Overexpression of immune-responsive gene 1 prevents acute otitis media due to *streptococcus pneumoniae* infection through Nrf2- Hmox1 pathway

Peizhen Yang
Linyi People's Hospital
Yufen Li
Linyi People's Hospital
Dongqing Wang
Linyi People's Hospital
Zhipeng Chen (chen_chenzhipeng@126.com)
Linyi People's Hospital https://orcid.org/0000-0002-7400-7382

Research

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Abstract

Background: Acute otitis media (AOM) is one of the most commonly diagnosed childhood diseases that are associated with influenza infection. Immunoresponsive gene 1 (Irg1) is overexpressed in mammalian macrophage during inflammation process. Here, the present study aims to investigate the role of Irg1 in streptococcus pneumoniae infection-induced inflammatory response in AOM through Nrf2 signaling pathway.

Methods: Positive rate of streptococcus pneumoniae and expression of Irg1, Nrf2 and Hmox1 were determined in the middle ear lavage fluid from 85 AOM patients infected with streptococcus pneumoniae and 48 healthy subjects. After the successful establishment of mice model with AOM, macrophage was harvest from the middle ear lavage fluid of mice. At last, to investigate the effect of Irg1 and Nrf2 signaling pathway on inflammatory response and streptococcus pneumoniae infection by accumulating macrophage, Irg1, sh-Irg1 and retinoic acid (an inhibitor of Nrf2 signaling pathway) were injected into AOM mice.

Results: Irg1 exhibited a high level and activated Nrf2 signaling pathway was detected in AOM. Besides, positive rate of streptococcus pneumoniae was increased in AOM. Furthermore, in the mice model with AOM, Irg1 could repress inflammatory response by downregulating expression of TNF-α, IL-6 and IL-1β and inhibit streptococcus pneumoniae infection by stimulating accumulation of macrophage to increase its endocytosis through activation of Nrf2 signaling pathway.

Conclusion: Taken together, Irg1 contributes to inhibiting inflammatory response and streptococcus pneumoniae infection in AOM by promoting accumulation of macrophage through activation of Nrf2 signaling pathway, which provides novel therapeutic targets for AOM therapy.

Background

Otitis media (OM) is one of the most prevalent childhood diseases that remain to be a serious health burden for children with chronic or recurrent middle ear infections [1]. Acute otitis media (AOM) is universal and is one major type of OM [2]. AOM patients, especially those diagnosed in the emergency department, usually suffer ear pain, increased cry, poor sleep and hearing loss, and they are often received traditional antibiotic therapy [3, 4]. As an ordinary upper respiratory tract infection complication, AOM has a complex pathogenesis that consists of the host inflammatory response and interactions between viruses and bacteria. Streptococcus pneumoniae is identified as one of the most common pathogens of AOM [5]. As we know streptococcus pneumoniae is a major pathogen of bacterial infections in infants, children and older adults, which accounts for a high percentage of mortality and morbidity caused by pneumococcal diseases [6]. Both nasopharyngeal inflammation and streptococcus pneumoniae are associated with AOM progression [7]. Macrophages serve as critical cellular regulators of the host homeostasis and primary defenders against those pathogens, which can facilitate immune responses in response to infection and promote tissue repair following immune responses [8]. Recently,

the critical role of immunoresponsive gene 1 (Irg1) has been identified to be associated with innate immunity including antiviral effect, secretion of inflammatory factors and response to reactive oxygen species [9].

Irg1 is primarily recognized from a murine macrophage cell line stimulated by lipopolysaccharide, and one of the most overly expressed genes in proinflammatory environments [10]. Irg1 exerts its prominent effect on neurodegeneration and embryonic implantation, and its localization to the mitochondria demonstrates a vital correlation between metabolic and immunological progression [11]. Moreover, Irg1 is linked to lung injury and inflammatory cell infiltration in respiratory syncytial virus infection [12]. NF-E2-related factor 2 (Nrf2) is a transcription factor that protects against xenobiotic or oxidative stresses response, and its activation can ameliorate inflammation in myeloid cells [13]. Nrf2 exerts a regulatory effect on host defense in mice with pneumonia induced by streptococcus pneumoniae [14]. Nrf2 might be regulated by Irg1. Irg1 generates metabolite itaconate by decarboxylating cis-aconitate that is intermediate in the TCA cycle when affected by inflammatory factors [15] [16]. And itaconate is demonstrated as an activator of the anti-inflammatory transcription factor Nrf2 both in mouse and human macrophages [17]. In addition, it is found that LPS-induced AOM caused increased production of ROS as well as upregulation of some immune factors including Nrf2 [18]. Based on the above discussed, it is hypothesized that Irg1 exerts its effect on AOM via accumulation of macrophage through Nrf2 signaling pathway. Therefore, the present study was designed to elucidate the intrinsic regulatory mechanism of Irg1 in streptococcus pneumoniae infection-induced inflammatory response of AOM by means of Nrf2 signaling pathway, with the intention of presenting a theoretical foundation for an enhanced understanding of AOM treatment.

