fig. 2e-2 g). By contrast, 7 days after LPS injection, deletion of Mrgprd resulted in the significant increase in the MWT values (Fig. 2e) and the significant decrease of numbers of paw lifts (Fig. 2f), but without altering the withdrawal latency of hind paw to heat stimulus when compared with saline-injected groups (Fig. 2g). These data indicate that MrgprD is required for maintaining the mechanical and cold allodynia, but not heat hyperalgesia, in a mouse model of LPS-induced inflammatory pain.

Mrgprd knockout attenuates LPS-triggered neuroinflammation in DRG

We next assessed the activations of LPS-triggered signaling pathways in DRGs derived from WT or Mrgprd$^{-/-}$ mice. In saline-treated DRGs, the protein levels of TLR4 and the phosphorylation levels of IKKα/β, ERK, and p38 MAPK were comparable between WT and Mrgprd$^{-/-}$ mice (Fig. 3a). However, in LPS-treated DRGs, deletion of Mrgprd substantially suppressed the up-regulation of TLR4 and the phosphorylation of IKKα/β, without affecting the phosphorylation of ERK and p38 MAPK (Fig. 3b). We further compared the expression levels of pro-inflammatory cytokines in DRGs between LPS-injected WT and Mrgprd$^{-/-}$ mice. Indeed, the LPS injection-induced expressions of IL-1β and TNF-α were significantly reduced in DRGs of Mrgprd$^{-/-}$ mice compared with those of WT counterparts (Fig. 3c, d). During the inflammation, chemokines such as MCP-1 have been implicated in peripheral neuroinflammation and chronic pain sensitization [41]. We then compared the expression levels of MCP-1 in DRGs from WT and Mrgprd$^{-/-}$ mice after saline or LPS administration. As expected, LPS administration caused the up-regulation of MCP-1 in DRGs, and the deletion of Mrgprd substantially attenuated MCP-1 up-regulation in LPS-treated DRG (Fig. 3e). Consistently, the protein levels of IL-1β, TNF-α, and MCP-1 were significantly reduced in DRGs from LPS-injected Mrgprd$^{-/-}$ mice, compared to those from LPS-injected WT mice (Fig. 3f-h). These results indicate a pivotal role of MrgprD in LPS-induced neuroinflammation in DRG.

MrgprD Overexpression Promotes LPS-triggered Activation Of NF-κB

After establishing a correlation between MrgprD and neuroinflammation, we further investigated the underlying mechanism. LPS is well known to stimulate pro-inflammatory gene expression by engaging TLR4 complex that activates bifurcating signaling pathway leading to the activations of MAPK and NF-κB signaling cascades [17, 42]. Of note, TLR4 expression has been recently illustrated in sensory neurons rather than in satellite glial cells in DRG [43]. We therefore first examined whether MrgprD is co-localized with TLR4 in DRG neurons using immunohistochemistry. In the DRG of $Mrgprd^{-/-}$ mice, the expression of EGFP was clearly distributed in a subset of DRG neurons and the nerve fibers, while the immunostaining of TLR4 was readily detected in the cell bodies of DRG neurons (Fig. 4a). As expected, MrgprD-positive neurons were identified with TLR4 expression. Moreover, using Western blotting, the expression of TLR4 and the activation of NF-κB signaling upon LPS stimulation were confirmed in the primary cultured DRG neurons (Additional file 1: Figure S3).

Next, we utilized a HEK-Blue mTLR4 cell model, in which mouse TLR4 and MD2/CD14 were stably overexpressed [44], to assess the impact of MrgprD on LPS-triggered signaling. HEK-Blue mTLR4 cells were transiently transfected with MrgprD expression construct and empty vector, respectively. Confocal microscopy was used to verify the localization of ectopically expressed MrgprD in cell membrane (Additional file 1: Figure S4). At 36 h post transfection, the cells were stimulated with LPS for 30 min and subjected to Western blot analyses. We found that MrgprD overexpression facilitated the LPS-induced activation of NF-κB, but not ERK, JNK or p38 MAPK in HEK-Blue mTLR4 cells (Fig. 4b-d). Moreover, the ectopic expression of MrgprD substantially enhanced the LPS-triggered nuclear translocation of NF-κB p65 subunit (Fig. 4e, f), demonstrating that MrgprD promotes the LPS-triggered signaling that leads to NF-κB activation.

MrgprD Overexpression Enhances Nf-κb P65 Transactivation Activity

NF-κB transcription factors are expressed throughout the peripheral and central nervous systems [20]. The activation of NF-κB is well known to transactivate a variety of genes, especially those encoding pro-inflammatory cytokines and chemokines [45]. Our observation that deletion of Mrgprd attenuated the expressions of IL-1β, TNF-α, and MCP-1 in LPS-challenged DRGs led us to examine whether MrgprD is able to enhance the NF-κB binding capability to target promoter DNA. To this end, HEK293-Blue mTLR4 cells were transiently transfected with MrgprD expression plasmid or empty vector. After 36 h, the cells were stimulated with LPS for 30 min and subjected to chromatin immunoprecipitation (ChIP) to analyze the recruitment of NF-κB p65 to the promoter region of known NF-κB target gene, TNF-α. Indeed, overexpression of MrgprD enhanced the LPS-induced enrichment of p65 to the κB region of TNF-α promoter (Additional file 1: Figure S5a). The p65 enrichment was specific, as there was negligible

recruitment of p65 to the β-Actin promoter that does not contain any κB sites (Additional file 1: Figure S5b). Consistently, the amount of the LPS-induced production of TNF-α in the culture medium was significantly increased in MrgprD overexpression group (Additional file 1: Figure S5c). Hence, our results demonstrate that ectopic expression of MrgprD substantially augments the LPS-induced NF-κB transactivation of certain pro-inflammatory cytokine genes, in line with the enhanced NF-κB activation signaling cascade (Fig. 4).

MrgprD mediates NF-κB signaling through interaction with TAK1 and IKKs

We sought to elucidate the mechanism through which MrgprD promotes the cellular signaling that leads to NF-κB activation in DRG. The observation that deletion of Mrgprd markedly suppressed LPS-induced phosphorylation of IKKα/β (Fig. 3a) led us to investigate the interaction of MrgprD with the components in NF-κB signaling pathway upstream of the IKK complex. By co-immunoprecipitation using MrgprD antibody, we found that MrgprD physically associated with TAK1, but not MyD88, TRAF6, or TLR4, in mouse DRGs (Fig. 5a). Such interaction was confirmed in Flag-MrgprD-overexpressing HEK293T cells by immunoprecipitation using anti-Flag (MrgprD) and anti-TAK1 antibodies (Fig. 5b).

