Title

Expression of macrophage migration inhibitory factor and CD74 in the inner ear and middle ear in lipopolysaccharide-induced otitis media

Running Head

MIF and CD74 in otitis media

Authors

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Abstract:

Conclusion: Significant expression of macrophage migration inhibitory factor and its receptor (CD74) was observed in both the middle ear and inner ear in experimental otitis media in mice. Modulation of macrophage migration inhibitory factor and its signaling pathway might be useful in the management of inner ear inflammation due to otitis media.

Objectives: The inner ear dysfunction secondary to otitis media has been reported. However, the specific mechanisms involved are not clearly understood. The aim of this study is to investigate the expression of macrophage migration inhibitory factor and CD74 in the middle ear and inner ear in lipopolysaccharide-induced otitis media.

Method: BALB/c mice received a transtympanic injection of either lipopolysaccharide or phosphate-buffered saline (PBS). The mice were sacrificed 24 h after injection, and temporal bones were processed for polymerase chain reaction (PCR) analysis, histologic examination, and immunohistochemistry.

Results: PCR examination revealed that the lipopolysaccharide-injected mice showed a

significant up-regulation of macrophage migration inhibitory factor in both middle ear and inner ear as compared with the PBS-injected control mice. Immunohistochemical study showed positive reactions for macrophage migration inhibitory factor and CD74 in infiltrating inflammatory cells, middle ear mucosa, and inner ear in the lipopolysaccharide-injected mice.

Keywords

hearing loss; tinnitus; cochlea; hair cell; spiral ganglion cells; stria vascularis; spiral ligament; cytokine; inflammation; labyrinthitis

Introduction

Otitis media is a common childhood disease and is the major cause of hearing loss in children. Multiple factors including infection, Eustachian tube dysfunction, and inflammatory cytokines are involved in the pathogenesis of otitis media [1]. Inflammatory mediators in the middle ear cavity can spread from the middle ear into the inner ear through the round window during the course of otitis media [2, 3]. This condition can lead to cochlear pathology including the loss of hair cells in the cochlea, resulting in sensorineural hearing loss [4-6].

Macrophage migration inhibitory factor is an inflammatory cytokine, and is also an essential factor for neural development [7]. The expression of macrophage migration inhibitory factor in middle ear effusion in patients with otitis media and the effect of macrophage migration inhibitory factor in cochlear function have been reported [8-10]. However, the role of macrophage migration inhibitory factor in the inner ear is still under debate.

Up-regulation of macrophage migration inhibitory factor in the middle ear in

experimental otitis media has been reported [11]. In addition, the blocking macrophage migration inhibitory factor activity relieves middle ear inflammation in lipopolysaccharide-induced otitis media [12]. However, to the best of our knowledge, no previous study reported the finding of macrophage migration inhibitory factor and its receptor (CD74) in the inner ear during the course of otitis media. The purpose of this study is to determine the expressions of macrophage migration inhibitory factor and CD74 in the middle ear and inner ear in experimental otitis media in mice.

Material and Methods

Animals

BALB/c mice were used in this study. The mice were deeply anesthetized with an intraperitoneal injection of a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). An otoscopic examination was performed on all mice prior to treatment in order to ensure that the tympanic membranes were normal and that no middle ear effusion was present.

The mice were randomly divided into two groups. The experimental group (n=10) received lipopolysaccharide (1.0 mg/mL; Sigma-Aldrich, St Louis, Missouri, USA) via transtympanic injection using a 30-gauge needle. Phosphate-buffered saline (PBS) at 0.01 M was injected into the middle ear of the animals in the control group (n=10). The mice were sacrificed 24 hours after injection of the lipopolysaccharide or PBS. The temporal bones were removed immediately after sacrifice and processed for polymerase chain reaction (PCR) analysis, histologic examination, and immunohistochemistry.

This study conformed to the current laws of Japan and was performed in accordance with the relevant animal protection rules. The Animal Research Control Committee of Okayama University approved the study (Approval number, OKU-2013121).

