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Blocking macrophage migration inhibitory factor activity alleviates mouse acute otitis media *in vivo*

Jin Zhang^a, Min Xu^{a,*}, Qing Yin Zheng^b, Yan Zhang^a, Weijun Ma^a, and Zhaoqiang Zhang^b

^aDepartment of Otorhinolaryngology-HNS, The Second Affiliated Hospital, Xi'an Jiaotong University, School of Medicine, Xi'an 710049, China

^bDepartment of Otolaryngology-HNS, Case Western Reserve University, Cleveland, OH 44106, USA

Abstract

This study was to investigate the role of macrophage migration inhibitory factor (MIF) in mouse acute otitis media (AOM), we hypothesize that blocking MIF activity will relieve mouse AOM. A mouse AOM model was constructed by injecting lipopolysaccharide (LPS) into the middle ear of C57BL/6 mice through the tympanic membrane (TM). MIF levels were measured by real-time PCR (RT-PCR) and ELISA after LPS application. Normal or AOM mice were given PBS or ISO-1 (MIF antagonist) every day for 10 days and the hearing levels were determined by measuring auditory brainstem response (ABR) threshold. After the ABR test finished, H&E staining was conducted and the inflammation was also measured by detecting interleukin (IL)-1 β , tumor necrosis factor (TNF)- α and vascular endothelial growth factor (VEGF) levels with RT-PCR and ELISA. TLR-4 expression was determined by western blotting and NF- κ B activation was determined by electrophoretic mobility shift assays. Compared with the normal control, MIF levels in the middle ear of LPS-induced AOM mice were significant increased. The ABR results showed that mean ABR thresholds in ISO-1 treated AOM mice were significantly reduced compared with PBS treated AOM mice since day 7, indicating that ISO-1 treatment potentially improved the hearing levels of AOM mice. H&Estaining showed that ISO-1 treatment could reduce the mucosal thickness of AOM mice. In ISO-1 treated mice, TLR-4 expression and levels of IL-1 β , TNF- α and VEGF were significantly lower compared with PBS treated AOM mice. ISO-1 treatment also significantly inhibited NF- κ B activation in AOM mice compared with PBS treated AOM mice. These results suggested that blocking the activity of MIF by ISO-1 could reduce the inflammation in AOM mice in which process TLR-4 and NF- κ B were involved. The reduction in MIF activity is conducive to alleviate mouse AOM, which may serve as a potential therapeutic target for the treatment of AOM.

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*Corresponding author at: Department of Otorhinolaryngology-HNS, The Second Affiliated Hospital, Xi'an Jiaotong University, School of Medicine, No. 28, Xian Ning Western Road, Xi'an 710049, China. Tel.: +86 13991163858; fax: +86 21 8727 5892. minxu first@163.com (M. Xu).

Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contribution

Jin Zhang and Min Xu designed the study and prepared the manuscript; Jin Zhang, Qingyin Zheng and Yan Zhang performed experiments, Weijun Ma and Zhaoqiang Zhang processed the data.

Keywords

AOM; ISO-1; MIF; IL-1 β ; TNF- α ; VEGF

1. Introduction

Otitis media (OM) is one of the most common diseases caused by infection with viral, bacterial, or fungal pathogens. The pathogenesis and pathophysiology of OM are affected by multiple factors, including inflammatory mediators, Eustachian tube dysfunction, and pathogenicity of pathogens [1]. Copious studies have also indicated that a broad array of cytokines and chemokines are involved in the pathogenesis of OM. Acute otitis media (AOM), a subtype of OM, is one of the most frequently reported childhood diseases and is the primary reason for physician visits and antibiotic use in children in industrialized countries [2]. Perforation of the tympanic membrane (TM) can occur as a consequence of AOM.

Macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine, was first described in 1966. MIF was cloned and found to be a homotrimer of 12.5-kDa subunits, containing oxidoreductase and tautomerase enzymatic activity [3,4]. MIF was originally reported as a cytokine preventing random macrophage migration in tissue with inflammation; however, it has now been reported that MIF has multiple functions, both within and outside of immune system, on pituitary hormone, growth factor, and pro-inflammatory cytokines [5]. Lipopolysaccharides (LPS) from gram-negative bacteria have been shown to be powerful inducers of MIF secretion by macrophages, and autocrine and paracrine signaling of MIF induces the release of tumor necrosis factor (TNF)- α from macrophages in otitis media with effusion (OME) in adults [6]. It has been shown that TNF- α can modulate the expression of Toll-like receptors (TLR) and is also essential for TLR signaling, including nuclear factor (NF)- κ B, via both positive [7] and negative [8] feedback mechanisms. Recent data illustrate a critical role for TLR2 and TLR4 signaling via a MyD88-dependent pathway in the middle ear inflammatory responses to bacteria, and they are particularly important during the recovery process from OM [9]. Thus, MIF can indirectly trigger inflammatory responses in OM through TNF- α .