We then assessed the impact of MrgprD on the phosphorylation of TAK1 at Thr-187, which is highly correlated with the TAK1 kinase activity [46], in DRGs isolated from WT and Mrgprd$^{-/-}$ mice injected with either saline or LPS. We found that Mrgprd knockout did not alter the LPS-triggered phosphorylation of TAK1 in DRGs (Fig. 5c). In HEK-Blue mTLR4 cells, the stimulation with LPS induced the phosphorylation of TAK1 (Fig. 5d), while MrgprD overexpression did not affect the LPS-induced phosphorylation of TAK1 and p38 MAPK (Fig. 5e). Since it has been proposed that TAK1 can phosphorylate IKKβ and increase its enzyme activity [47], we thus determined whether MrgprD forms a complex with TAK1 together with IKKβ. In DRGs, TAK1 and the IKK complex (IKKα, IKKβ, and IKKγ) were both immunoprecipitated with MrgprD (Fig. 5f). By contrast, p38, ERK, and JNK did not co-precipitated with MrgprD-TAK1 complex (Fig. 5g). These data suggest that MrgprD is involved in TAK1-mediated phosphorylation of IKKs in LPS-triggered NF-κB activation, via forming a complex with TAK1 and IKKs.

Activation Of MrgprD Elicits The Activation Of NF-κB Signaling

MrgprD is activated by β-alanine [30] and has been demonstrated to mediate β-alanine-evoked itch [32]. MrgprD has also been identified as the receptor for alamandine, a new component of renin-angiotensin system (RAS) on the regulation of cardiovascular homeostasis [33, 48]. Recently, we illustrated the expression of MrgprD in mouse peripheral peritoneal macrophages and macrophage-like RAW 264.7 cells [34]. We, therefore, determined the signaling pathways downstream of MrgprD upon the stimulation with β-alanine or alamandine in RAW 264.7 cells. The treatment with β-alanine alone elicited the activation of

NF-κB signaling, and the addition of β-alanine reinforced the LPS-induced activation of NF-κB signaling (Fig. 6a, b). By contrast, β-alanine treatments did not alter the phosphorylation levels of ERK, p38 MAPK and JNK in the absence or presence of LPS (Fig. 6a, c). Consistently, the activation of MrgprD by alamandine also induced the activation of NF-κB signaling in the absence of LPS, whilst it had no effect on the phosphorylation of ERK, p38 MAPK and JNK (Additional file 1: Figure S6).

Co-immunoprecipitation assays further confirmed the binding of MrgprD to TAK1 and IKK complex in RAW 264.7 cells (Fig. 6d). We also observed that MrgprD interacted with TAK1 and IKK complex in the absence of LPS. Further, to determine whether LPS stimulation could alter phosphorylated TAK1 and phosphorylated IKKα/β present in MrgprD protein complexes, we immunoprecipitated MrgprD from either resting or LPS stimulated cells. Immunoblotting with phosphorylation-specific antibodies showed that the comparable amounts of phosphorylated IKKα/β and phosphorylated TAK1 interacted with MrgprD between LPS stimulated and unstimulated groups (Fig. 6e-g). Taken together, we speculate that the MrgprD-mediated activation of NF-κB signaling pathway is independent of LPS-TLR4 pathway in RAW 264.7 cells.

Discussion

The Mrgpr family proteins have been suggested to play an important role in pathological pain [28]. Among them, MrgprD, reported to be mainly expressed in small-diameter sensory neurons of DRG [25], is encoded by a single copy Mrgprd gene with defined orthologues in rodent and human and constitutes an experimentally attractive therapeutic target for pain [28]. In the present study, we provided the first evidence, to our best knowledge, that MrgprD is able to promote the activation of NF-κB signaling pathway and enhance the production of pro-inflammatory mediators in DRGs, thereby exacerbating LPS-triggered inflammatory response and inflammatory hyperalgesia. We also demonstrated that MrgprD is a novel regulator of canonical NF-κB activation via interaction with TAK1 and IKK complex in mouse DRG and RAW 264.7 cells.

Intraperitoneal injection of pathogenic LPS has been known to contribute to inflammatory pain [6]. Consistent with previous reports [8, 49], our study demonstrated that LPS administration induced neuroinflammation in mouse DRG. Neuroinflammation is often the result of a peripheral damage and excessive neuronal activity of primary sensory neurons [5]. Here, we found that the expression of MrgprD in DRGs was increased after intraperitoneal injection of LPS. In comparison with saline-injected $Mrgprd^{-/-}$ mice ($Mrgprd\Delta^{EGFP}$), the percentages of MrgprD-positive ($MrgprD^{+}$) neurons labeled by EGFP were significantly increased in LPS-treated DRGs (Fig. 2c, d). Previous study using ex vivo skin/nerve/DRG/spinal cord preparation has demonstrated that deletion of Mrgprd gene resulted in a significantly reduced excitability in the sensory afferents consisting of a decreased sensitivity to thermal and mechanical stimuli [31]. Here we revealed an increased number of $MrgprD^{+}$ neurons in DRGs during the LPS-induced neuroinflammation, indicating the increased sensitivities of inflamed animals to noxious stimuli.

Behaviorally, mice lacking MrgprD$^+$ neurons exhibit reduced mechanical but not thermal pain, suggesting MrgprD$^+$ neurons are mainly involved in mechano-sensation in vivo [50]. However, mice lacking Mrgprd gene did not exhibit any thermal or mechanical behavioral phenotype compared to their WT littermates [50]. Here, we also observed that Mrgprd$^{-/-}$ and WT mice did not display substantial behavioral differences in response to mechanical, heat and cold stimulations as shown in Fig. 2E-G. However, in LPS-induced inflammatory hyperalgesia model, deletion of Mrgprd significantly reduced the LPS-induced behavioral hypersensitivities to mechanical and cold stimuli, but not heat stimulus, in comparison to WT littermates. In our recent work, we demonstrated that MrgprD is functionally coupled to cold-sensing channel TRPA1 to modulate the chronic constriction injury (CCI)-induced neuropathic pain [36], in which we also found that the CCI operation-induced hypersensitivity to cold but not heat stimulation was significantly different between Mrgprd$^{-/-}$ and WT mice. In murine DRG, MrgprD has been identified to be expressed exclusively in TRPV1-negative neurons [25, 37], while it is colocalized with TRPA1 in DRG neurons [36]. TRPV1 is a heat-activated ion channel highly expressed in nociceptors and is required for inflammatory sensitization to noxious heat [51, 52]. Beaudry et al. recently reported that optogenetic activation of MrgprD$^+$ or TRPV1$^+$ subset of C-fibers evokes distinct nocifensive behaviors in freely moving mice, suggesting that these two neuronal subsets are relate to distinct components of pain [53]. In light of these findings, we reason that Mrgprd knockout does not affect heat signal transduction presumably due to non-overlapping expression of MrgprD and TRPV1 in DRG neurons. However, a detailed comparative transcriptome analysis of MrgprD$^+$ and Mrgprd deleted DRG neurons should be conducted to identify any changed genes involved in sensory neuron functions, through which more mechanisms underlying the MrgprD-mediated pain will be elucidated.