Polymerase chain reaction

Inner ear (cochlea and vestibular end organ) was dissected from the tympanic

bulla. Tympanic bulla harvested for analysis included middle ear mucosa, bulla wall, and ossicles. Inner and middle ear RNA collected separately from the BALB/c mice (experimental group, n=6, 12 ears; control group, n=6, 12 ears) were subjected to quantitative PCR. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, California, USA) according to the manufacturer's protocol. PCR was performed with a TaqMan® gene expression assay, using a specific pre-made TaqMan® probe for macrophage migration inhibitory factor gene (Mm01611157_gH; CATCAGCCCGGACCGGGTCTACATC; Applied Biosystems, Foster City, California, USA), TaqMan® One-step RT-PCR Master Mix Reagents (Applied Biosystems), and Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mm99999915_g1, GAACGGATTTGGCCGTATTGGGCGC, Applied Biosystems) was used as an endogenous control in all analyses.

Histologic examination

The temporal bone specimens (experimental group, n=2, 4 ears; control group,

n=2, 4 ears) were placed in 4% paraformaldehyde for 72 hours and decalcified in 10% ethylenediaminetetraacetic acid for 3 weeks at 4 ℃. 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 tissues (experimental group, n=2, 4 ears; control group, n=2, 4 ears) 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. Non-specific protein binding was blocked with goat serum albumin (Vector Laboratories, Inc., Burlingame, California, USA) for 1 hour at room temperature. Immunohistochemical staining was performed using rabbit anti-macrophage migration inhibitory factor antibody (sc-20121; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) and rabbit anti-CD74

antibody (NBP1-33109; Novus Biologicals, Littleton, Colorado, USA) overnight at 4 ℃. Rabbit Immunoglobulin Fraction (X0903, Dako, Glostrup, Denmark) was used as a negative control. For visualization, a VECTASTAIN Elite ABC Kit (Vector Laboratories, Inc.) and 3, 3'-diaminobenzidine (DAB) reagent (K3467, Dako) were used according to the manufacturer's instructions.

Statistical analysis

Data are presented as means ± standard deviation. For statistical analysis, the non-parametric Mann-Whitney U test was used for comparison between the two groups. Significant differences were established at a level of $P < 0.05$ (IBM SPSS Statistics; IBM, New York, USA).

Results

Histologic examination

The lipopolysaccharide-injected mice showed a remarkable infiltration of

inflammatory cells (polymorphonuclear leukocyte and monocyte) in the middle ear cavity (Figure 1a). Labyrinthitis with inflammatory cell infiltration was observed in the cochlea of the lipopolysaccharide-injected mice (Figure 1b). No significant inflammatory finding was detected in the middle ear and inner ear of the PBS-injected mice.

Quantitative PCR

The expressions of macrophage migration inhibitory factor gene relative to GAPDH in the middle ear and inner ear tissues are shown in Figure 2. The lipopolysaccharide-injected mice showed a significant increase in the gene expression of macrophage migration inhibitory factor in both the middle ear ($P < 0.05$) and inner ear ($P < 0.05$) as compared with the PBS-injected control mice.

Expression of macrophage migration inhibitory factor

The PCR findings suggest the possibility of a role played by macrophage migration inhibitory factor in the middle ear and inner ear in

lipopolysaccharide-induced otitis media. Next, we examined the localization of the macrophage migration inhibitory factor. 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 mice (Figure 3a). Positive immunostaining for macrophage migration inhibitory factor was observed in the spiral ligament, stria vascularis, spiral limbus, spiral ganglion cells, organ of Corti, and infiltrating inflammatory cells in the cochlea of the lipopolysaccharide-treated mice (Figure 3b, 3c). Macrophage migration inhibitory factor was not detected in the middle ear mucosa of the PBS-treated mice (Figure 3d). Positive immunostaining for macrophage migration inhibitory factor was observed in the spiral ligament, stria vascularis, spiral limbus, spiral ganglion cells, and organ of Corti in the PBS-treated mice (Figure 3e). Macrophage migration inhibitory factor was strongly expressed in the saccular macula, utricular macula, crista ampullaris, and cells lining the membranous labyrinth, but not in the facial nerve in the PBS-treated mice. There was no significant immunostaining in the middle ear and inner ear in the negative controls using Rabbit Immunoglobulin Fraction in the PBS-treated mice (Figure 3f, 3g).

Expression of CD74

Up-regulation of macrophage migration inhibitory factor was observed in lipopolysaccharide-induced otitis media in both the mRNA level and protein level. Therefore, we next examined the presence of the receptor for macrophage migration inhibitory factor (CD74) in lipopolysaccharide-induced otitis media. CD74 was expressed in the infiltrating inflammatory cells in the middle ear cavity and middle ear mucosa (Figure 4a). CD74 was also detected in fibrocytes of the spiral ligament, stria vascularis, spiral limbus, spiral ganglion cells, and organ of Corti in lipopolysaccharide-induced otitis media (Figure 4b, 4c). Unlike macrophage migration inhibitory factor, CD74 was detected in middle ear mucosa in PBS-treated mice (Figure 4d). CD74-positive cells were also detected in inner ear (spiral ligament, stria vascularis, spiral limbus, spiral ganglion cells, and organ of Corti) in PBS-treated mice (Figure 4e).