MIF is up-regulated in experimental OME in mice [10]. Research has also shown that increased concentrations of MIF in middle ear effusion are positively correlated with the levels of LPS, interleukin (IL)-1 β and TNF- α in OME of adults [6]. MIF has been considered as an important factor in many inflammatory diseases, such as rheumatoid arthritis, SLE, Wegener's granulomatosis [11], but its role in mouse AOM has not been fully understood.

(S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) is a MIF small molecule antagonist. ISO-1 significantly improves survival and reduces disease progression and/or severity in multiple murine models where MIF is implicated [12]. Therefore, to investigate the role of MIF in mouse AOM, ISO-1 was applied, and the hearing levels and inflammation of AOM mouse model were determined.

2. Materials and methods

2.1. Mice

Seventy-eight C57BL/6 mice (male, 8–10 weeks old) were used for this study. Mice were housed under specific pathogen-free conditions and kept at constant temperature (25°C) and relative humidity (55%) with a 12 h light/dark cycle in the Department of Animal Resources at Medical School of Xi'an Jiaotong University. All the mice were allowed free access to chow and water. All study procedures were approved by the Animal Research Control Committee of Medical School of Xi'an Jiaotong University.

2.2. Mouse model of AOM

The model was made according to Kariya's method [10]. Briefly, to ensure that TMs were normal, all mice were performed otoscopic examination prior to treatment. No middle ear effusion was present. Mice were anesthetized with avertin (0.5 mg/g) via intraperitoneal injection.

The AOM mouse model was created by injecting LPS (Sigma, St. Louis, MO) into the middle ear of mice through TM only in the right ear. The untreated mice served as the control. Forty-four of forty-six AOM mice were successfully induced. Two mice failed to be induced. After LPS application, fifteen AOM mice were used to measure the MIF levels at three time-points (24 h, 48 h, 72 h, for each time point, $n = 5$). Five AOM mice were used to evaluate the AOM model at 72 h ($n = 5$). The remaining twenty-four AOM mice were divided into two groups, randomly. Twelve AOM mice and six normal mice were given 20 mg/kg of ISO-1 (in 5% DMSO in PBS) (EMD Chemicals Inc., Gibbstown, NJ) via intraperitoneal injection every day for 10 days, and the other twelve AOM mice and six normal mice were given 0.2 mL PBS containing 5% DMSO (PBS, for short) via intraperitoneal injection every day for 10 days. Hearing level was examined every day after ISO-1 treatment for 10 days. After the ABR test finished, 3 mice of all groups were killed for pathological observation (H&E staining). All the remaining mice were sacrificed to determine the levels of inflammatory factors (Fig. 1).

2.3. Evaluation of the mouse AOM model

The animals were anesthetized with avertin (0.5 mg/g) on the 3rd day of LPS injection ($n = 5$) or after ABR test finished ($n = 3$). To examine the TM under otoscopy and to determine the color and position of the TM as well as the presence of fluid, the bulla was opened through TM and the effusion was drawn into a syringe.

Temporal bones were removed immediately after sacrifice and processed for histological examination. Bone specimens were fixed in 4% paraformaldehyde for 48 h and decalcified in 10% ethylene diaminetetraacetic acid (EDTA) for 10 days at 4°C. After dehydration, specimens were embedded in paraffin and sectioned at 7 μ m thickness. Sectioned specimens were then mounted on glass slides, stained with hematoxylin and eosin (H&E) and evaluated under light microscopy.

The mucosal thickness was measured for 3 times. To avoid the cochlea and the region close to the Eustachian tube, blinded assessment of a standard 1000 μm length of middle ear mucosa was used for morphometric evaluation.