LPS is the most potent immunostimulatory cue produced by Gram-negative bacteria and binds to TLR4 in immune cells, which triggers the synthesis of pro-inflammatory cytokines and induces inflammation and pain [54]. The recent identification of TLR4/MD2/MyD88 in sensory neurons suggests LPS can directly stimulate sensory afferents and activate NF-κB [15]. We showed that LPS stimulation directly activated the NF-κB signaling in the cultured primary DRG neurons (Additional file 1: Figure S3). Therefore, besides systemic inflammatory response, LPS can induce neurogenic inflammation in DRG neurons. Recently, Meseguer et al. revealed that LPS can directly activate TRPA1 channel in nociceptive sensory neurons and cause acute neurogenic inflammation independent of TLR4 signaling [55]. As we previously found a functional link between MrgprD and TRPA1 [36], it is thus intriguing to further investigate whether MrgprD is also involved in LPS-induced neurogenic inflammation via TRPA1.

Emerging data demonstrate that activation of NF-κB signaling pathway contributes to the development of both neuropathic and inflammatory pain [19, 20]. The expressions of NF-κB targets, such as IL-1β and TNF-α, have been reported to be upregulated in DRGs of several different pain models in mice and have been supposed to be associated with persistent pain states [56–61]. Herein, we found that deletion of Mrgprd significantly suppressed the intraperitoneal injection of LPS-triggered activation of NF-κB and upregulation of IL-1β and TNF-α in DRGs. Conversely, overexpression of MrgprD promoted the LPS-induced activation of NF-κB and enhanced the transactivation activity of NF-κB as well as the production

of TNF-α. This means that MrgprD promotes the activation of NF-κB signaling pathway in mouse DRG and facilitate the development of LPS-induced inflammatory hyperalgesia.

TAK1 (Transforming growth factor β-activated kinase 1) is one of the most important regulatory components in the NF-κB signaling pathway, which phosphorylates IKKs upon activated by pro-inflammatory stimuli [47, 62]. In this study, we showed that TAK1 physically interacted with MrgprD and its phosphorylation induced by LPS did not altered by MrgprD overexpression or deletion, whilst the phosphorylation of IKKs did. These observations thus suggest that MrgprD modulates NF-κB signaling pathway at the stage of TAK1-mediated phosphorylation of IKKs. TAK1 was originally identified as a member of MAPK kinase kinase (MAPKKK) family in TGF-β signaling [63]; however, MrgprD deletion or overexpression showed the differential effects on the activation of NF-κB and MAPKs in response to LPS challenge, raising a question of how MrgprD selectively regulate the downstream signaling pathways. Herein, co-immunoprecipitation showed that MrgprD formed a protein complex with TAK1 and IKK complex while MrgprD did not interact with MAPKs in DRGs, which may explain the differential effects of MrgprD on the phosphorylations of IKKα/β and MAPKs.

MrgprD is initially identified in a subset of nociceptive DRG neurons while the expression and function of MrgprD outside the nerves system has been reported recently. MrgprD is expressed in aortic endothelia cells and has been identified as a receptor for alamandine [33, 48]. Additionally, MrgprD has been found expressed in mouse primary neutrophils [35], the first sign of presence of MrgprD in immune cells. Recently, we illustrated the expression of MrgprD not only in the intestinal resident macrophages and lymphocytes but also in the peritoneal macrophages and the splenic T lymphocytes [34]. We showed here, for the first time, activation of MrgprD by its agonist β-alanine or alamandine elicited the activation of NF-κB signaling pathway in macrophage-like RAW 264.7 cells, probably via the complex formation among MrgprD, TAK1, and IKKs. Interestingly, we found that LPS stimulation did not alter the binding of MrgprD to phosphorylated TAK1 and phosphorylated IKKα/β which are activated forms of these kinases. Given that MrgprD did not interact with TLR4, MyD88 or TRAF6 determined by co-immunoprecipitation, we therefore suggest that MrgprD receptor acts as a novel regulator to activate canonical NF-κB independent of LPS-TLR4 pathway although the detailed mechanism remains to be fully investigated.

Conclusion

In summary, we elucidate that MrgprD facilitates the development of LPS-triggered inflammatory pain via promoting the activation of canonical NF-κB signaling pathway. As the widespread expression of MrgprD detected in animals, it may be fruitful to explore more comprehensive role of MrgprD-mediated NF-κB activation in other physiological processes, such as innate immune responses and vasodilation.

Abbreviations

MrgprD: Mas-related G-protein-coupled receptor D; DRG: Dorsal root ganglion; NF-κB: Transcription factors of the nuclear factor kappa B; TLR: Toll-like receptor; LPS: Lipopolysaccharide; ChIP: Chromatin

immunoprecipitation; PWT: Paw withdrawal threshold; MWT: mechanical withdrawal threshold; IKK: I-kappaB kinase; MD2: Lymphocyte antigen 96; MyD88: Myeloid differentiation primary response protein 88; TRAF6: Transforming growth factor receptor-associated factors 6; TAK1: Transforming growth factor β-activated kinase 1; p38 MAPK: p38 mitogen-activated protein kinase; ERK: Extracellular regulated MAP kinase; JNK: c-Jun N-terminal kinase.

Declarations

Ethical Approval and Consent to participate

All animal procedures performed in this study were reviewed and approved by Animal Use and Care Committee in Nanjing Normal University (Nanjing, China), and were conducted in accordance with the guidelines of the International Association for the Study of Pain.

Consent for publicationConsent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available within the article and its additional files and from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Lei Lan, Miao Xu, and Fengyi Wan conceived and designed research; Lei Lan, Miao Xu, Jia Li, Lin Liu, Min Xu, Chenxing Zhou, and Lei Shen performed the experiments; Lei Lan, Miao Xu, Fengyi Wan and Zongxiang Tang analyzed and interpreted data; Lei Lan and Fengyi Wan wrote the manuscript. All authors read and approved the final manuscript.

Additional Files

Additional file 1

additional materials and methods

additional figure and figure legends

Figure S1. The genotyping of *Mrgrpd* knockout mice using PCR amplification.

