Title

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

Short running head

Role of MIF in NLRP3 inflammasome production

Name of authors

Shin Kariya, MD, PhD[1)]; Mitsuhiro Okano, MD, PhD[1,2)]; Pengfei Zhao, MD[1)]; Yukihide Maeda, MD, PhD[1)]; Yuko Kataoka, MD, PhD[1)]; Takaya Higaki, MD, PhD[1)]; Seiichiro Makihara, MD, PhD[3)]; Jun Nishihira, MD, PhD[4)]; Tomoyasu Tachibana, MD, PhD[5)]; Kazunori Nishizaki, MD, PhD[1)]

Author's affiliations

1) Department of Otolaryngology-Head and Neck Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

2) Department of Otorhinolaryngology, International University of Health and Welfare School of Medicine, Narita, Japan

3) Department of Otolaryngology-Head & Neck Surgery, Kagawa Rosai Hospital, Marugame, Japan

4) Department of Medical Bioinformatics, Hokkaido Information University, Sapporo, Japan

5) Departments of Otolaryngology, Japanese Red Cross Society Himeji Hospital, Himeji City, Japan

Correspondence author

Shin Kariya, MD, PhD

Corresponding mailing address

Department of Otolaryngology-Head and Neck Surgery

Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences

2-5-1 Shikata-cho, Kita-ku, Okayama, 700-8558, Japan

E-mail: skariya@cc.okayama-u.ac.jp

Conflicts of Interest and Source of Funding

The authors report no conflict of interest to disclose. This work was supported by JSPS KAKENHI (Grants-in-Aid for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology of Japan) (Grant Numbers, JP17K11329).

Abstract

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 $MIF^{-/-}$ 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 $MIF^{-/-}$ 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:

37 infection; Toll-like receptor; NOD-like receptor; cytokine; interleukin; inflammation

38

39

40 Text

41

42 Introduction

43

44 Otitis media is one of the most common diseases, especially in children. Otitis
45 media associated with bacterial infection is frequently treated with antibiotics all over
46 the world (1). Repeated use of antibiotics for frequent recurrence of otitis media might
47 be related to microbial antibiotic resistance (2). Multiple inflammatory mediators have
48 been reported in the pathophysiology of otitis media, and regulation of these factors
49 may become a novel therapeutic option for otitis media without the administration of
50 antibiotics (3,4). Interleukin (IL)-1β is a pro-inflammatory cytokine with important
51 roles in the innate immune system. IL-1β is involved in the pathogenesis of otitis media,
52 and activated caspase-1 is required for the processing of pro-IL-1β into mature IL-1β
53 (3).

54 The inflammasome is a protein complex, and several subtypes of
55 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 ($MIF^{-/-}$) 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

88 targeted disruption of the macrophage migration inhibitory factor gene, $MIF^{-/-}$ mice in
89 the BALB/c background were established (23). The study was performed in accordance
90 with the relevant animal protection rules, and the Animal Research Control Committee
91 approved the study (application number, OKU-2016541; the name of the principal
92 investigator, S.K.). Before the experiment, an otoscopic examination was performed on
93 the ears of all the mice to ensure that the tympanic membranes were normal and that no
94 middle ear inflammation was present. An intraperitoneal injection of a mixture of
95 ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) was
96 administered for anesthesia during all experimental procedures. Both the wild-type mice
97 and $MIF^{-/-}$ mice were randomly divided into two groups. The otitis media group
98 received lipopolysaccharide (1.0 mg/mL; 10 μl/ear; both ears in each mouse;
99 Sigma-Aldrich, St. Louis, Missouri, USA) via transtympanic injection using a 30-gauge
100 needle. Phosphate-buffered saline (PBS) (10 μl/ear) was injected into both middle ears
101 of the animals in the control group. The mice were sacrificed 24 hours after injection of
102 the lipopolysaccharide or PBS. The middle ears were then washed transtympanically
103 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 $MIF^{-/-}$ 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

168 the middle ear of the lipopolysaccharide-injected wild-type mice. NLRP3, ASC, and
169 caspase-1 were also observed in inflammatory cells and middle ear mucosa of the
170 lipopolysaccharide-treated wild-type mice. There was no significant immunostaining in
171 the middle ear in the negative controls using Rabbit Immunoglobulin Fraction in the
172 PBS-treated wild-type mice (Figure 1).
173 The rating scores of immunopositive cells for macrophage migration
174 inhibitory factor, NLRP3, ASC, and caspase-1 were summarized in Table 1. The
175 lipopolysaccharide-injected wild-type mice had the increased number of
176 immunopositive cells as compared with PBS-injected control mice (macrophage
177 migration inhibitory factor, $P<0.05$; NLRP3, $P<0.05$; ASC, $P<0.05$; caspase-1, $P<0.05$).
178
179 *Role of macrophage migration inhibitory factor in lipopolysaccharide-induced otitis*
180 *media*
181 Next, we examined the effect of deficiency of the macrophage migration
182 inhibitory factor gene in lipopolysaccharide-induced otitis media. Administration of
183 lipopolysaccharide into the middle ear cavity induced remarkable infiltration of