Discussion

Gram-negative bacteria are the major pathogens in otitis media. Lipopolysaccharide, also known as endotoxin, is the structural component of the outer membrane of gram-negative bacteria, and is a critical pathogenic mediator of inflammatory diseases. A previous study reported that lipopolysaccharide was detected in the middle ear in 96% of patients with otitis media [8]. The injection of bacterial lipopolysaccharide into the middle ear can cause labyrinthitis, and induces cochlear damage [13]. We showed here for the first time that macrophage migration inhibitory factor and CD74 were significantly expressed in inner ear in lipopolysaccharide-induced otitis media.

The significant role of macrophage migration inhibitory factor in acute infections and chronic inflammatory diseases has been reported. For example, the role of macrophage migration inhibitory factor in sepsis has been extensively examined. The plasma level of macrophage migration inhibitory factor in patients with sepsis correlated positively with the severity of sepsis, and was significantly higher in septic patients who died than in those who survived [14]. In the middle ear, down-regulation of macrophage migration inhibitory factor activity reduced middle ear inflammation in

experimental otitis media [12]. Macrophage migration inhibitory factor is also an important factor in maintaining normal cochlear function [7]. We showed that macrophage migration inhibitory factor was expressed in inner ear in the PBS-treated control mice. Macrophage migration inhibitory factor-positive inflammatory cell infiltration might be related to the significant expression of macrophage migration inhibitory factor shown by PCR in inner ear in lipopolysaccharide-treated mice.

CD74, also known as a MHC class II invariant chain, is a type II transmembrane protein and is a major component of the macrophage migration inhibitory factor receptor complex. Macrophage migration inhibitory factor binds to cell surface CD74, and induces p44/p42 MAPK phosphorylation and cell proliferation [15]. Macrophage migration inhibitory factor induces neutrophilic inflammation in rodent lung, and administration of anti-CD74 antibody inhibits the infiltration of neutrophils and the production of inflammatory cytokine and chemokine [16]. On the other hand, CD74 is required for the maintenance of normal alveolar structure in mice [17]. We showed here that CD74 was expressed in both the middle ear and the inner ear in lipopolysaccharide-induced otitis media.

In conclusion, middle ear injection of lipopolysaccharide induced significant expression of macrophage migration inhibitory factor, and the receptor (CD74) for macrophage migration inhibitory factor was observed in the middle ear and inner ear of the lipopolysaccharide-injected mice. Macrophage migration inhibitory factor and CD74 might play some roles in both the middle ear and inner ear in lipopolysaccharide-induced otitis media in mice. Our findings suggest that macrophage migration inhibitory factor and its signaling pathway serve as a therapeutic target in the management of inner ear damage induced by otitis media.