2.4. Real-time polymerase chain reaction

MIF levels were measured at 24 h, 48 h and 72 h after LPS injection. AOM mice and normal mice were given PBS or ISO-1 every day for 10 days and IL-1 β , TNF- α and VEGF levels were measured. Real-time PCR was performed to detect the mRNA expression levels. RNA was extracted from mice dissected middle ear tissue using the Pure-Link™ Micro-to-Midi Total RNA Purification System according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). RT-PCR was done according to the manufacture's instruction (Invitrogen, Carlsbad, CA). The primer information is as follows:

GAPDH: Forward 5'-AAC TTT GGC ATT GTG GAA GG-3', Reverse 5'-GGA GAC AAC CTG GTC CTC AG-3'; MIF: Forward 5'-GTG CCA GAG GGG TTT CTG T-3', Reverse 5'-AGG CCA CAC AGC AGC TTA CT-3'; IL-1 β : Forward 5'-TCA TGG GAT GAT GAT GAT AAC CTG CT-3', Reverse 5'-CCC ATA CTT TAG GAA GAC ACG GAT T-3'; TNF- α : Forward 5'-CCT TGA AGA GAA CCT GGG AGT-3', Reverse 5'-CCA CCA CGC TCT TCT GTC TAC-3'; VEGF: Forward 5'-GAG GGC AGA ATC ATC ACG AAG T-3', Reverse 5'-GGC TTG AAG ATG TAC TCG ATC TCA T-3'.

2.5. Western blot analysis

AOM mice and normal mice were given PBS or ISO-1 every day for 10 days, and TLR-4 levels were determined by western blotting. Briefly, tissues were collected and samples were lysed with the RIPA lysis buffer (Beyotime, Shanghai, China) to extract the total protein. Equal amounts of total proteins were separately electrophoresed on a 12% polyacrylamide gel, and then were transferred onto a polyvinylidene difluoride membrane. After blocking with a buffer containing 5% non fat dry milk in Tris-buffered saline with Tween (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4) at 4°C overnight, the nitrocellulose membrane was incubated with the primary antibodies against TLR-4 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h, following incubation with secondary antibodies conjugated with horseradish peroxidase. The bound antibodies were visualized by the LumiGlo Reagent (Pierce, Rock-ford, IL).

2.6. Auditory brainstem response (ABR) procedure

While mice were given ISO-1 or PBS treatment every day for 10 days, hearing levels were measured on each day. The hearing levels of each mouse were determined by measuring ABR thresholds using a computer-aided evoked potential system (Intelligent Hearing Systems). Clicks were presented to the mice. Right ear of each mouse was stimulated with an ear probe sealed into the ear canal. To elicit ABR, the click sounds were generated with 1 ms rise/fall times and 2 ms of plateau. The recording electrode was placed on the cranial-parietal line of the mouse. The reference electrode was placed in the post auricular position, and the zero electrode was placed on the nasal root. ABR waveforms were recorded in 10 dB steps by decreasing stimulus intensity from the maximum level of 90 dB sound pressure level.

All wave-forms were stored for off-line analysis by a blind observer to obtain average thresholds for click sounds. Hearing thresholds are shown as mean $\pm$ standard deviation (SD).

2.7. Measurement of MIF, IL-1 β , TNF- α and VEGF by ELISA method

Concentrations of MIF, IL-1 β , TNF- α and VEGF in the middle ear effusions were measured with enzyme-linked immunosorbent assay (ELISA) using mouse Opt enzyme immunoassay sets (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's instructions. All samples were run in duplicate and averaged.

2.8. Nuclear extract preparation and electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared from tissues with ice-cold hypotonic buffer. The subsequent pellet was lysed and placed on ice for 10 min. Nuclear-associated proteins were extracted and maintained on ice for 20 min. Following centrifugation, the supernatant was mixed with storage buffer and used immediately, or frozen at -20°C . In the EMSA, nuclear extracts were incubated for 30 min with 10,000 cpm of a 22-bp DNA fragment oligonucleotide (5'-GGG ACT TTC CGC TGG GGA CTTT-3') containing the NF- κ B consensus sequence previously been labeled with [γ - ^{32}P] ATP, in the presence of poly(dI-dC) and 10 $\times$ binding buffer. Incubated mixtures were subjected to electrophoresis on native 5% (w/v) polyacrylamide gels, which were subsequently dried and autoradiographed. Competition studies were performed by addition of 100-fold excess unlabeled oligonucleotide to the binding reaction.