Figure S2. Intraperitoneal injection with LPS induced the overexpression of COX2 protein in DRGs.

Figure S3. LPS treatment stimulated the activation of NF-κB signaling in primary DRG neurons.

Figure S4. Distribution of ectopically overexpressed MrgprD in HEK-Blue mTLR4 cells.

Figure S5. MrgprD overexpression enhanced NF-κB p65 transactivation activity.

Figure S6. MrgprD activation by alamandine induced the activation of NF-κB signaling in RAW 264.7 cells.

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Figures

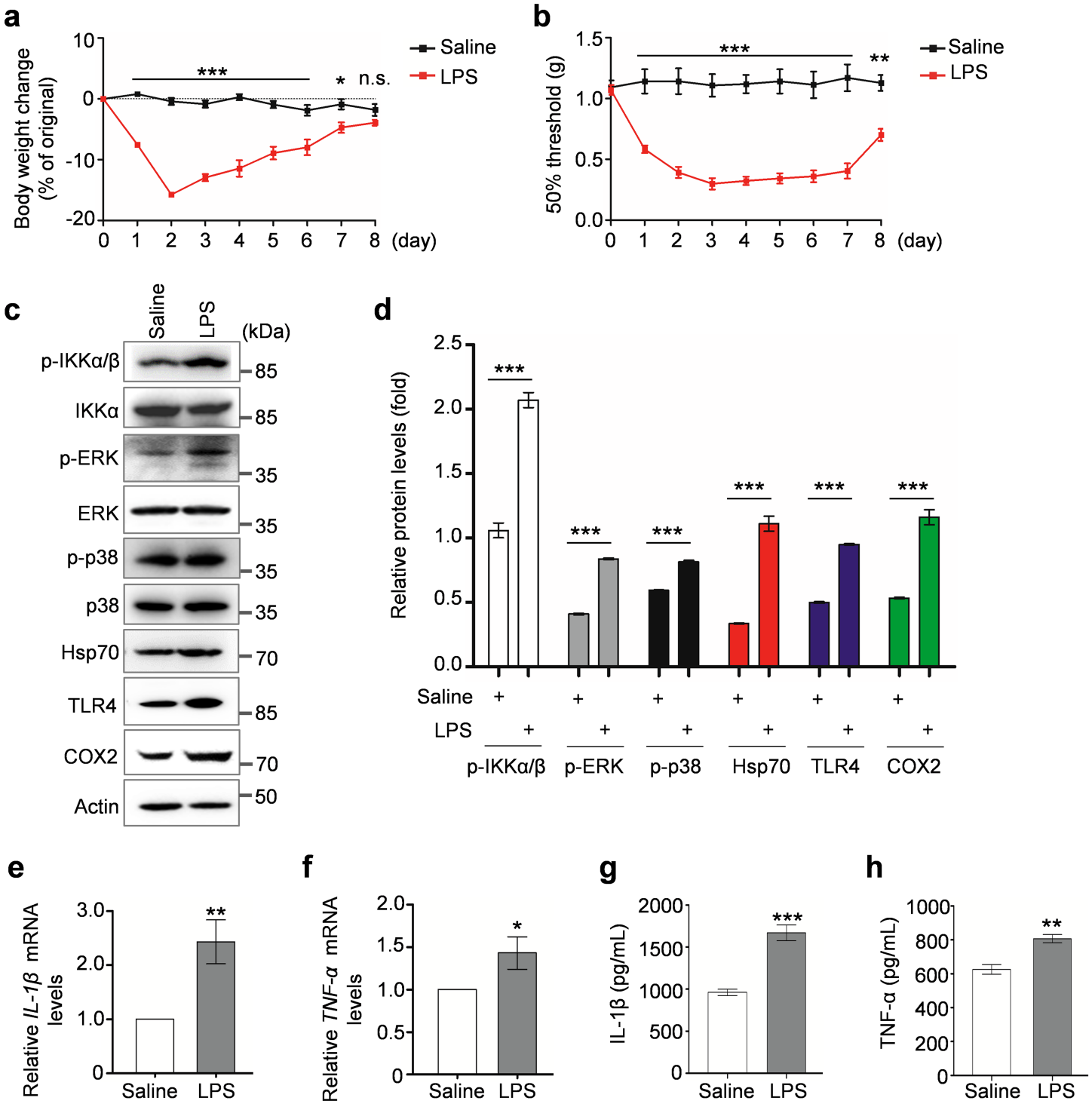

Figure 1

A single intraperitoneal injection of LPS induced inflammatory hyperalgesia and neuroinflammation. Animals were injected with either saline or LPS (2.5 mg/kg) via intraperitoneal on day 0 (n = 10 mice/group). Weight loss (a) and paw withdrawal thresholds to mechanical stimulation (b) of mice treated with saline or LPS were monitored every day until day 8. c On day 7, DRGs were isolated from all spinal levels, pooled per mouse and subjected to Western blotting with the indicated antibodies to show

the upregulation of inflammation-related proteins and the activations of signaling pathways (n = 5 mice/group). d The protein levels of indicated proteins were quantified by Image J software. p-IKKα/β, p-ERK, and p-p38 were normalized to their basal protein levels, respectively. Hsp70, TLR4, and COX2 were normalized to β-actin. e-h On day 7 after injection of either saline or LPS, all spinal levels of DRGs were isolated and pooled per mouse, and total RNA or proteins were extracted. The qRT-PCR was used to determine the mRNA expression levels of cytokine IL-1β (e) and TNF-α (f) relative to β-Actin in DRGs from saline- and LPS-treated group (n = 3 mice/group). ELISA assays were used to determine the protein levels of IL-1β (g) and TNF-α (h) (n = 5 mice/group). All values represent mean ± SD of at least three biological replicates, *P<0.05, **P<0.01, ***P<0.001, n.s., non-significance.