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Figure captions

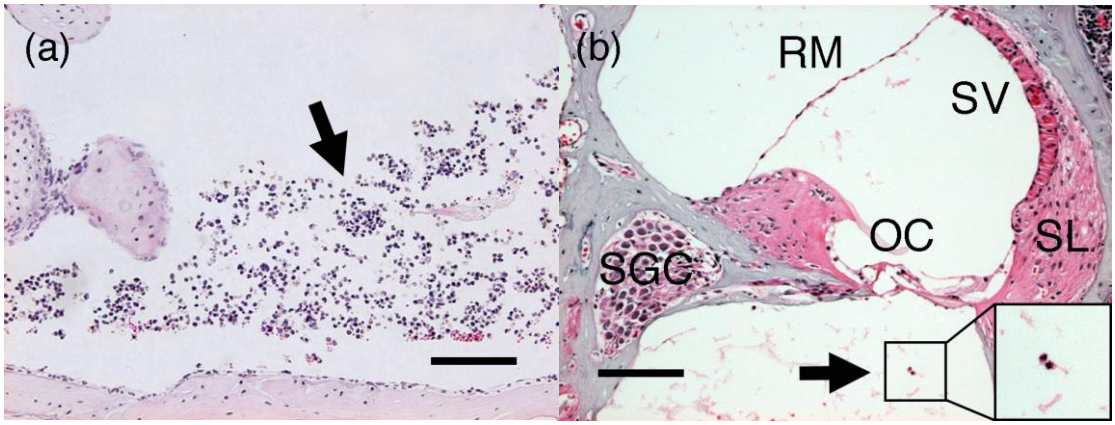


Figure 1: Histopathological findings of the (a) middle ear and (b) inner ear in lipopolysaccharide (LPS)-injected mice. Infiltration of inflammatory cells (arrow) was observed. (hematoxylin and eosin staining; OC, organ of Corti; RM, Reissner's membrane; SV, stria vascularis; SL, spiral ligament; SGC, spiral ganglion cell; Scale bar, 100 μm).

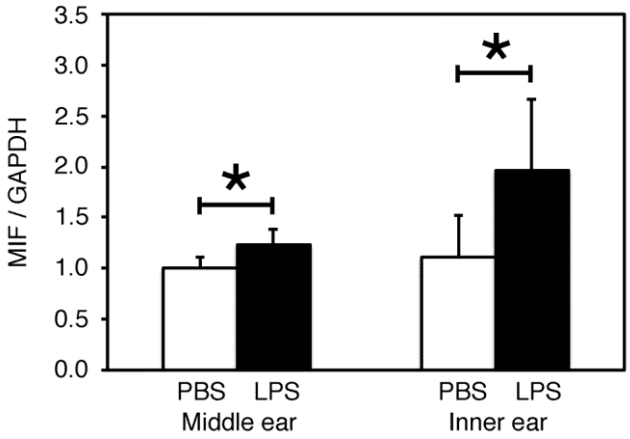
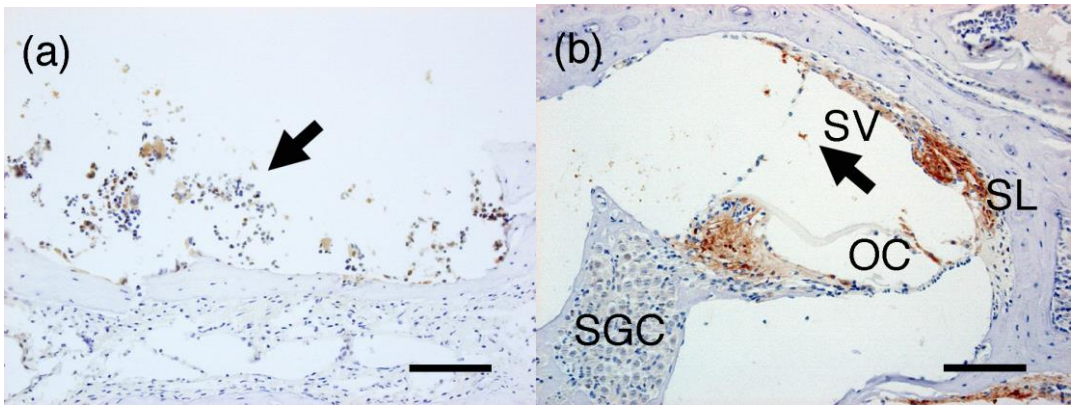


Figure 2: Mean expression of macrophage migration inhibitory factor mRNA by

real-time reverse transcription polymerase chain reaction (RT-PCR) assays. Quantitative analysis of the real-time RT-PCR results revealed that macrophage migration inhibitory factor mRNA expression was significantly up-regulated in the lipopolysaccharide (LPS)-injected mice both in the middle ear and inner ear as compared with normal control ear tissues from mice with middle ear injection of phosphate-buffered saline (PBS). (LPS, lipopolysaccharide; PBS, phosphate-buffered saline; MIF, macrophage migration inhibitory factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *, $P<0.05$).