Materials And Methods

Ethics statement

Written informed consent was obtained from all patients and their guardians prior to the study. Study protocols according to the ethical principles for medical research involving human subjects of the Helsinki Declaration were approved by Ethic Committee of Linyi People's Hospital. The animal experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol of animal experiments was approved by the Institutional Animal Care and Use Committee of Linyi People's Hospital. The animal experiments were conducted based on minimized animal number and the least pains on experimental animals.

Study subjects

The AOM specimens were collected from 85 AOM patients infected with *streptococcus pneumoniae* who received treatment in Linyi People's Hospital from January 2015 to December 2016. The clinical diagnostic criteria of those patients were as follow: acute occurrence (duration $\leq$ 48 h); fever, earache, purulent discharge in ear and other infection; tinnitus, aural fullness and hearing loss; puncture of

eardrum and observed shining point detected by otoscope. All patients lived in the local area more than 3 months. Patients with symptoms for a duration over 8 weeks and immune deficiency or those who had received antibiotics therapy yet diagnosed with combined infection in other parts should be excluded. Among those patients, there were 49 male patients and 36 female patients (aged: 15 days - 10 years; average age: 4.4 ± 2.2 years). Meanwhile, the specimens of middle ear lavage fluid were collected from 40 healthy subjects as control, among which there were 21 boys and 19 girls (aged: 3 - 11 years; average age: 5.0 ± 2.0 years).

Establishment of mice model with AOM

A total of 56 specific pathogen-free (SPF) grade healthy C57BL/6 mice (half male and half female; aged: 6 - 8 weeks; weighing: 16 - 20 g) were provided by animal center. Mice were subjected to general anesthesia by intraperitoneal injection with 3% pentobarbital solution. Afterwards, bilateral external auditory canals and tissues surrounding ear were disinfected by 75% ethanol. Then a 20 μL micro pipette was used to extract 5 μL *streptococcus pneumoniae* suspension and inoculated into tympanum for binaural modeling after auripuncture by otoscope. Mice in the control group were injected with equal volume of phosphate buffer saline (PBS) or other prepared solutions and labeled properly. Subsequently, mice were housed in a cage in the prone position so as not to be suffocated by disturbance in respiration. Mice were further well fed, and their weight and temperature were recorded. Later, mice were collected at corresponding time points for following experiments.

Mice treatment with lentivirus and chemical

Eight mice were set as normal control, and rest 48 mice were used for establishing model. A total of 40 mice were successfully modeled. Next, 36 modeled mice were assigned into 6 groups (6 mice per group), and other 4 mice were reserved for later use. Lentivirus vector LV5-GFP was synthesized by GenePharma Company (Shanghai, China). Then lentivirus was packaged by 293T cells, which were incubated in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS) and passaged every other day. Subsequently, 2 weeks before the inoculation of *streptococcus pneumoniae*, mice were transduced with lentivirus vector solution (5×10^8 pfu/100 μL) or retinoic acid (RA, an inhibitor of Nrf2 signaling pathway, 40 μM) twice a week for consecutive 4 weeks [19].

Collection of middle ear lavage fluid

After mice model was successfully established, at least 3 mice were randomly collected at the 1st and 5th day. Mice underwent general anesthesia by intraperitoneal injection with 5% pentobarbital solution. Next, mice were euthanized by opening chests to collect heart blood. After the surrounding area of mouse ear was disinfected by 75% ethanol, the operating scissors sterilized under high pressure were used to cut off its auricle and expose middle ear. Subsequently, PBS containing 1% FBS was utilized to lavage middle ears of mice (10 μL per time; 6 times each ear), and then about 50 μL lavage fluid was collected and labeled. Afterwards, 5 μL lavage fluid of each ear was applied for serial dilution and bacteria counting, and rest lavage fluid was centrifuged at 4℃ and 350 × g for 5 min. The supernatant was sub-packaged,

labeled and preserved at 80℃ for detection of cytokines. Cell precipitation was lysed on ice by red blood cell lysis buffer at 4℃ and 350 × g for 5 min. The supernatant was removed, and cell precipitation was resuspended by precooling PBS containing 1% FBS for cell counting, flow cytometry and the cover glasses preparation.