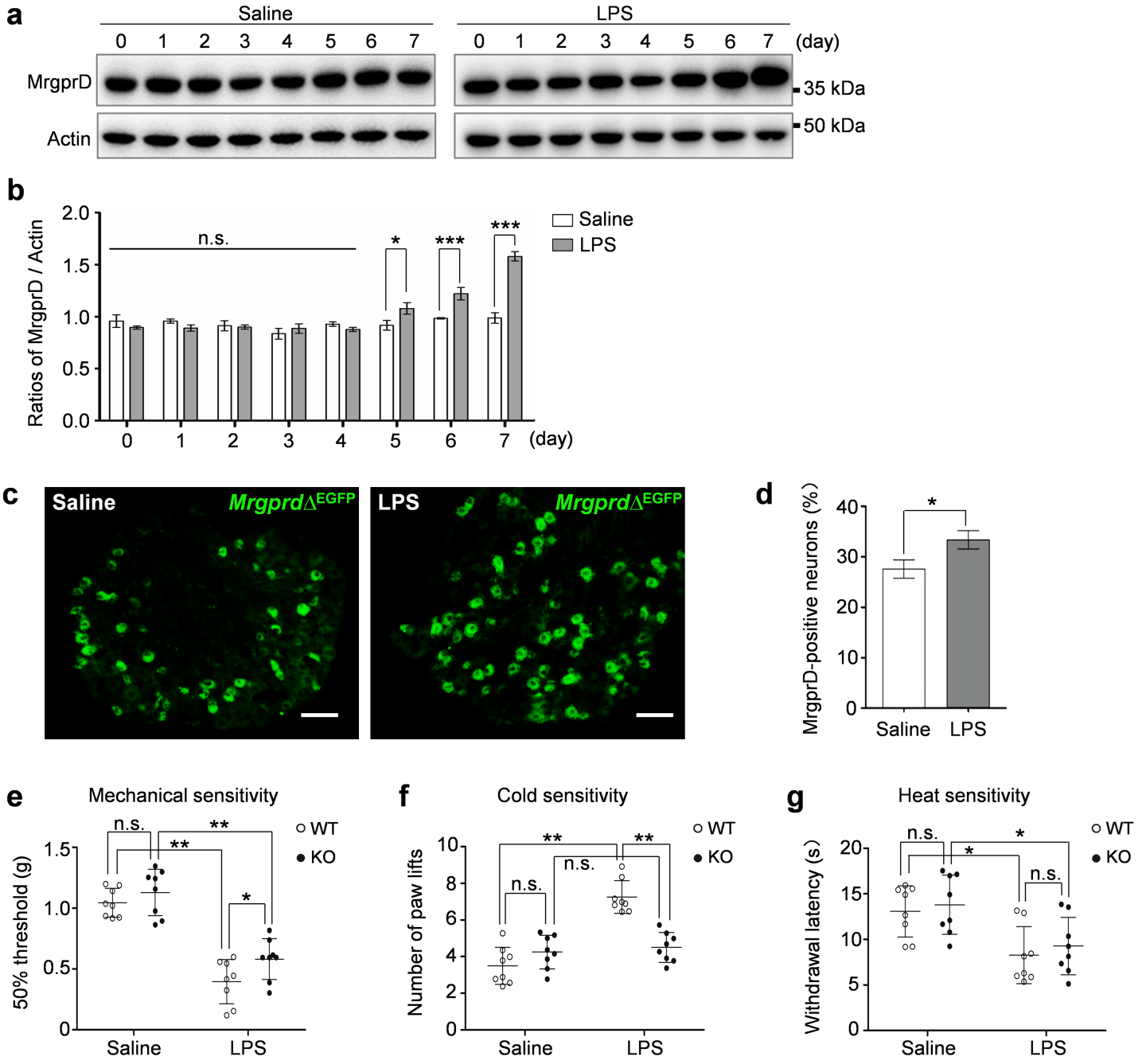

Figure 2

MrgprD was up-regulated in inflamed DRGs and participated in LPS-triggered inflammatory pain. a Mice were injected with either saline or LPS (2.5 mg/kg) via intraperitoneal on day 0 (n = 8 mice/group). One mouse from each group was sacrificed at the indicated day and DRGs from all spinal levels were collected and lysed. The protein levels of MrgprD in DRGs were determined by Western blotting. b The protein levels of MrgprD relative to β-actin were quantified by Image J software from three independent experiments. c Mrgprd-/- mice (MrgprdΔEGFP) were injected with either saline or LPS (2.5 mg/kg) via intraperitoneal. On day 7, L4 DRGs were separately isolated from saline- or LPS-treated Mrgprd-/- mice, cut into cryosections, and imaged under fluorescence and bright-field illumination, respectively. Representative images are shown, scale bars 50 μm. d Percentages of MrgprD-positive neurons were quantified. The numbers of EGFP-positive neurons and the total neurons were enumerated on each section using Image J. n = 3 mice/group. e-g WT and Mrgprd-/- mice (KO) were injected intraperitoneally with either saline or LPS. After 7 days, animal behaviors to noxious stimuli were determined (n = 8 mice/group). Paw withdrawal thresholds to mechanical stimulation in WT and Mrgprd-/- mice were determined using von Frey testing (e). The numbers of the left hind paw lifts of WT and Mrgprd-/- mice in response to cold were counted over 5 min and compared (f). The withdrawal latencies of the left hind paw of WT and Mrgprd-/- mice in response to radiant heat were recorded and compared (g). All values represent mean ± SD of at least three biological replicates, *P<0.05, **P<0.01, n.s., non-significance.