2.9. Statistical analysis

Data are given as mean $\pm$ standard deviation (SD). Statistical analyses were performed using Student's *t* test or two-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Evaluation of mouse AOM model after LPS application

To confirm successful construction of the mouse AOM model, the TM, mucosa and effusion in middle ear were observed. From otoscopic examination, the AOM ears showed signs of inflammation. Compared with the normal control, TMs in AOM group were thick and opaque in appearance. By visual assessment, effusion was observed in all AOM mice ears, but the normal control ears had a clear middle ear cavity without signs of inflammation. The white malleus was clearly visible in both groups (Fig. 2A and B).

The photomicrographs in Fig. 2C and D show the middle ear mucosa of the middle ear cavity roof in the normal control and AOM mice ears on the 3rd day. The mucosa in the roof of the AOM mice ear was thicker and edematous compared with the normal control, and significant differences were observed (Fig. 2E, $P < 0.05$).

3.2. MIF level was increased in LPS-induced mouse AOM model

MIF was originally described as a factor that inhibits the random migration of macrophages and its up-regulation has now been linked to a number of inflammatory diseases [5]. We

wondered whether MIF expression was up-regulated in the mouse AOM model. Results were analyzed with 2-way ANOVA, and shown in Fig. 3. mRNA accumulation levels of MIF at 24 h, 48 h and 72 h were significantly higher in mouse AOM group than in normal control (Fig. 3A, $P < 0.05$). ELISA analysis showed that MIF protein expression was significantly higher in the mouse AOM group than in normal control ($P < 0.05$) (Fig. 3B), which was consistent with real-time PCR results.

3.3. Effect of MIF on mouse ABR threshold shifts

During the first 6 days, the average hearing levels of AOM ISO-1 treatment group ($n = 12$) and AOM PBS treatment group ($n = 12$) showed virtually no change. Hearing levels of these two groups were 59.8 ± 4 dB and 61.1 ± 3 dB on the 6th day, respectively. Student's *t* test analysis showed that there is no significant difference between the two groups ($P > 0.05$) during the first 6 days. On the 7th day of ISO-1 treated AOM mice, the ABR hearing threshold decreased to 50.4 ± 4 dB. In PBS treated AOM mice, hearing level was 61.1 ± 3 dB on the 7th day. The ABR thresholds in ISO-1 treated mice were significantly lower than those in PBS treated mice at click stimulus frequencies ($P < 0.05$) (Fig. 4). In short, hearing levels were statistically improved in ISO-1-treated mice than in PBS treated group ($P < 0.05$) on the 7th day. From the 8th to the 10th day, similar results were obtained, which indicated that hearing levels were truly improved after ISO-1 treatment in AOM mice from the 7th day.

However, there was no significant difference between PBS treated normal mice and ISO-1 treated normal mice ($P > 0.05$) ($n = 6$). In addition, from the 9th day and the 10th day, there was no significant difference between ISO-1 treated normal or AOM mice and normal mice (2-way ANOVA, $P > 0.05$) (Fig. 4), which means that ISO-1 had a potential role in improving the AOM mice hearing levels.

3.4. ISO-1 reduced the inflammatory reaction in mouse AOM model

Pathological observation showed that the mucosal thickness of ISO-1 treated AOM mice was significantly smaller than that of PBS treated AOM group (Fig. 5B and D). While in ISO-1 or PBS treated normal mice, the mucosal thickness barely changed (Fig. 5A and C). The mucosal thickness of ISO-1 treated mice was reduced to a level that had no significant difference with the normal groups ($P > 0.05$) (Fig. 5E). To further investigate whether ISO-1 can reduce the biological activity of MIF and lessen inflammatory reaction, downstream inflammatory genes of MIF (IL-1 β , TNF- α , VEGF) were evaluated. Factors in middle ear effusion fluid were measured by ELISA method. Factors in middle ear dissected tissue were measured by real-time PCR method. GAPDH-corrected real-time PCR results showed that the mRNA accumulations of IL-1 β , TNF- α and VEGF in ISO-1 treated AOM mice ears were significantly lower than in PBS AOM treated mice ears ($P < 0.05$) (Fig. 6B, D and F). However, there was no significant difference between the ISO-1 treated normal or AOM mice and normal mice ($P > 0.05$, 2-way ANOVA) (Fig. 6A, C and E). To confirm that the decrease in protein expression levels of inflammatory factors were consistent with the mRNA accumulation, the protein expression were measured by ELISA method. The result also revealed a significant reduction in protein levels in ISO-1 treated AOM mice ($P < 0.05$), which was close to the normal groups (Fig. 6A, C and E). All these results suggested that

inflammatory reaction was relieved in ISO-1 treated AOM mice compared with PBS treated AOM mice.