Isolation and culture of macrophage

Macrophages were harvested from middle ear lavage fluid, which were centrifuged at 500 × g. Next, the harvested cells were resuspended in red blood cell lysis buffer, washed with cold PBS twice and centrifuged at 500 × g for 5 min to obtain macrophages. Then cells (1×10^5 cells/mL) were incubated with dulbecco's modified eagle's medium (DMEM) (Gibco Company Grand Island, NY, USA) containing 10% FBS in a 24-well plate at 37℃ and with 5% CO_2 for 4 - 6 h.

Endocytosis analysis

Fluorescein isothiocyanate (FITC; 0.5 mg/mL; Sigma, St. Louis, MO, USA) was incubated at 37℃ for 20 min to prepare FITC-labeled *streptococcus pneumoniae*. Next, macrophages of mice (1×10^5 cells) were infected by FITC-labeled sodium dodecyl sulfate (SDS) (multiplicity of infection, 10). Then cell nucleus was stained by 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen Inc., Carlsbad, CA, USA), and the images were visualized by confocal laser scanning microscope (LSM 510, Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). The ratio of endocytosis on bacteria (determined by the overlapping of green bacteria) was quantified by independent researchers based on 100 random counted cells and expressed as the percentage of cells containing bacteria.

Detection of bacterial load

The middle ear lavage fluid (0.8 mL) was rotated at 4℃ and 260 × g for 10 min, and the precipitation was resuspended in 0.5 mL sterile PBS. Next, the suspension was continuously diluted for 10 times, and 50 mL final diluent was added on a sheep blood agar plate and fixed at 37℃ and with 5% CO_2. After 16 h, the colony formation unit was counted.

Colorimetric assay

The specimens were centrifuged at 1610 × g for 5 min. The supernatant was respectively added with 50 μL, 0.1 or 2 mL and 0.1 mL superoxide dismutase (SOD), nitric oxide (NO) and malondialdehyde (MDA) detection kits (20091105, NanJing JianCheng Bioengineering Institute, Nanjing, China) in strict accordance with their instructions. Initially, the specimens were fully mixed with SOD by a vortex blender and water-bathed at 37℃ for 40 min for colorimetric determination (light wavelength λ = 550 nm). Next, for NO detection, the testing, standard and blank tubes were set, completely mixed with corresponding reaction solution and allowed to stand at 37℃ for 60 min, which were then rotated for 30 s, cooled down to room temperature and centrifuged. The supernatant was visualized and allowed to stand for 10 min. The optical density (OD) value was determined at the light wavelength of 550 nm. Then the specimens

were uniformly mixed with MDA by a vortex blender, water-bathed at 95℃ for 80 min and centrifuged at 2191 - 2862 × g for 10 min. The colorimetric determination was conducted for acquiring the OD value (light wavelength λ = 532 nm).

Reverse transcription quantitative polymerase chain reaction (RT-QPCR)

Total RNA was extracted using Trizol RNA kit (Invitrogen Inc., Carlsbad, CA, USA). Next, the integrity of RNA was assessed by agarose gel electrophoresis. If 28 S and 18 S bands were bright, clear and sharp, and the grey value of 28S band was twice than that of 18 S band, RNA fragment should be complete. Also, the ratio of A260/A280 between 1.8 and 2.1 indicated higher purity of RNA. Primescript^{TM} RT reagent Kit (RRO37A, Takara Biotechnology Ltd., Dalian, China) was applied for reverse transcription. Fluorescence quantitative PCR instrument (ABI 7500, Applied Biosystems, Inc., CA, USA) was used to amplify target gene and internal reference. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was regarded as internal reference, and $2^{-\Delta\Delta Ct}$ method was utilized to calculate mRNA expression of genes [20]. The experiment was repeated 3 times, which was also applicable for animal experiment. The primer sequences were shown in Table 1.