184 inflammatory cells (polymorphonuclear leukocyte and monocyte) in the middle ear in
185 wild-type mice (Figure 2A). In contrast, a small number of infiltrating inflammatory
186 cells was detected in the middle ear in lipopolysaccharide-treated $MIF^{-/-}$ mice (Figure
187 2B). No significant number of inflammatory cells was found in the middle ear in either
188 the wild-type mice or $MIF^{-/-}$ mice in the PBS-injected control group.
189
190 *Quantification of IL-1β and NLRP3 inflammasome*
191 The histological findings showed the inflammatory response reduced in $MIF^{-/-}$
192 mice by lipopolysaccharide as compared with wild type mice. Thus, we examined the
193 levels of IL-1β, NLRP3, ASC, and caspase-1 in lipopolysaccharide-induced otitis media.
194 The protein levels of IL-1β, NLRP3, ASC, and caspase-1 in the supernatant of the
195 middle ear lavage from both the wild-type mice and $MIF^{-/-}$ mice are shown in Figure 3.
196 Compared with the PBS-injected wild-type mice, the
197 lipopolysaccharide-injected wild-type mice showed a significant increase in the protein
198 concentration of IL-1β in the middle ear ($P < 0.05$). In the $MIF^{-/-}$ mice,
199 lipopolysaccharide induced a lower level of IL-1β than in the wild-type mice. There was

200 a significant difference in the concentration of IL-1β between the lipopolysaccharide
201 group and PBS group of $MIF^{-/-}$ mice (Figure 3).
202 Compared with the PBS-injected wild-type mice, the
203 lipopolysaccharide-injected wild-type mice showed significant up-regulation of NLRP3
204 ($P < 0.05$), ASC ($P < 0.05$), and caspase-1 ($P < 0.05$) in the middle ear. There were
205 significant differences between the wild-type mice and $MIF^{-/-}$ mice in the concentrations
206 of NLRP3 ($P < 0.05$), ASC ($P < 0.05$), and caspase-1 ($P < 0.05$) induced by
207 lipopolysaccharide. In addition, no statistically significant difference was observed in
208 the concentrations of NLRP3, ASC, and caspase-1 between the lipopolysaccharide
209 group and PBS group of $MIF^{-/-}$ mice (Figure 3).
210
211 Discussion
212
213 Otitis media is one of the most common middle ear diseases, and patients with
214 otitis media frequently have hearing impairment. Numerous factors are associated with
215 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

232 hearing function (14,19). Inhibition of macrophage migration inhibitory factor resulted
233 in the reduction of inflammatory responses in experimental otitis media (20). However,
234 the mechanism was not revealed. This study shows for the first time that production of
235 IL-1β and the NLRP3 inflammasome by lipopolysaccharide is remarkably suppressed in
236 $MIF^{-/-}$ mice. There were several limitations in this study including small sample size and
237 the use of a single time point. However, our findings suggest that the reduced
238 inflammation in histological findings and the decreased secretion of IL-1β in $MIF^{-/-}$
239 mice are the result of down-regulation of the NLRP3 inflammasome.
240 Investigations are just starting to examine the relationship between
241 macrophage migration inhibitory factor and the NLRP3 inflammasome. A recent study
242 showed that macrophage migration inhibitory factor is required for the interaction
243 between NLRP3 and the intermediate filament protein vimentin, which is critical for
244 NLRP3 activation (22). Another study showed that macrophage migration inhibitory
245 factor has an upstream role in the inflammatory pathway by regulating NLRP3
246 inflammasome activation (21). The possible mechanism of macrophage migration
247 inhibitory factor and NLRP3 inflammasome on the induction of IL-1β is shown in