3.5. ISO-1 reduced TLR-4 levels and inhibited NF- κ B activation in AOM mice

Studies have shown that MIF plays vital roles in the regulation of responses to stimuli acting via TLR-4 and NF- κ B. So we asked whether TLR-4 and NF- κ B were involved in ISO-1 induced reduction of inflammatory reaction in mouse AOM model. As shown in Fig. 7A, in ISO-1 treated AOM mice, TLR-4 levels were significantly decreased compared with PBS treated AOM mice, to a level that had no significant differences with PBS treated normal mice. Fig. 7B demonstrates that ISO-1 treatment significantly inhibited NF- κ B activation in AOM mice compared with PBS treated AOM mice (Fig. 7B, line 2–5). The specificity of NF- κ B DNA-binding complex was further confirmed by competition analyses using 100-fold excess unlabeled NF- κ B oligonucleotide (Fig. 7B, line 1).

4. Discussion

Injection of LPS into the middle ear can mimic the pathological changes of AOM: mucosal inflammation, leukocytosis, edema, middle ear pressure abnormalities and an infiltrate of macrophages into the sub epithelial space [13]. In this study, an AOM model was created by injection of LPS into the middle ear of mice to investigate the role of MIF in AOM. Middle ear effusion volume and mucosal lining thickness are typical characteristics of middle ear inflammation severity [14]. Increased middle ear effusion, thickened mucosa and opaque TM confirmed the successful construction of mouse AOM.

In this study, we found that the mRNA and protein levels of MIF were up-regulated in mouse AOM model, which was also observed in the pathogenesis of OM [6,10]. ISO-1 is the most frequently studied MIF small molecule antagonist. It binds to the pro-inflammatory pocket and competitively inhibits MIF tautomerase and biologic activities [12]. Decreased ABR thresholds for clicks stimulation in AOM mice were observed after ISO-1 treatment, however, it was only observed after ISO-1 treatment for 7 days. And from the 9th day to 10th day, the hearing levels of ISO-1 treated mice were closed to the normal group. This result showed that blocking MIF activity can improve the hearing levels of AOM mice. Previous research has reported that ISO-1 that inhibits the MIF pro-inflammatory activities is protective against sepsis and septic shock. The inhibitory effect of ISO-1 on MIF could suppress LPS responses in macrophages [15]. MIF may be a new target for the therapeutic treatment of OM induced by LPS [10]. To explore whether the repression of MIF in the ISO-1 treatment group will lead to the significant suppression in the inflammatory responses, associated with AOM, IL-1 β , TNF- α , and VEGF were measured by real-time PCR and ELISA.

We found that inflammatory response of AOM mouse treated with ISO-1 was suppressed and the levels of inflammatory factors in ISO-1 treated AOM mouse had no significant difference with that of normal groups, suggesting that AOM was significantly alleviated by inhibiting MIF activity. We also found that NF- κ B and TLR-4 were involved in these process, which was consistent with previous studies [16]. MIF is an immune-regulatory cytokine and has an effect on arresting random immune cell movement [11]. TNF- α can

trigger the production of MIF, and subsequently MIF induces the release of TNF- α from macrophages through autocrine and paracrine mechanisms [17]. TNF- α is an inflammatory cytokine and it can facilitate the initiation of the host defense response [16]. These may explain that the reduced TNF- α expression and lightened inflammation by MIF activity inhibition in our study. OME is also a subtype of OM and often follows an episode of AOM, and is simply a collection of fluid that occurs within the middle ear space. In a murine model of OME, the concentration of IL-1 β was elevated [18] and resulted in fluid retention [19]. In this study, IL-1 β was decreased due to MIF activity inhibition. This result indicated that IL-1 β was a downstream gene and involved in inflammatory response in AOM. VEGF is an angiogenic growth factor and it plays a vital role in the neovascularization of the middle ear mucosa during OM in mice and also an important role in inflammatory events together with other inflammatory cytokines [20,21]. Increasing expression of VEGF protein in middle ear of OME model induced by endotoxin and hypoxia indicated that VEGF might be associated with the pathogenesis of OME [22,23]. Studies have reported that both VEGF receptor-1 and VEGF receptor-2 are up-regulated during experimental OM in the rat [24]. In our study, VEGF expression was down-regulated in AOM after ISO-1 treatment because of the decreased MIF activity. Study has reported that there was positive correlation among concentrations of MIF, IL-1 β and TNF- α in mouse OME [10]. This is similar to our result in AOM mice. In short, blocking MIF activity was helpful to alleviate the AOM inflammation in mice.