Western blot analysis

Cells were washed by precooling PBS and lysed by Radio-Immunoprecipitation assay (RIPA) lysis buffer supplemented with protease inhibitor. Next, lysis of cells was removed by a cell scraper, transferred into a centrifuge tube and ice-bathed after vibration for full lysis. The supernatant was collected after centrifugation at 4℃ and 25764 × g for 5 min and the precipitate was abandoned. Protein concentration was determined by bicinchoninic acid (BCA) method. Afterwards, 20 μg of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane, which was then sealed with 5% skim milk for 2 h. Subsequently, the membrane was probed with primary antibodies against immunoresponsive gene 1 (Irg1) (ab222411, 1 : 1000), NF-E2-related factor 2 (Nrf2) (ab137550, 1 : 2000) and heme oxygenase-1 (Hmox1) (ab13243, 1 : 2000) overnight at 4℃.After washed with 0.1% tris-buffered saline with Tween-20 (TBST) 3 times, the membrane was incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (IgG) (ab205718, 1 : 5000) at 37℃ for 1 h. The above antibodies were all purchased from Abcam Inc. (Cambridge, MA, USA). Afterwards, the membrane was washed 3 times and visualized by developing solution. The images were captured by enhanced chemiluminescence (ECL). Bio-Rad imaging analysis system (Bio-Rad, Inc., Hercules, CA, USA) was used to photograph, and Quantity One v4.6.2 software was utilized to analyze. The gray value ratio of target protein bands to GAPDH protein band was regarded as the relative expression of proteins. The experiment was run in triplicate.

Enzyme-linked immunoassay (ELISA)

The middle ear lavage fluid of mice was collected for determining expression of tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-1β based on the manuals of TNF-α, IL-6 and IL-1β ELISA detection kits (RapidBio, Tucson, AZ, USA). The antigen was diluted by coating diluent at the ratio of 1 : 20, and each

well was reacted with 100 μL standard diluent at 4℃ overnight. After the solution was discarded, the specimens were washed 3 times (1 min per time), dried and preserved at 4℃. The diluted specimens were added into reaction wells of the ELISA plate (100 μL each well), with negative and positive controls as well as duplicate wells set. Afterwards, each well was reacted with 100 μL specimen diluent diluted by certain multiple of enzyme conjugate at 37℃ for 30 min. Subsequently, the specimens were added with 100 μL HRP substrate solution and visualized in a dark place at 37℃ for 10 - 20 min. When obvious color change was observed in positive control or slight color change was observed in negative control, each well was added with 50 μL stop buffer to terminate the reaction. Within 20 min, a microplate reader (SpectraMax M5, Molecular Devices, Shanghai China) was used to determine the OD value at the light wavelength of 450 nm.

Hematoxylin-eosin (HE) staining

The middle ear tissues of mice were fixed with 10% neutral formaldehyde solution overnight, embedded in paraffin and sectioned into slices. Next, the slices were dehydrated by 100% xylene I and II respectively for 10 min, immersed in ethanol with a descending concentration (5 min each) and washed in distilled water for 3 min. Cell nucleus stained with Hansen hematoxylin for 7 - 10 min turned into bluish violet. Saline was prepared with ethanol at the ratio of 1 : 1. The slices that were quickly dipped into ammonium hydroxide turned into dark blue. After stained with eosin for 6 min, the slices were washed under running water to remove surface dyeing, dehydrated by ethanol with an ascending concentration (5 min each), cleared with 100% xylene I and II respectively for 5 min and mounted with neutral gum. At last, the cover glasses were dried, photographed and observed under an optical microscope.

Statistical analysis

All data were analyzed using a Statistic Package for the Social Science (SPSS) 21.0 statistical software (IBM Corp. Armonk, NY, USA). The enumeration data were expressed as ratio or percentage, and comparisons among groups were analyzed by *chi*-square test. The measurement data that conformed to normal distribution were described as mean ± standard deviation. Comparisons between two groups were analyzed by non-paired *t* test, while comparisons among multiple groups were analyzed by one-way analysis of variance (ANOVA) with Tukey's post hoc test. Comparisons at different time points were analyzed by ANOVA of repeated measurement with bonferroni's post hoc test. The level of significant difference was set as $p < 0.05$.

