Role of macrophage migration inhibitory factor in NLRP3 inflammasome expression in otitis media

Role of MIF in NLRP3 inflammasome production

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Hypothesis: Macrophage migration inhibitory factor plays an important role in the expression of interleukin (IL)-1β and the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome in lipopolysaccharide-induced otitis media.

Background: NLRP3 inflammasome and macrophage migration inhibitory factor are critical molecules mediating inflammation. However, the interaction between the NLRP3 inflammasome and macrophage migration inhibitory factor has not been fully examined.

Methods: Wild-type mice and macrophage migration inhibitory factor gene-deficient (MIF-/-) mice received a transtympanic injection of either lipopolysaccharide or phosphate-buffered saline. The mice were sacrificed 24 h after the injection. Concentrations of IL-1β, NLRP3, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain and a pyrin domain), and caspase-1 in the
middle ear effusions were measured by enzyme-linked immunosorbent assay. Temporal
bones were processed for histologic examination and immunohistochemistry.

Results: In the immunohistochemical study using the wild-type mice, positive staining
of macrophage migration inhibitory factor, NLRP3, ASC, and caspase-1 were observed
in infiltrating inflammatory cells induced by lipopolysaccharide in the middle ear. The
number of inflammatory cells caused by lipopolysaccharide administration decreased
remarkably in the MIF<sup>−/−</sup> mice as compared with the wild-type mice. The concentrations
of IL-1β, NLRP3, ASC, and caspase-1 increased in the lipopolysaccharide-treated
wild-type mice. The MIF<sup>−/−</sup> mice with lipopolysaccharide had decreased levels of IL-1β,
NLRP3, ASC, and caspase-1 as compared with the wild-type mice.

Conclusion: Macrophage migration inhibitory factor has an important role in the
production of IL-1β and the NLRP3 inflammasome. Controlling the inflammation by
modulating macrophage migration inhibitory factor and the NLRP3 inflammasome may
be a novel therapeutic strategy for otitis media.

Keywords:
infection; Toll-like receptor; NOD-like receptor; cytokine; interleukin; inflammation
Otitis media is one of the most common diseases, especially in children. Otitis media associated with bacterial infection is frequently treated with antibiotics all over the world (1). Repeated use of antibiotics for frequent recurrence of otitis media might be related to microbial antibiotic resistance (2). Multiple inflammatory mediators have been reported in the pathophysiology of otitis media, and regulation of these factors may become a novel therapeutic option for otitis media without the administration of antibiotics (3,4). Interleukin (IL)-1β is a pro-inflammatory cytokine with important roles in the innate immune system. IL-1β is involved in the pathogenesis of otitis media, and activated caspase-1 is required for the processing of pro-IL-1β into mature IL-1β (3).

The inflammasome is a protein complex, and several subtypes of inflammasome have been reported. The nucleotide-binding oligomerization domain...
(NOD)-like receptor protein 3 (NLRP3) inflammasome is an important inflammatory factor discovered at the beginning of the 2000s (5,6). The components of the NLRP3 inflammasome are NLRP3, ASC (adaptor apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) and a pyrin domain (PYD)), and pro-caspase-1 (7). The NLRP3 inflammasome controls the production of IL-1β and IL-18 in collaboration with Toll-like receptors and nuclear factor kappa B (NF-κB). When the NLRP3 inflammasome is formed, it causes caspase-1 activation, resulting in the maturation of IL-1β (8). The role of the NLRP3 inflammasome has been extensively examined in numerous diseases, and has also been reported as a critical factor controlling inflammation in otitis media, both in human and animal models (9-12).

Macrophage migration inhibitory factor is an inflammatory and stress-regulating cytokine with multiple functions (13). The significant role of macrophage migration inhibitory factor in middle ear and inner ear diseases has been reported (14-19). The reduction in macrophage migration inhibitory factor activity by intraperitoneal injection of a macrophage migration inhibitory factor antagonist can
decrease inflammatory responses in the middle ear cavity in lipopolysaccharide-induced otitis media (20). The inhibition of macrophage migration inhibitory factor pathway reduces cytokine production (13). However, the mechanism of inflammation through macrophage migration inhibitory factor has not been fully revealed.