248 Figure 4. In this study, the concentration of IL-1β induced in the middle ear by
249 lipopolysaccharide was low in the $MIF^{-/-}$ mice as compared with the wild-type mice.
250 Down-regulation of the caspase-1 may be the major factor in the reduced production of
251 IL-1β in the $MIF^{-/-}$ mice. However, there was still a significant difference in the
252 expression of IL-1β between the lipopolysaccharide-injected $MIF^{-/-}$ mice and
253 PBS-injected $MIF^{-/-}$ mice. Lipopolysaccharide may also induce IL-1β through a
254 different signaling pathway independent of macrophage migration inhibitory factor and
255 the NLRP3 inflammasome.
256 Otitis media is a common disease, and the management of intractable otitis
257 media is a challenging problem. Macrophage migration inhibitory factor and NLRP3
258 inflammasome have an important role in immune response. For example, the inhibition
259 of macrophage migration inhibitory factor activity attenuated lethality in endotoxic
260 shock (31). In addition, macrophage migration inhibitory factor genetic variants are a
261 clinically important risk factor for the development of several diseases (32). Currently,
262 there is no clinically available targeted therapy that can effectively inhibit macrophage
263 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

This work was supported by JSPS KAKENHI Grant Number JP17K11329 (Grants-in-Aid for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology of Japan).

281 Disclosure of Interest

282 The authors report no conflict of interest.

283

284

285 References:

286

287 1. Monasta L, Ronfani L, Marchetti F, et al. Burden of disease caused by otitis media:

288 systematic review and global estimates. *PLoS One* 2012;7:e36226.

289 2. Vergison A, Dagan R, Arguedas A, et al. Otitis media and its consequences: beyond

290 the earache. *Lancet Infect Dis* 2010;10:195-203.

291 3. Juhn SK, Jung MK, Hoffman MD, et al. The role of inflammatory mediators in the

292 pathogenesis of otitis media and sequelae. *Clin Exp Otorhinolaryngol*

293 2008;1:117-38.

294 4. Hirai H, Kariya S, Okano M, et al. Expression of toll-like receptors in chronic otitis

295 media and cholesteatoma. *Int J Pediatr Otorhinolaryngol* 2013;77:674-6.

296 5. Franchi L, Muñoz-Planillo R, Núñez G. Sensing and reacting to microbes through

297 the inflammasomes. *Nat Immunol* 2012;13:325-32.

298 6. Afonina IS, Zhong Z, Karin M, Beyaert R. Limiting inflammation-the negative

299 regulation of NF-κB and the NLRP3 inflammasome. *Nat Immunol* 2017;18:861-9.

300 7. Strowig T, Henao-Mejia J, Elinav E, et al. Inflammasomes in health and disease.

301 *Nature* 2012;481:278-86.

302 8. Lamkanfi M, Dixit VM. Mechanisms and functions of inflammasomes. *Cell*

303 2014;157:1013-22.

304 9. Granath A, Cardell LO, Uddman R, et al. Altered Toll- and Nod-like receptor

305 expression in human middle ear mucosa from patients with chronic middle ear

306 disease. *J Infect* 2011;63:174-6.

307 10. Kurabi A, Lee J, Wong C, et al. The inflammasome adaptor ASC contributes to

308 multiple innate immune processes in the resolution of otitis media. *Innate Immun*

309 2015;21:203-14.

310 11. Kariya S, Okano M, Zhao P, et al. Activation of NLRP3 inflammasome in human

311 middle ear cholesteatoma and chronic otitis media. *Acta Otolaryngol*

312 2016;136:136-40.

313 12. Kariya S, Okano M, Zhao P, et al. NLRP3 inflammasome expression in

314 lipopolysaccharide-induced otitis media. *Acta Otolaryngol* 2018;138:1061-5.

315 13. Trivedi-Parmar V, Jorgensen WL. Advances and insights for small molecule

316 inhibition of macrophage migration inhibitory factor. *J Med Chem* 2018

27;61:8104-19.

14. Ishihara H, Kariya S, Okano M, Zhao P, Maeda Y, Nishizaki K. Expression of macrophage migration inhibitory factor and CD74 in the inner ear and middle ear in lipopolysaccharide-induced otitis media. *Acta Otolaryngol* 2016;136:1011-6.

15. Kariya S, Okano M, Fukushima K, et al. Expression of inflammatory mediators in the otitis media induced by Helicobacter pylori antigen in mice. *Clin Exp Immunol* 2008;154:134-40. Erratum in: *Clin Exp Immunol* 2008;154:432.

16. Kariya S, Schachern PA, Cureoglu S, et al. Up-regulation of macrophage migration inhibitory factor induced by endotoxin in experimental otitis media with effusion in mice. *Acta Otolaryngol* 2008;128:750-5.

17. Kariya S, Okano M, Aoji K, et al. Role of macrophage migration inhibitory factor in otitis media with effusion in adults. *Clin Diagn Lab Immunol* 2003;10:417-22.