Results

Irg1 expresses highly and nrf2 signaling pathway is activated in AOM

Initially, bacterial pathogens in the middle ear lavage fluid from AOM patients and healthy subjects were detected. The results showed that positive rate of *streptococcus pneumoniae* was obviously increased in the middle ear lavage fluid from AOM patients (Table 2). Next, to investigate whether the inflammatory response of AOM patients was associated with Irg1 and Nrf2 signaling pathway, protein expression of

Irg1, Nrf2 and Hmox1 in the middle ear lavage fluid from AOM patients and healthy subjects was determined by western blot analysis. The results found that compared with healthy subjects, Irg1 was elevated and Nrf2 signaling pathway was activated in the middle ear lavage fluid from AOM patients ($p < 0.05$; Fig. 1A-B). Then to verify whether mice model with AOM was successfully established, *streptococcus pneumoniae* capacity in the middle ear lavage fluid was assessed first. The *streptococcus pneumoniae* capacity increased on the 5^{th} day in the middle ear lavage fluid relative to that on the 1^{st} day ($p < 0.05$; Fig. 1C). Afterwards, ELISA was conducted to determine expression of inflammatory cytokines (TNF-α, IL-6 and IL-1β), which identified elevated expression of TNF-α, IL-6 and IL-1β in the middle ear lavage fluid of AOM mice (Fig. 1D). Subsequently, HE staining was carried out to examine the histopathology of the middle ear tissues 4 weeks after infection. The results revealed that increased inflammatory cell infiltration, thickened mucous membrane and aggravated damage were observed in the middle ear tissues of AOM mice (Fig. 1E). The above results demonstrated that mice model with AOM was successfully established. Overall, Irg1 was elevated and Nrf2 signaling pathway was activated both in AOM patients and mice models.

Irg1 promotes accumulation of macrophage to eliminate streptococcus pneumoniae in AOM

Next, to prove that whether upregulation of Irg1 could drive inflammatory cells, the percentage of macrophages in the middle ear lavage fluid of mice was detected by flow cytometry. The results showed that the middle ear lavage fluid of AOM mice contained 16% $CDIIb^+F4/80^+$ macrophages. No significance difference was found in $CDIIb^+F4/80^+$ cells in the middle ear lavage fluid of AOM mice treated with Irg1-NC relative to AOM mice ($p > 0.05$). $CDIIb^+F4/80^+$ cells in the middle ear lavage fluid of AOM mice treated with Irg1 was 29% showing an increased trend, while that in the middle ear lavage fluid of AOM mice treated with sh-Irg1 was 9% exhibiting a decreased tendency (all $p < 0.05$; Fig. 2A). Then macrophages were harvested. mRNA and protein expression of Irg1 in macrophages was determined by RT-qPCR and western blot analysis, which revealed that macrophages in the middle ear lavage fluid of both AOM mice or AOM mice treated with Irg1-NC showed no obvious difference in mRNA and protein expression of Irg1 ($p > 0.05$). However, expression of Irg1 was found to increase in macrophages in the middle ear lavage fluid of AOM mice treated with overexpression of Irg1 and decreased in macrophages in the middle ear lavage fluid of AOM mice treated with sh-Irg1 (all $p < 0.05$; Fig. 2B-D). Afterwards, expression of TNF-α, IL-6 and IL-1β in macrophages was evaluated by ELISA. The results demonstrated that the treatment of Irg1 overexpression weakened expression of TNF-α, IL-6 and IL-1β, while the treatment of Irg1 silencing achieved reverse effect (all $p < 0.05$). The treatment of Irg1-NC displayed no difference in expression of TNF-α, IL-6 and IL-1β when compared to AOM mice ($p > 0.05$; Fig. 2E). As we know, phagocytosis of macrophage against foreign agents includes identification, combination, internalization and endocytosis. Endocytosis analysis was conducted to examine phagocytosis of macrophage. Endocytosis elevated following the treatment of Irg1 overexpression and reduced following the treatment of sh-Irg1 (all $p < 0.05$), while did not alter following the treatment of Irg1-NC ($p > 0.05$; Fig. 2F). Taken together, overexpression of Irg1 could reduce *streptococcus pneumoniae* via accumulation of macrophage in the middle ear tissue of AOM cases.