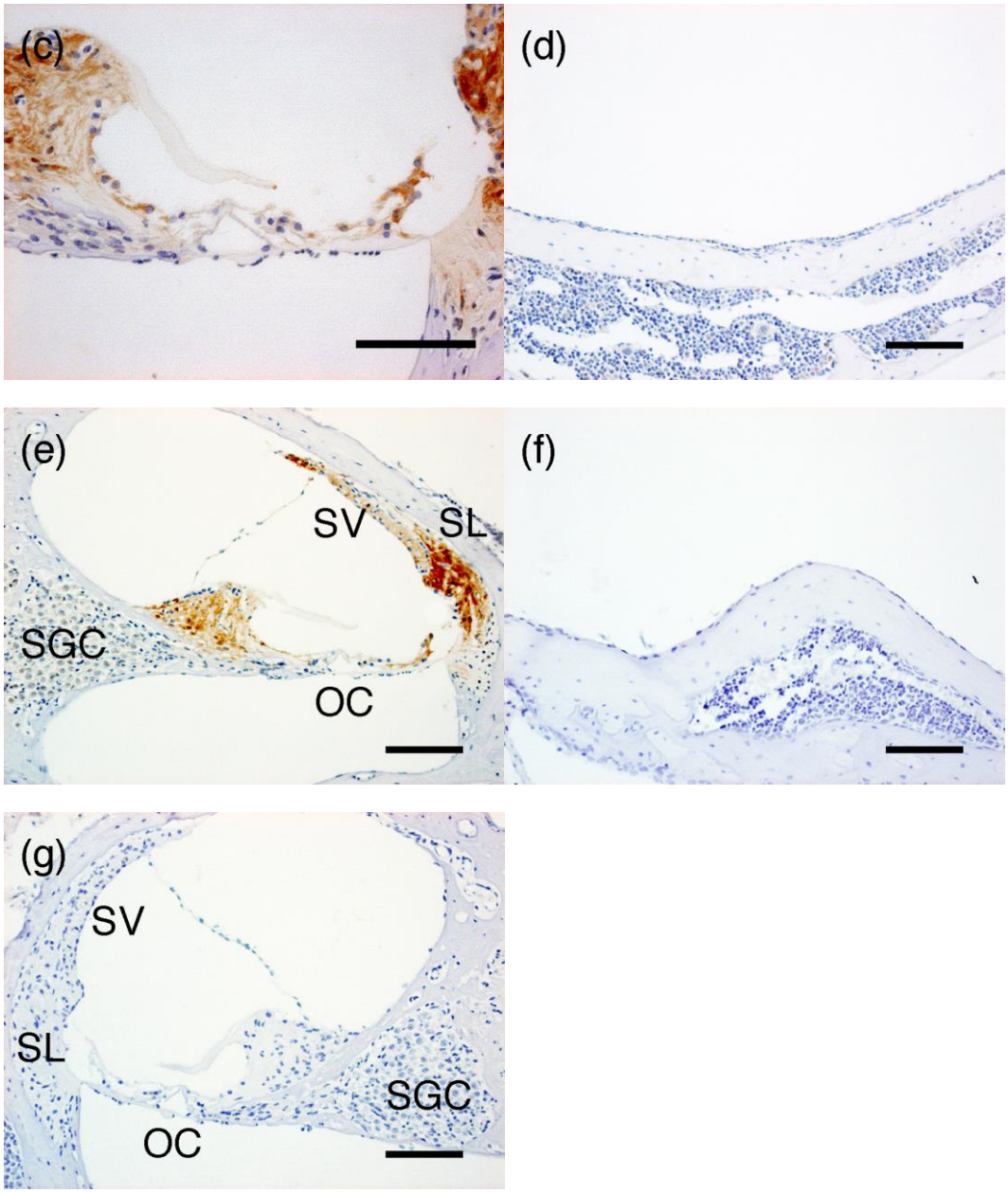
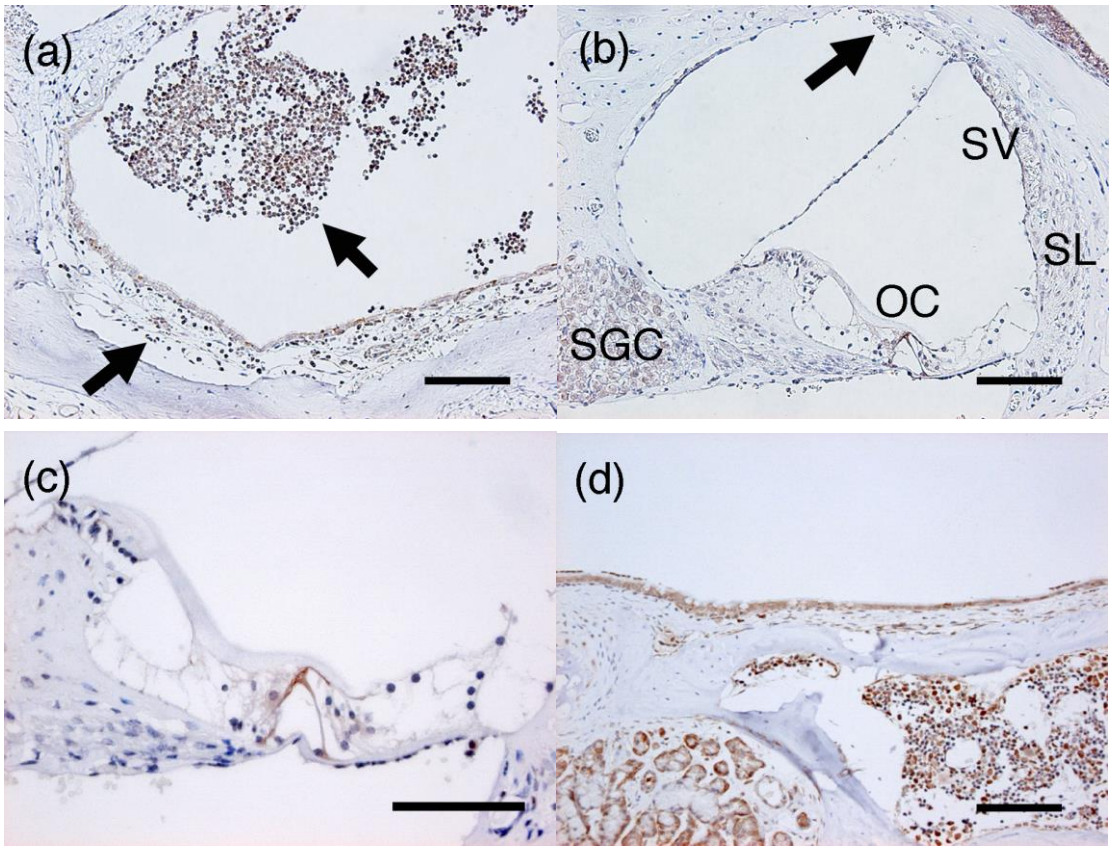