18. Kariya S, Okano M, Maeda Y, et al. Macrophage migration inhibitory factor deficiency causes prolonged hearing loss after acoustic overstimulation. *Otol Neurotol* 2015;36:1103-8.

19. Kariya S, Okano M, Maeda Y, et al. Role of macrophage migration inhibitory factor

in age-related hearing loss. *Neuroscience* 2014;279:132-8.

20. Zhang J, Xu M, Zheng Q, Zhang Y, Ma W, Zhang Z. Blocking macrophage migration inhibitory factor activity alleviates mouse acute otitis media in vivo. *Immunol Lett* 2014;162:101-8.

21. Shin MS, Kang Y, Wahl ER, et al. Macrophage migration inhibitory factor regulates U1 small nuclear RNP immune complex-mediated activation of the NLRP3 inflammasome. *Arthritis Rheumatol* 2019;71:109-20.

22. Lang T, Lee JPW, Elgass K, et al. Macrophage migration inhibitory factor is required for NLRP3 inflammasome activation. *Nat Commun* 2018;9:2223.

23. Honma N, Koseki H, Akasaka T, et al. Deficiency of the macrophage migration inhibitory factor gene has no significant effect on endotoxaemia. *Immunology* 2000;100:84-90.

24. Szczepański M, Szyfter W, Jenek R, Wróbel M, Lisewska IM, Zeromski J. Toll- like receptors 2, 3 and 4 (TLR-2, TLR-3 and TLR-4) are expressed in the microenvironment of human acquired cholesteatoma. *Eur Arch Otorhinolaryngol* 2006;263:603–7.

25. Zhang J, Chen S, Hou Z, Cai J, Dong M, Shi X. Lipopolysaccharide-induced middle ear inflammation disrupts the cochlear intra-strial fluid-blood barrier through down-regulation of tight junction proteins. *PLoS One* 2015;10:e0122572.

26. Man SM, Kanneganti TD. Converging roles of caspases in inflammasome activation, cell death and innate immunity. *Nat Rev Immunol* 2016;16:7-21.

27. Man SM, Karki R, Kanneganti TD. Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. *Immunol Rev* 2017;277:61-75.

28. Jesic S, Jotic A, Tomanovic N, et al. Expression of toll-like receptors 2, 4 and nuclear factor kappa B in mucosal lesions of human otitis: pattern and relationship in a clinical immunohistochemical study. *Ann Otol Rhinol Laryngol* 2014;123:434-41.

29. Lee HY, Park MS, Byun JY, et al. Expression of pattern recognition receptors in cholesteatoma. *Eur Arch Otorhinolaryngol* 2014;271:245-53.

30. Jankauskas SS, Wong DWL, Bucala R, Djudjaj S, Boor P. Evolving complexity of MIF signaling. *Cell Signal* 2019 Jan 22. pii: S0898-6568(19)30015-4.

365 31. Sparkes A, De Baetselier P, Brys L, et al. Novel half-life extended anti-MIF
366 nanobodies protect against endotoxic shock. *FASEB J* 2018;32:3411-22.
367 32. Das R, Koo MS, Kim BH, et al. Macrophage migration inhibitory factor (MIF) is a
368 critical mediator of the innate immune response to Mycobacterium tuberculosis.
369 *Proc Natl Acad Sci USA* 2013;110:E2997-3006.
370
371
372
373

374 Figure Captions

375

376 Figure 1

377 Immunohistochemical staining for (A) macrophage migration inhibitory factor, (B)

378 nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3), (C)

379 apoptosis-associated speck-like protein containing a caspase recruitment domain and a

380 pyrin domain (ASC), and (D) caspase-1 in lipopolysaccharide-injected wild-type mice.

381 Strong positive staining (brown color) was observed in inflammatory cells (black arrow).

382 (E) Immunohistochemical staining using Rabbit Immunoglobulin Fraction in

383 phosphate-buffered saline (PBS)-treated control mice. (*, middle ear cavity; Scale bar,

384 100 μm)

385

386 Figure 2

387 Histological findings of the middle ear cavity in (A) wild-type mice and (B) $MIF^{-/-}$ mice

388 with transtympanic injection of lipopolysaccharide. Numerous inflammatory cells

389 (polymorphonuclear leukocyte and monocyte) infiltrated into the middle ear cavity in

390 the lipopolysaccharide-injected wild-type mice. In contrast, a small number of

391 inflammatory cells were found in the lipopolysaccharide-injected $MIF^{-/-}$ mice.

392 (Hematoxylin and eosin staining; scale bar, 100 μm) (black arrow, inflammatory cells; *,

393 middle ear cavity; MIF, macrophage migration inhibitory factor).