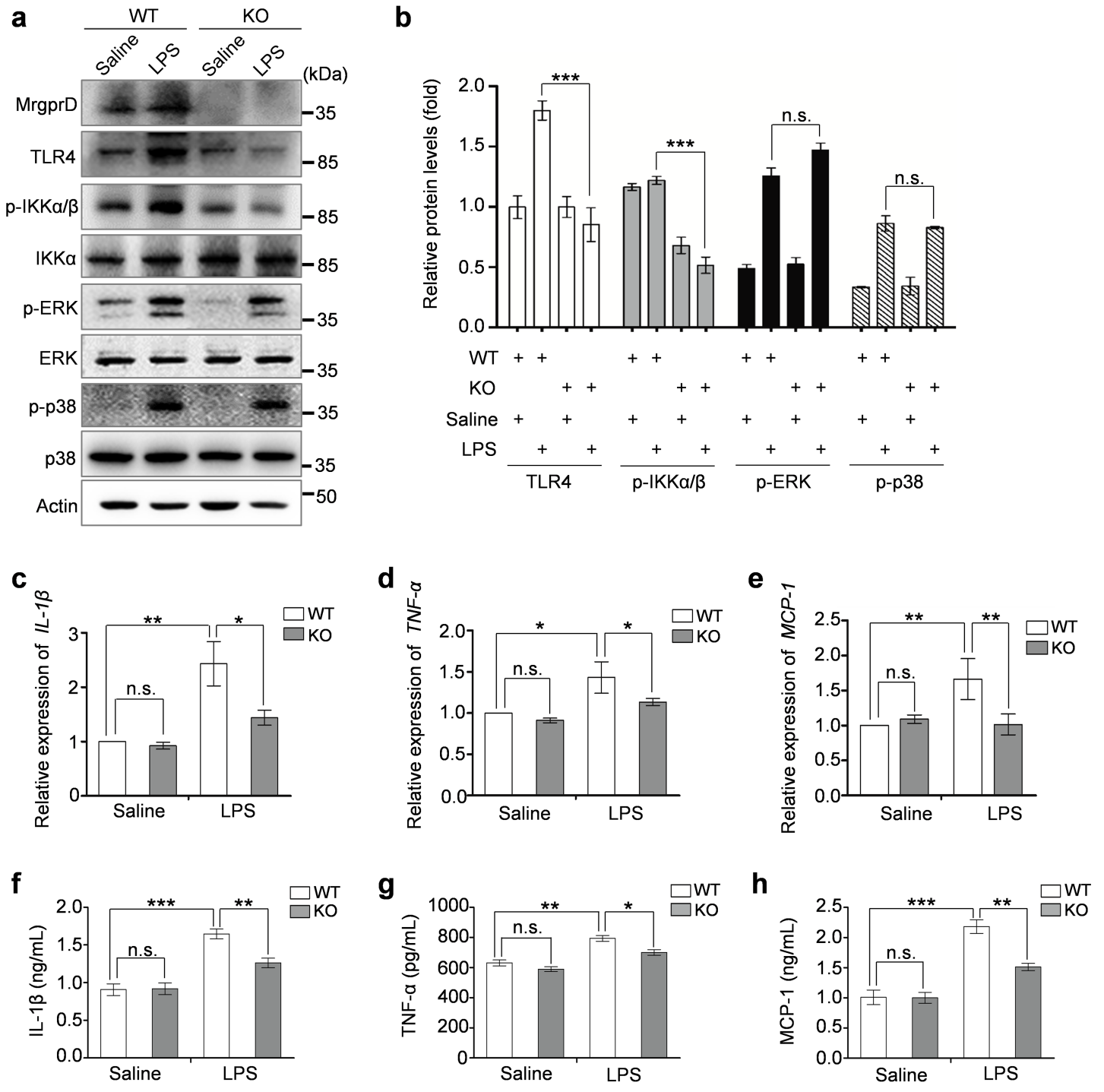

Figure 3

The deletion of Mrgprd resulted in an attenuation of LPS-induced inflammatory responses in DRGs. a WT and Mrgprd-/- mice (KO) were injected intraperitoneally with either saline or LPS. 7 days after injection, DRGs from all spinal levels were isolated and pooled per mouse (n = 5 mice/group). Proteins were extracted and subjected to Western blotting using the indicated antibodies. β-actin was used as an internal control. b The relative expression levels of TLR4 (normalized to β-actin) or phosphorylation levels of IKKα/β, ERK, p38 (normalized to their basal protein levels, respectively) in each group were quantified from three independent experiments. c-h 7 days after injection, all spinal levels of DRGs were isolated and

pooled per mouse, and total RNA or proteins were extracted. The qRT-PCR was used to determine the expression levels of pro-inflammatory cytokines, IL-1β (c) and TNF-α (d), and chemokine MCP-1 (e) (n = 3 mice/group). ELISA assays were used to determine the protein levels of IL-1β (f), TNF-α (g), and MCP-1 (h) (n = 5 mice/group). All values represent mean ± SD, *P<0.05, **P<0.01, ***P<0.001, n.s., non-significance.