To the best of our knowledge, only two recent studies have reported the interaction between the NLRP3 inflammasome and macrophage migration inhibitory factor (21,22). In addition, no previous study has shown the role of macrophage migration inhibitory factor in expression of the NLRP3 inflammasome in otitis media. Using macrophage migration inhibitory factor-deficient (MIF<sup>−/−</sup>) mice, the purpose of this study is to reveal the definitive effect of macrophage migration inhibitory factor in the induction of the NLRP3 inflammasome in lipopolysaccharide-induced otitis media.

Materials and Methods

Induction of otitis media by lipopolysaccharide

Male BALB/c mice at 6-10 weeks of age were used in this study. Through
targeted disruption of the macrophage migration inhibitory factor gene, MIF\(^{-/-}\) mice in
the BALB/c background were established (23). The study was performed in accordance
with the relevant animal protection rules, and the Animal Research Control Committee
approved the study (application number, OKU-2016541; the name of the principal
investigator, S.K.). Before the experiment, an otoscopic examination was performed on
the ears of all the mice to ensure that the tympanic membranes were normal and that no
middle ear inflammation was present. An intraperitoneal injection of a mixture of
ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) was
administered for anesthesia during all experimental procedures. Both the wild-type mice
and MIF\(^{-/-}\) mice were randomly divided into two groups. The otitis media group
received lipopolysaccharide (1.0 mg/mL; 10 μl/ear; both ears in each mouse;
Sigma-Aldrich, St. Louis, Missouri, USA) via transtympanic injection using a 30-gauge
needle. Phosphate-buffered saline (PBS) (10 μl/ear) was injected into both middle ears
of the animals in the control group. The mice were sacrificed 24 hours after injection of
the lipopolysaccharide or PBS. The middle ears were then washed transtympanically
using 200 μl of PBS. The collected washings from the middle ear lavage were
centrifuged. The supernatant was transferred to microcentrifuge tubes (Treff AG, Degersheim, Switzerland) and stored at -30°C until analysis. The temporal bones were removed immediately after sacrifice and processed for histologic examination.

Levels of IL-1β, NLRP3, ASC, and caspase-1

The concentrations of IL-1β, NLRP3, ASC, and caspase-1 in the supernatant of the middle ear lavage (otitis media group, n=6; control group, n=6) were measured using enzyme-linked immunosorbent assay (ELISA) (IL-1β, 559603, BD OptEIA Mouse IL-1β ELISA Set, BD Biosciences, San Jose, CA, USA; NLRP3, CSB-EL015871MO, Mouse NLRP3 ELISA Kit, CUSABIO, College Park, MD, USA; ASC, CSB-EL019114MO, Mouse Apoptosis-associated speck-like protein containing a CARD (PYCARD) ELISA kit, CUSABIO; Caspase-1, SEB592Mu, ELISA Kit for Caspase 1, Cloud-Clone Corp., Houston, TX, USA). All samples were examined in duplicate, and measured values were averaged.

Histologic examination
Temporal bone specimens from both the wild-type mice and MIF<sup>−/−</sup> mice (otitis media group, n=4; control group, n=4) were placed in 4% paraformaldehyde for 72 hours and decalcified in 10% ethylenediaminetetraacetic acid for 3 weeks at 4°C. After dehydration, the specimens were embedded in paraffin and sectioned at a thickness of 10 μm, then mounted on glass slides, processed using hematoxylin and eosin staining, and evaluated under light microscopy.

**Immunohistochemistry**

The paraffin-embedded temporal bone specimens from the wild-type mice (otitis media group, n=6; control group, n=6) were sectioned at a thickness of 4 μm and mounted on glass slides. The sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature. Antigen retrieval was performed by microwave heating. Goat serum albumin (S-1000, Vector Laboratories Inc., Burlingame, CA, USA) was used for 1 hour at room temperature to block non-specific protein binding. Rabbit anti-macrophage migration inhibitory factor antibody (sc-20121; Santa Cruz)
Biotechnology, Inc., Santa Cruz, CA), rabbit anti-NLRP3 antibody (bs-10021R, Bioss Antibodies Inc., Woburn, MA, USA), rabbit anti-ASC antibody (NBP1-78977, Novus Biologicals, Littleton, CO, USA), and rabbit anti-caspase-1 antibody (NB100-56564, Novus Biologicals) were applied overnight at 4°C as the primary antibodies for immunohistochemical staining. Rabbit Immunoglobulin Fraction (X0903, Dako, Glostrup, Denmark) was used as a negative control. For visualization, a VECTASTAIN Elite ABC Kit (PK-6100, Vector Laboratories Inc.) and 3,3’-diaminobenzidine (DAB) reagent (K3467, Dako) were used according to the manufacturers’ instructions. The reaction was assessed by blinded investigators under light microscopy according to the method of previous study (24). Briefly, the rating score was classified as: (-), no positive reaction; (+), 1-10 positive cells; ( ++ ), 11-100 positive cells; and ( +++ ), over 100 positive cells per high power field (×400).