394

395 Figure 3

396 Concentrations of (A) interleukin-1β (IL-1β), (B) nucleotide-binding oligomerization

397 domain-like receptor protein 3 (NLRP3), (C) apoptosis-associated speck-like protein

398 containing a caspase recruitment domain and a pyrin domain (ASC), and (D) caspase-1

399 in lipopolysaccharide (LPS)-injected and phosphate-buffered saline (PBS)-treated mice.

400 (n = 6 (12 ears); median ± standard error; *, $P < 0.05$) (MIF, macrophage migration

401 inhibitory factor; n.s., not significant).

402

403 Figure 4

404 Potential molecular mechanism of macrophage migration inhibitory factor and NLRP3

405 inflammasome on the production of IL-1β. Signal 1 (Toll like receptor/NF-κB pathway)

406 is needed to induce pro-IL-1β. Signal 2 with macrophage migration inhibitory factor,

407 vimentin, and NLRP3 inflammasome has a critical role in the production of caspase-1.

408 The active caspase-1 released from the NLRP3 inflammasome is responsible for the

409 conversion of inactive IL-1β precursor into its biological active form.

410 LPS: lipopolysaccaride

411 TLR4: Toll-like receptor 4

412 NF-κB: nuclear factor-kappa B

413 NLRP3: the nucleotide-binding oligomerization domain (NOD)-like receptor protein 3

414 ATP: adenosine triphosphate

415 PAMPs: pattern-associated molecular patterns

416 DAMPs: danger-associated molecular patterns

417 ROS: reactive oxygen species

418 MIF: macrophage migration inhibitory factor

419 ASC: adaptor apoptosis-associated speck-like protein containing a caspase activation

420 and recruitment domain (CARD) and a pyrin domain (PYD)

421

Figure 1

Click here to access/download;Figure (TIFF, PDF, Word Doc, PPT, or EPS files are acceptable);Fig 1.tif