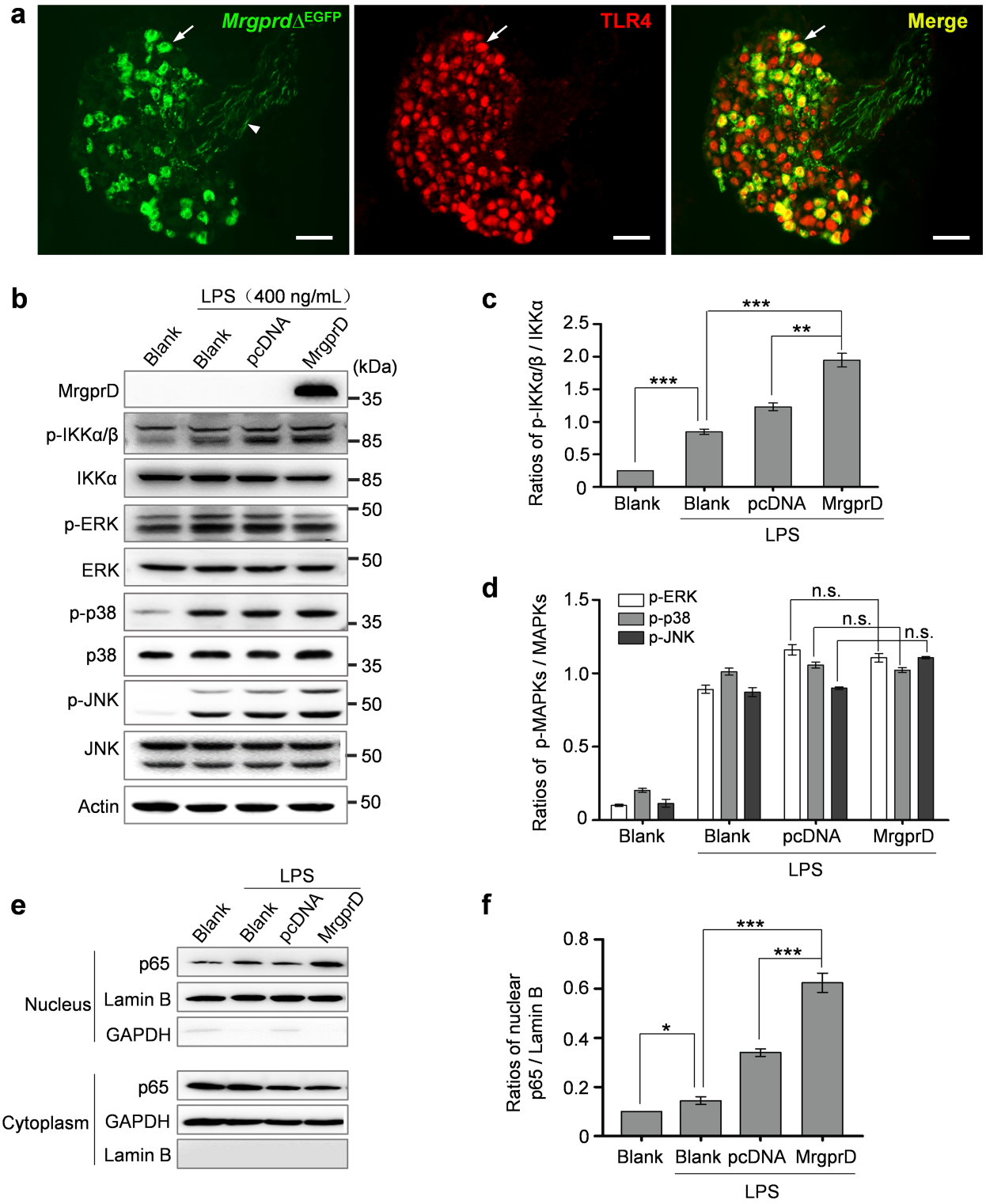

Figure 4

MrgprD overexpression promoted LPS-triggered activation of NF-κB signaling. a Immunostaining of mouse TLR4 (red) in L4 DRGs of Mrgprd-/- mice (MrgprdΔEGFP) showing the co-localization of TLR4 with MrgprD (EGFP-positive) in a subset of DRG neurons, scale bars 50 μm. b HEK-Blue mTLR4 cells were transfected with pcDNA3.1-Flag-MrgprD and pcDNA3.1 empty vector, respectively. After transfection for 36 h, the cells were stimulated with LPS for 30 min and subjected to Western blotting using the indicated antibodies. The un-transfected cells were used as blank control. c The relative expressions of p-IKKα/β to IKKα in different treatments in (b) were quantified. d The expression levels of p-ERK, p-p38, and p-JNK relative to their basal levels in different treatments in (b) were quantified. e After transfection for 36 h, the cells were stimulated with LPS for 30 min and the cytoplasmic/nuclear fractions were separately extracted and analyzed for the presence of p65 by Western blotting. The un-transfected cells were used as blank control. The purity of fraction was determined by the expression of Lamin B in nucleus and its absence in cytoplasm. The blots are representative of three independent experiments. f Ratios of p65 relative to Lamin B in the nuclear fractions were quantified. The ratios were calculated from the band intensities measured by densitometry and are means ± SD of three independent assays. **P<0.01, ***P<0.001, n.s., non-significance.

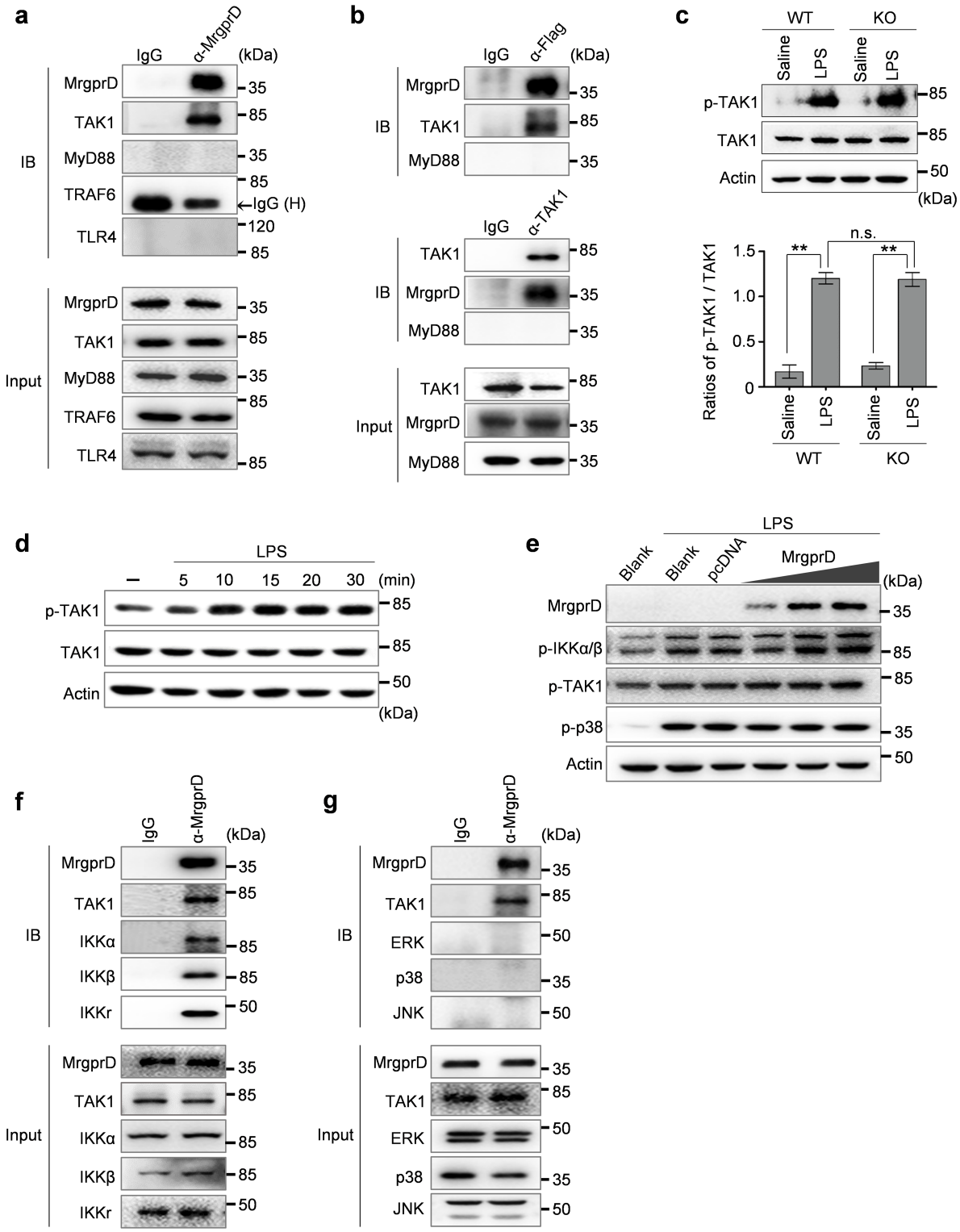

Figure 5

MrgprD interacted with TAK1 and IKK complex in mouse DRG. a Co-immunoprecipitation showing the physical interaction of MrgprD and TAK1 in mouse DRGs. The samples precipitated using antibody specific against MrgprD were subjected to Western blotting and probed with the indicated antibodies, respectively. Non-specific immunoglobulin G (IgG) was used as control. Inputs indicated pre-immunoprecipitated samples of DRG extracts. b Co-immunoprecipitation showing the complex formation