Statistical analysis

Data are presented as median ± standard error. For statistical analysis, the non-parametric Mann-Whitney U test was used for comparison of continuous variables.
between the two groups. The chi-square test was applied to compare categorical variables. Significant differences were established at a level of $P < 0.05$ (IBM SPSS Statistics; IBM, New York, USA).

Results

Expression of macrophage migration inhibitory factor and NLRP3 inflammasome by lipopolysaccharide

Lipopolysaccharide is a component of the outer membrane of gram-negative bacteria that is a major causative pathogen of otitis media, and it is a potent inflammatory molecule (14). Lipopolysaccharide induces an increased infiltration of inflammatory cells in middle ear (25). As a first step, we examined the expression and localization of macrophage migration inhibitory factor, NLRP3, ASC, and caspase-1 in the middle ear cavity as induced by lipopolysaccharide in wild-type mice.

Strong positive immunostaining was found for macrophage migration inhibitory factor in the infiltrating inflammatory cells as well as mucosal epithelium in...
the middle ear of the lipopolysaccharide-injected wild-type mice. NLRP3, ASC, and caspase-1 were also observed in inflammatory cells and middle ear mucosa of the lipopolysaccharide-treated wild-type mice. There was no significant immunostaining in the middle ear in the negative controls using Rabbit Immunoglobulin Fraction in the PBS-treated wild-type mice (Figure 1).

The rating scores of immunopositive cells for macrophage migration inhibitory factor, NLRP3, ASC, and caspase-1 were summarized in Table 1. The lipopolysaccharide-injected wild-type mice had the increased number of immunopositive cells as compared with PBS-injected control mice (macrophage migration inhibitory factor, \( P<0.05 \); NLRP3, \( P<0.05 \); ASC, \( P<0.05 \); caspase-1, \( P<0.05 \)).

Role of macrophage migration inhibitory factor in lipopolysaccharide-induced otitis media

Next, we examined the effect of deficiency of the macrophage migration inhibitory factor gene in lipopolysaccharide-induced otitis media. Administration of lipopolysaccharide into the middle ear cavity induced remarkable infiltration of
inflammatory cells (polymorphonuclear leukocyte and monocyte) in the middle ear in wild-type mice (Figure 2A). In contrast, a small number of infiltrating inflammatory cells was detected in the middle ear in lipopolysaccharide-treated MIF−/− mice (Figure 2B). No significant number of inflammatory cells was found in the middle ear in either the wild-type mice or MIF−/− mice in the PBS-injected control group.

Quantification of IL-1β and NLRP3 inflammasome

The histological findings showed the inflammatory response reduced in MIF−/− mice by lipopolysaccharide as compared with wild type mice. Thus, we examined the levels of IL-1β, NLRP3, ASC, and caspase-1 in lipopolysaccharide-induced otitis media. The protein levels of IL-1β, NLRP3, ASC, and caspase-1 in the supernatant of the middle ear lavage from both the wild-type mice and MIF−/− mice are shown in Figure 3. Compared with the PBS-injected wild-type mice, the lipopolysaccharide-injected wild-type mice showed a significant increase in the protein concentration of IL-1β in the middle ear (P < 0.05). In the MIF−/− mice, lipopolysaccharide induced a lower level of IL-1β than in the wild-type mice. There was
a significant difference in the concentration of IL-1β between the lipopolysaccharide
group and PBS group of MIF−/− mice (Figure 3).