In this study, we also found an interesting result: ISO treatment improves hearing level from day 7, and does not show significant difference with normal control group on day 10, suggesting that ISO-1 only have an effect from day 7. MIF is rapidly produced following LPS treatment, and studies have shown that MIF induce inflammation in an earlier phase [10]. Increased MIF thus induced the expression of several inflammation cytokines such as IL-1 β and TNF- α . These cytokines after MIF production might be the reason of the delayed improvement of hearing level.

5. Conclusion

Our results indicated that MIF levels were markedly increased in AOM mouse model. MIF blockade by ISO-1 significantly decreases the levels of inflammation cytokines and improves the hearing level. Therefore, inhibiting MIF is a promising approach to the treatment of AOM.

Abbreviations

MIF	macrophage migration inhibitory factor
AOM	acute otitis media
OME	otitis media with effusion
LPS	lipopolysaccharide
TLR	Toll-like receptors

TM	tympanic membrane
ABR	auditory brainstem response
IL	interleukin
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
ISO-1	(S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester

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Fig. 1.
The flow chart of experiment design.

Fig. 2. Photographs of the tympanic membrane and H&E staining mucosa in mice middle ears on the 3rd day after LPS application. Photographs of control group (A and C) and AOM group (B and D) and the mucosa thickness (E). (TM = tympanic membrane, MEC = middle ear cavity, M = malleus. scale bar = 500 μm in (A and B), scale bar = 50 μm in (C and D). * P < 0.05, significant differences were observed between control group and LPS treated group) (n = 5).

Fig. 3.

Expression of MIF in AOM and control mice ears at three time points after LPS application. (A) mRNA accumulation levels of MIF were measured by real-time PCR. (B) ELISA method analyzed the MIF protein expression. The error bars represent standard deviation in the figures (* $P < 0.05$ vs. Control) ($n = 5$).

Fig. 4.

Effect of ISO-1 on mice ABR threshold shifts. Hearing levels of AOM mice or normal mice giving ISO-1 or PBS treatment were measured every day for 10 days. The error bars represent standard deviation in the Figures (PBS: PBS containing 5% DMSO; * $P < 0.05$ vs. PBS control).

Fig. 5. Photographs of H&E staining mucosa in mice middle ear of AOM group and normal group after ISO-1 treatment for 10 days. Photographs of normal group (A and C) and AOM group (B and D) and the mucosa thickness (E). scale bar = 50 μm. * $P < 0.05$, significant differences were observed between PBS-treated AOM group and ISO-1-treated AOM group (PBS: PBS containing 5% DMSO).

Fig. 6. Detection of inflammatory cytokine levels in different groups. ISO-1 treated mice show decreased levels of inflammatory cytokine and the levels were closed to the normal groups. (A), (C) and (E) ELISA method analysis for the protein levels of inflammatory cytokine. (B), (D) and (F) Inflammatory cytokine mRNA levels were measured by real-time PCR after ABR test. The error bars represent standard deviation in the figures. * $P < 0.05$, significant differences were observed between PBS-treated AOM group and ISO-1-treated AOM group (PBS: PBS containing 5% DMSO).

Fig. 7.

TLR-4 levels and NF-κB activation in middle ear tissue dissected from mice of different groups. (A) Western blotting analysis of TLR-4 levels. (B) NF-κB activation by EMSA.

Line 1: 100-fold excess unlabeled NF-κB oligonucleotide; line 2–4: NF-κB oligonucleotide labeled with [γ - 32 P] ATP. * $P < 0.05$, significant differences were observed between PBS-treated AOM group and ISO-1-treated AOM group (PBS: PBS containing 5% DMSO).