between MrgprD and TAK1 in HEK293T cells overexpressed with Flag-tagged MrgprD. The samples precipitated using anti-Flag antibody or anti-TAK1 antibody were subjected to Western blotting and probed with the indicated antibodies, respectively. c The WT and Mrgprd-/- mice (KO) were injected intraperitoneally with saline or LPS. 7 days after injection, DRGs were isolated and pooled per mouse. Upper panel, proteins were extracted and subjected to Western blotting using the indicated antibodies. Lower panel, the expression levels of phosphorylated TAK1 (Thr-187) relative to basal levels of TAK1 in each group were quantified. All values represent mean ± SD of three independent assays, **P<0.01, n.s., non-significance. d LPS stimulation induced the phosphorylation of TAK1 at the site of Thr-187. HEK-Blue mTLR4 cells were treated with 400 ng/mL of LPS for the indicated times and subjected to Western blotting using the indicated antibodies. e HEK293-Blue mTLR4 cells were transfected with pcDNA3.1 vector (1.5 μg) and pcDNA3.1-Flag-MrgprD (0.5 μg, 1.0 μg, or 1.5 μg), respectively. After transfection for 36 h, the cells were stimulated with LPS for 30 min and subjected to Western blotting to show the phosphorylation levels of TAK1, IKKα/β, and p38 MAPK. f Co-immunoprecipitation using anti-MrgprD showed the physical interaction of MrgprD with TAK1 and the IKK complex (IKKα, IKKβ, and IKKγ) in mouse DRGs. g Co-immunoprecipitation using anti-MrgprD showed that p38, ERK, and JNK did not precipitated along with MrgprD-TAK1 complex in mouse DRGs.

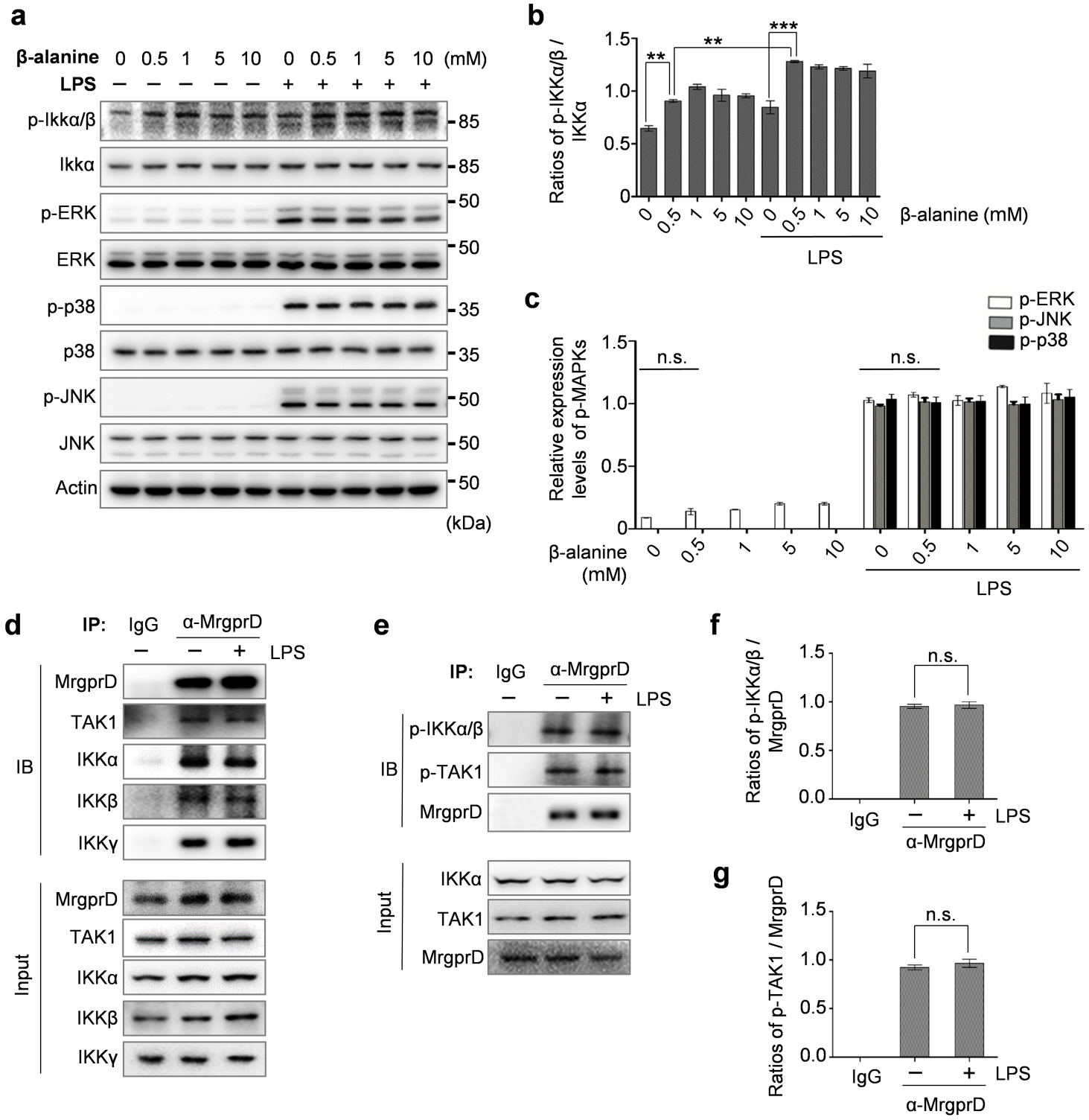

Figure 6

MrgprD activation by β-alanine induced the activation of NF-κB signaling through binding to TAK1-IKKs in RAW 264.7 cells. a Cells were treated with β-alanine (at the indicated concentrations) alone or together with LPS (400 ng/mL) for 30 min. Then, cells were lysed and subjected to Western blotting with the indicated antibodies. b The expression levels of p-IKKα/β relative to IKKα in different treatments in (a) were quantified. c The expression levels of p-ERK, p-p38, and p-JNK relative to their basal levels in

different treatments in (a) were quantified. All values represent mean ± SD of three independent assays, **P<0.01, ***P<0.001, n.s., non-significance. d Whole cell lysates from RAW 264.7 cells stimulated with or without LPS were immunoprecipitated using MrgprD antibody and then immunoblotted with the indicated antibodies, respectively. Non-specific IgG was used as control. Inputs indicated pre-immunoprecipitated samples. e Whole cell lysates from RAW 264.7 cells stimulated with or without LPS were immunoprecipitated using MrgprD antibody and then immunoblotted with antibodies against p-IKKα/β, p-TAK1, and MrgprD to show the amount of p-IKKα/β or p-TAK1 in MrgprD protein complex. f, g The relative protein levels of p-IKKα/β or p-TAK1 to MrgprD in the immunoprecipitants from (e) were quantified. All values represent mean ± SD of three independent assays, n.s., non-significance.

Supplementary Files

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