Figure 3: Immunohistochemical staining for macrophage migration inhibitory factor expression in (a) middle ear cavity in lipopolysaccharide (LPS)-treated mice, (b) inner ear in LPS-treated mice, (c) organ of Corti in LPS-treated mice, (d) middle ear cavity in phosphate-buffered saline (PBS)-treated mice, and (e) inner ear in PBS-treated mice.

Negative control immunohistochemical staining using Rabbit Immunoglobulin Fraction in (f) middle ear cavity and (g) inner ear of PBS-treated mice. Positive immunostaining for macrophage migration inhibitory factor was observed in infiltrating inflammatory cells (arrow) of LPS-treated mice (a, b). Macrophage migration inhibitory factor-positive staining was also observed in the inner ear of PBS-treated mice (e). (OC, organ of Corti; SV, stria vascularis; SL, spiral ligament; SGC, spiral ganglion cell; Scale bar, 100 μm)



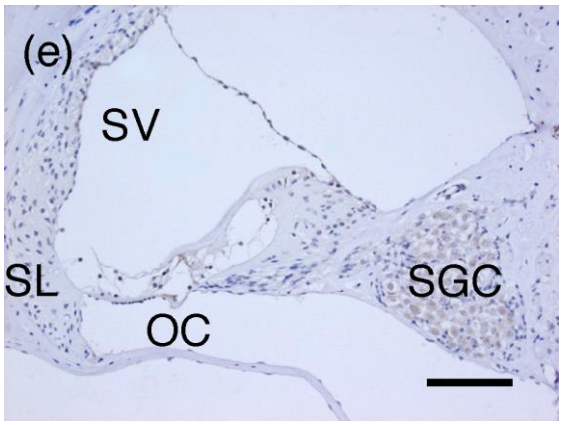


Figure 4: Immunohistochemical staining for CD74 expression in (a) middle ear cavity in lipopolysaccharide (LPS)-treated mice, (b) inner ear in LPS-treated mice, (c) organ of Corti in LPS-treated mice, (d) middle ear mucosa in phosphate-buffered saline (PBS)-treated mice, and (e) inner ear in PBS-treated mice. Positive immunostaining for CD74 was observed in infiltrating inflammatory cells (arrow). CD74-positive staining was also observed in the inner ear of LPS-treated mice (b, c). (OC, organ of Corti; SV, stria vascularis; SL, spiral ligament; SGC, spiral ganglion cell; Scale bar, 100 μm)