Compared with the PBS-injected wild-type mice, the lipopolysaccharide-injected wild-type mice showed significant up-regulation of NLRP3 (P < 0.05), ASC (P < 0.05), and caspase-1 (P < 0.05) in the middle ear. There were significant differences between the wild-type mice and MIF−/− mice in the concentrations of NLRP3 (P < 0.05), ASC (P < 0.05), and caspase-1 (P < 0.05) induced by lipopolysaccharide. In addition, no statistically significant difference was observed in the concentrations of NLRP3, ASC, and caspase-1 between the lipopolysaccharide group and PBS group of MIF−/− mice (Figure 3).

Discussion

Otitis media is one of the most common middle ear diseases, and patients with otitis media frequently have hearing impairment. Numerous factors are associated with the onset and development of otitis media. The presence of upper respiratory diseases
and Eustachian tube dysfunction are important factors, and inflammatory cytokines and chemokines including IL-1β are also involved in the pathogenesis of otitis media (3). Lipopolysaccharide from gram-negative bacteria activates Toll-like receptor 4, and induces IL-1β production through the NF-κB pathway (26). In addition, the maturation of pro-IL-1β protein into the secreted bioactive form of IL-1β requires a second signal via NLRP3 inflammasome (27). Lipopolysaccharide has been detected in the middle ear in almost all patients with otitis media (17). The expression of Toll-like receptor 4 in the middle ear tissues of patients with otitis media has been reported, and Toll-like receptors have been suggested to have an important role in the pathogenesis of otitis media (4,28,29). Recent studies have reported that NLRP3 was detected in middle ear tissues in patients with otitis media (9,11). In an animal model of otitis media, the NLRP3 inflammasome was induced by lipopolysaccharide in mouse middle ear, and ASC-deficient mice had reduced middle ear inflammation (10,12).

Macrophage migration inhibitory factor is a cytokine expressed in various cells, and has been associated with a multitude of diseases (30). Macrophage migration inhibitory factor has been reported to have a possible role in middle ear diseases and
hearing function (14,19). Inhibition of macrophage migration inhibitory factor resulted in the reduction of inflammatory responses in experimental otitis media (20). However, the mechanism was not revealed. This study shows for the first time that production of IL-1β and the NLRP3 inflammasome by lipopolysaccharide is remarkably suppressed in MIF−/− mice. There were several limitations in this study including small sample size and the use of a single time point. However, our findings suggest that the reduced inflammation in histological findings and the decreased secretion of IL-1β in MIF−/− mice are the result of down-regulation of the NLRP3 inflammasome.

Investigations are just starting to examine the relationship between macrophage migration inhibitory factor and the NLRP3 inflammasome. A recent study showed that macrophage migration inhibitory factor is required for the interaction between NLRP3 and the intermediate filament protein vimentin, which is critical for NLRP3 activation (22). Another study showed that macrophage migration inhibitory factor has an upstream role in the inflammatory pathway by regulating NLRP3 inflammasome activation (21). The possible mechanism of macrophage migration inhibitory factor and NLRP3 inflammasome on the induction of IL-1β is shown in
Figure 4. In this study, the concentration of IL-1β induced in the middle ear by lipopolysaccharide was low in the MIF<sup>−/−</sup> mice as compared with the wild-type mice. Down-regulation of the caspase-1 may be the major factor in the reduced production of IL-1β in the MIF<sup>−/−</sup> mice. However, there was still a significant difference in the expression of IL-1β between the lipopolysaccharide-injected MIF<sup>−/−</sup> mice and PBS-injected MIF<sup>−/−</sup> mice. Lipopolysaccharide may also induce IL-1β through a different signaling pathway independent of macrophage migration inhibitory factor and the NLRP3 inflammasome.

Otitis media is a common disease, and the management of intractable otitis media is a challenging problem. Macrophage migration inhibitory factor and NLRP3 inflammasome have an important role in immune response. For example, the inhibition of macrophage migration inhibitory factor activity attenuated lethality in endotoxic shock (31). In addition, macrophage migration inhibitory factor genetic variants are a clinically important risk factor for the development of several diseases (32). Currently, there is no clinically available targeted therapy that can effectively inhibit macrophage migration inhibitory factor and/or NLRP3 inflammasome. However, macrophage
migration inhibitory factor as well as NLRP3 inflammasome may be promising factors in future treatment strategies for otitis media.