Irg1 activated macrophage to repress streptococcus pneumoniae by activating Nrf2 signaling pathway

Subsequently, to evaluate the effect of Irg1 on Nrf2 signaling pathway, AOM mice was treated with Irg1-NC, Irg1, sh-Irg1, RA (an inhibitor of inflammation) or combined Irg1 and RA. According to RT-qPCR and western blot analysis, no significant difference was found in the expression of Nrf2 and Hmox1 after the introduction of Irg1-NC alone or combination of Irg1 and RA ($p > 0.05$). The introduction of Irg1 elevated expression of Nrf2 and Hmox1, but the introduction of either sh-Irg1 or RA caused apposite trends ($p < 0.05$; Fig. 3A-C). Based on the results of ELISA, there was no obvious difference in the expression of TNF-α, IL-6 and IL-1β after the introduction of Irg1-NC alone or Irg1 and RA together ($p > 0.05$). Besides, expression of TNF-α, IL-6 and IL-1β weakened after the introduction of Irg1 and strengthened after the introduction of either sh-Irg1 or RA ($p < 0.05$; Fig. 3D). The results of endocytosis analysis showed that endocytosis of macrophage increased after the introduction of Irg1 and reduced after the introduction of either sh-Irg1 or RA ($p < 0.05$), while did not differ after the introduction of Irg1-NC alone or Irg1 and RA together ($p > 0.05$; Fig. 3E). The aforementioned results collectively revealed that Irg1 could activate Nrf2 signaling pathway and promote expression of its downstream gene Hmox1, thus inducing accumulation of macrophage to eliminate *streptococcus pneumoniae*.

Irg1 activates Nrf2 signaling pathway to inhibit infection of streptococcus pneumoniae in vivo

Afterwards, to examine the roles of Irg1 and Nrf2 in infection of *streptococcus pneumoniae*, RA (Nrf2 signaling pathway inhibitor) and Irg1 lentiviral vectors were transduced into AOM mice by tail vein injection. The middle ear lavage fluid of mice was collected, from which the middle ear epithelial cells were isolated for determining *streptococcus pneumoniae* capacity. The results showed that the transduction of Irg1-NC or combined Irg1 and RA induced no obvious difference regarding *streptococcus pneumoniae* capacity ($p > 0.05$). The transduction of sh-Irg1 or RA alone increased *streptococcus pneumoniae* infection, while the transduction of Irg1 achieved reverse result ($p < 0.05$; Fig. 4A). The results of ELISA on inflammation showed that expression of TNF-α, IL-6 and IL-1β did not differ after the transduction of Irg1-NC or combined Irg1 and RA ($p > 0.05$). However, expression of TNF-α, IL-6 and IL-1β elevated after the transduction of sh-Irg1 or RA alone and decreased after the transduction of Irg1 ($p < 0.05$; Fig. 4B). HE staining was conducted to assess histopathology of the middle ear epithelial cells from mice, which revealed that no prominent difference was detected in histopathology of the middle ear epithelial cells after the transduction of Irg1-NC or combined Irg1 and RA. Furthermore, the transduction of sh-Irg1 or RA exhibited increased inflammatory cell infiltration, thickened mucous membrane and aggravated damage, while the transduction of Irg1 displayed reduced mucous membrane epithelial hyperplasia, decreased inflammatory cells and relieved damage (Fig. 4C). According to colorimetric assay, the transduction of sh-Irg1 or RA induced an increase of MDA and NO contents and a decrease of SOD activity, while the transduction of Irg1 led to an opposite trend ($p < 0.05$). The transduction of Irg1-NC or combined Irg1 and RA caused no significant change in those indicators ($p > 0.05$) (Table 3). All in all, Irg1 activated Nrf2 signaling pathway and inhibited oxidative stress, thus inhibiting infection of *streptococcus pneumoniae* in AOM mice.

Discussion

As a kind of polymicrobial disease, AOM is often regarded as a complication of upper respiratory tract infection by pathogens [21]. Streptococcus pneumoniae is often seen as a prevalent respiratory pathogen, which is majorly responsible for occurrence of childhood AOM [7]. Irg1 is known as one of the highest induced genes in mammalian macrophages under inflammation occurrence and is involved with function of macrophages [22] [23]. Therefore, the current study was designed to explore the regulatory role of Irg1 in the AOM progression. Collectively, the data of the study revealed that upregulation of Irg1 stimulated accumulation of macrophage to repress inflammatory response and streptococcus pneumoniae infection in AOM by activating Nrf2 signaling pathway.