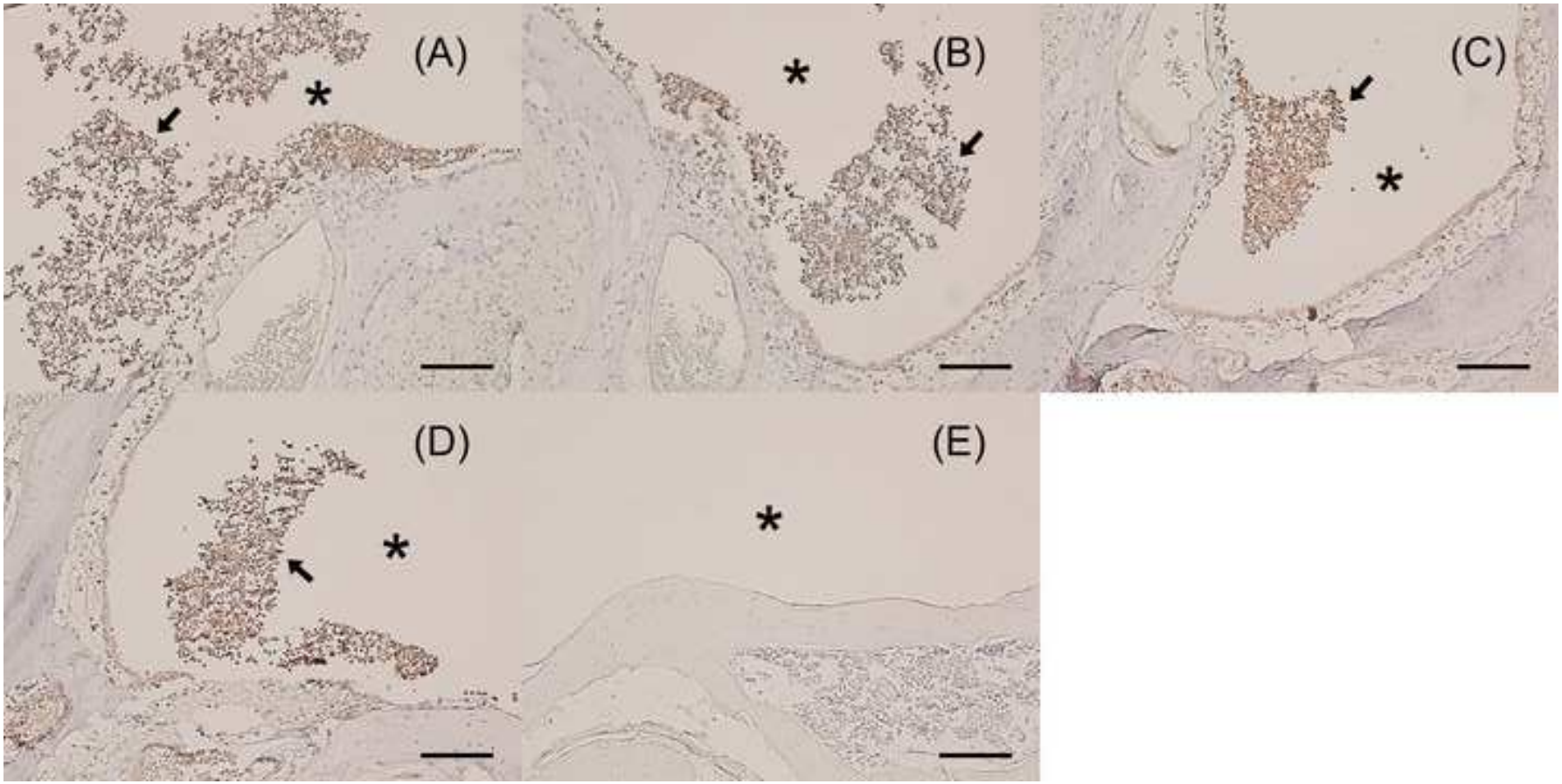

Figure 2

Click here to access/download;Figure (TIFF, PDF, Word Doc, PPT, or EPS files are acceptable);Fig 2.tif