In summary, the expression of IL-1β is markedly induced by lipopolysaccharide in mouse middle ear, and is significantly suppressed in MIF^-/- mice as compared with wild-type mice. The induction of NLRP3 inflammasome by lipopolysaccharide is also reduced in the MIF^-/- mice. Our findings suggest that regulation of macrophage migration inhibitory factor and the NLRP3 inflammasome may become a new therapeutic target for control of the inflammation from a different point of view.
Acknowledgments

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Disclosure of Interest

The authors report no conflict of interest.


Figure 1

Immunohistochemical staining for (A) macrophage migration inhibitory factor, (B) nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3), (C) apoptosis-associated speck-like protein containing a caspase recruitment domain and a pyrin domain (ASC), and (D) caspase-1 in lipopolysaccharide-injected wild-type mice. Strong positive staining (brown color) was observed in inflammatory cells (black arrow). (E) Immunohistochemical staining using Rabbit Immunoglobulin Fraction in phosphate-buffered saline (PBS)-treated control mice. (*, middle ear cavity; Scale bar, 100 μm)

Figure 2

Histological findings of the middle ear cavity in (A) wild-type mice and (B) MIF⁻/⁻ mice with transtympanic injection of lipopolysaccharide. Numerous inflammatory cells (polymorphonuclear leukocyte and monocyte) infiltrated into the middle ear cavity in
the lipopolysaccharide-injected wild-type mice. In contrast, a small number of inflammatory cells were found in the lipopolysaccharide-injected MIF-/- mice. (Hematoxylin and eosin staining; scale bar, 100 μm) (black arrow, inflammatory cells; *, middle ear cavity; MIF, macrophage migration inhibitory factor).

Figure 3

Concentrations of (A) interleukin-1β (IL-1β), (B) nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3), (C) apoptosis-associated speck-like protein containing a caspase recruitment domain and a pyrin domain (ASC), and (D) caspase-1 in lipopolysaccharide (LPS)-injected and phosphate-buffered saline (PBS)-treated mice. (n = 6 (12 ears); median ± standard error; *, P < 0.05) (MIF, macrophage migration inhibitory factor; n.s., not significant).

Figure 4

Potential molecular mechanism of macrophage migration inhibitory factor and NLRP3 inflammasome on the production of IL-1β. Signal 1 (Toll like receptor/NF-κB pathway)
is needed to induce pro-IL-1β. Signal 2 with macrophage migration inhibitory factor, vimentin, and NLRP3 inflammasome has a critical role in the production of caspase-1. The active caspase-1 released from the NLRP3 inflammasome is responsible for the conversion of inactive IL-1β precursor into its biological active form.

LPS: lipopolysaccharide
TLR4: Toll-like receptor 4
NF-κB: nuclear factor-kappa B
NLRP3: the nucleotide-binding oligomerization domain (NOD)-like receptor protein 3
ATP: adenosine triphosphate
PAMPs: pattern-associated molecular patterns
DAMPs: danger-associated molecular patterns
ROS: reactive oxygen species
MIF: macrophage migration inhibitory factor
ASC: adaptor apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) and a pyrin domain (PYD)
Figure 4: Schematic diagram showing the signaling pathways involving TLR4, MyD88, NF-κB, NLRP3, ROS, MIF, pro-caspase-1, and pro-IL-1β and pro-IL-18 in response to LPS and ATP/PAMPs/DAMPs. The diagram illustrates the activation of NF-κB in the nucleus, the interaction of NLRP3 with ASC, and the processing of pro-caspase-1 to caspase-1, leading to the production of IL-1β and IL-18.
Table 1: The rating scores of immunostaining for each protein in lipopolysaccharide (LPS)- or phosphate buffered saline (PBS)-injected wild-type mice.

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The rating score: (-), no positive reaction; (+), 1-10 positive cells; (++) 11-100 positive cells; and (+++), over 100 positive cells per high power field (×400).

MIF, macrophage migration inhibitory factor
NLRP3, The nucleotide-binding oligomerization domain (NOD)-like receptor protein 3
ASC, adaptor apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) and a pyrin domain (PYD)