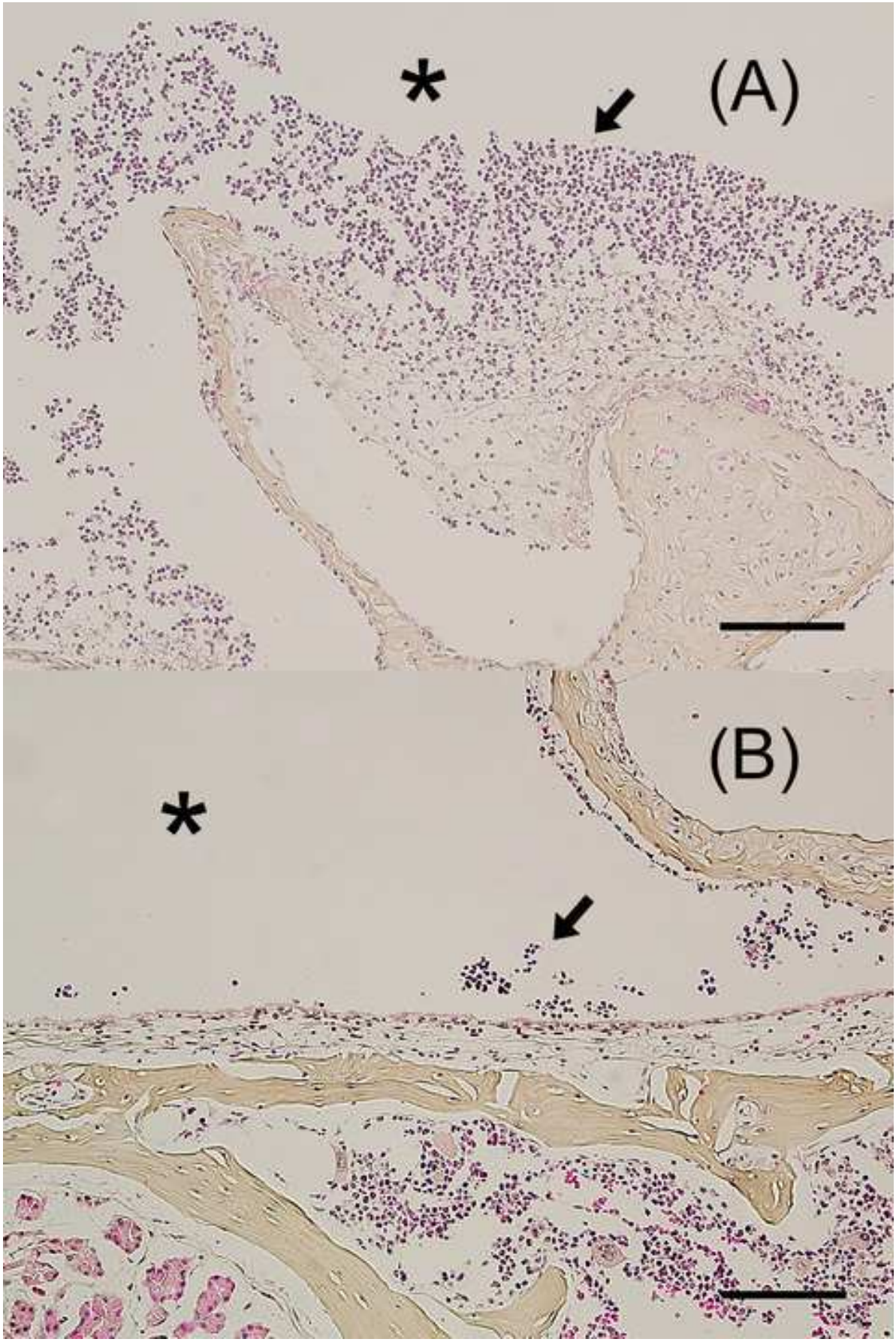

Figure 3

Click here to access/download;Figure (TIFF, PDF, Word Doc, PPT, or EPS files are acceptable);Fig 3.tif

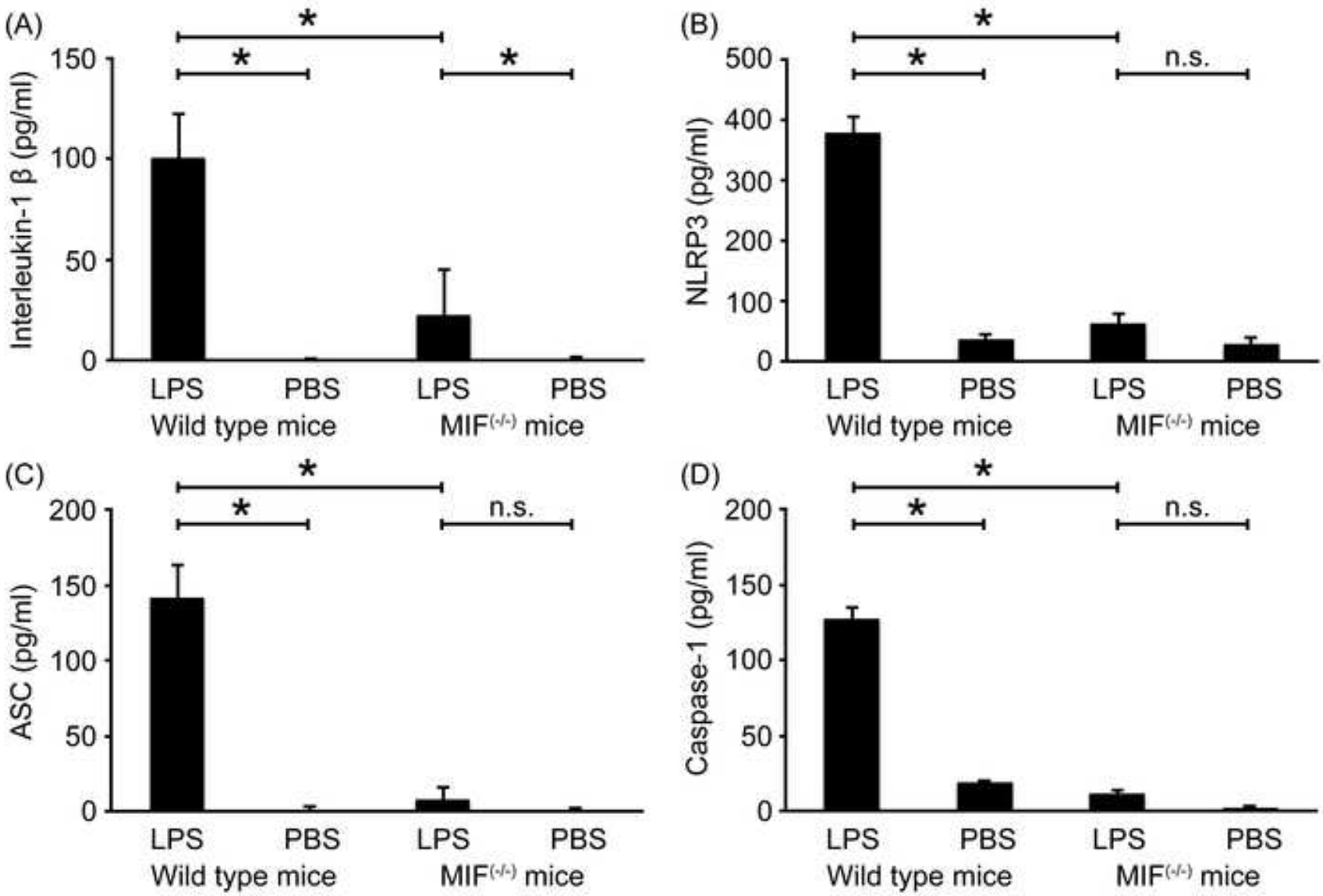

Figure 4

Click here to access/download;Figure (TIFF, PDF, Word Doc, PPT, or EPS files are acceptable);Fig 4.tif

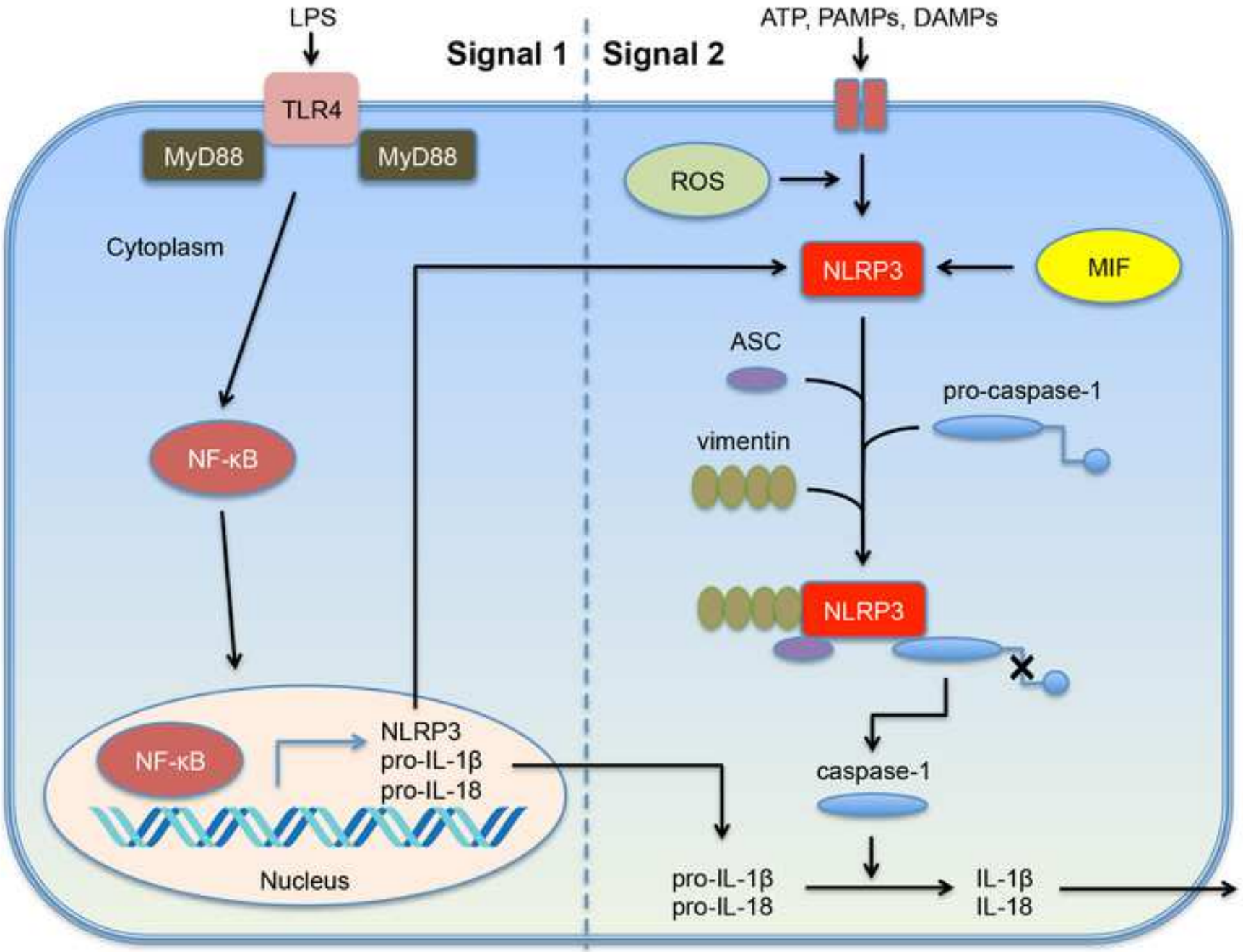

Table 1: The rating scores of immunostaining for each protein in lipopolysaccharide (LPS)- or phosphate buffered saline (PBS)-injected wild-type mice.

score	MIF		NLRP3		ASC		Caspase-1	
	LPS (n=6)	PBS (n=6)	LPS (n=6)	PBS (n=6)	LPS (n=6)	PBS (n=6)	LPS (n=6)	PBS (n=6)
-	0	0	0	0	0	1	0	0
+	0	6	0	5	0	5	0	6
++	1	0	1	1	1	0	2	0
+++	5	0	5	0	5	0	4	0

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)