The initial finding of our study was that Irg1was highly expressed in AOM and macrophage collected from the middle ear lavage fluid of AOM mice. AOM is an infection disease with inflammation symptoms [24]. According to gene expression profiling studies of microglial cells and murine macrophages, overexpressed Irg1 has been detected in pro-inflammatory conditions [11]. Similarly, during inflammation process, Irg1 has been reported to exhibit a high expression in macrophages of mammals [10]. In addition, high expression of Irg1 has been detected in the uterine luminal epithelium of the mouse at the initial period of pregnancy [25]. Additionally, our study found that Nrf2 signaling pathway was activated in AOM. Nrf2 activation protects arteries against a proinflammatory condition in endothelial cells [26]. Moreover, Nrf2-antioxidant signaling pathway activation promotes apoptosis and alleviates NF-kappa B-inflammatory response [27]. Hence, it seems that Nrf2 plays as an anti-inflammatory role against infection.

Second, our study showed that upregulation of Irg1 could attract macrophage to inhibit inflammatory response by decreasing expression of TNF-α, IL-6 and IL-1β via activated Nrf2 signaling pathway in AOM. A local inflammation in the middle ear is a major characteristic of AOM, and an inflammatory response to microbes promotes elimination of otopathogens in the middle ear in AOM [28] [29]. Macrophages are vital contributors for inflammatory immunopathology, which can produce lots of inflammatory cytokines and mediators, such as TNF-α, IL-6 and IL-1β, when exposed to bacterial products [8] [30]. Recurrent AOM has been reported to be associated with genetic polymorphisms in immunoresponse genes including TNFA, IL-6, TLR4 and IL-10 [31]. Increased expression of IL-6, IL-1β and TNF-α has been detected in AOM children with a bacterial otopathogen [24]. A prior study has reported that Irg1 induces decreased expression of TNF-α, IL-6, and IFN-β triggered by Toll-like receptor in lipopolysaccharide-tolerized macrophages [32]. In addition, Nrf2 inhibits macrophage inflammatory response by downregulating expression of IL-6 and IL-1β [13]. All these studies indicate that Irg1 and Nrf2 are linked to inflammatory response.

Last but not least, in our current work, we provided evidence that elevated Irg1 could decoy macrophage to suppress oxidative stress and streptococcus pneumoniae infection through activation of Nrf2 signaling pathway in response to increased expression of Nrf2, Hmox1 and SOD and decreased expression of MDA and NO. The reduction in activity of macrophage migration inhibitory factor can

relieve mouse AOM in vivo [33]. MDA is a final product of lipid peroxidation associated with oxidative stress, and NO is an extremely diffusible molecule that can establish firm oxidized metabolites [34]. SOD is linked to oxidative stress and various disease states such as inflammatory diseases [35]. Hmox1 is associated with oxidative stress defense and bilirubin homeostasis [36]. Streptococcus pneumoniae is a bacterial pathogen frequently localized in the human nasopharynx, and its transmission from the nasopharynx to other parts of the body can lead to mucosal infections like OM [37]. Irg1 has been reported to be linked to respiratory syncytial virus infection-associated disease and oxidative damage [12]. Moreover, it has been reported that Nrf2 is associated with lung inflammation caused by streptococcus pneumoniae [38]. Furthermore, Nrf2 plays a regulatory role in the transcriptional response to oxidative stress and in pneumonia caused by streptococcus pneumoniae [14]. Therefore, Irg1 and Nrf2 collaborate to fight against streptococcus pneumoniae infection.

Conclusion

Taken together, Irg1 confers an inhibitory role in inflammatory response and streptococcus pneumoniae infection of AOM by promoting the accumulation of macrophages through activation of Nrf2 signaling pathway. These findings identify Irg1 as a potential marker for the prognosis of AOM and a therapeutic target for this disease. However, the research is still at the preclinical stage, and due to the limited sample size, the exact mechanism of Irg1 in AOM is not fully clarified. Therefore, in the further study, large-scale studies should be included in the research to further determine the underlying role and functional mechanism of Irg1 and Nrf2 signaling pathway in AOM caused by other pathogens such as haemophilus influenzae, and moraxella catarrhalis.

Abbreviations

AOM:Acute otitis media; OM:Otitis media; PBS:phosphate buffer saline; FBS:fetal bovine serum; DMEM:dulbecco's modified eagle's medium; SDS:sodium dodecyl sulfate; SOD:superoxide dismutase.

Declarations

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Author's contribution

Peizhen Yang, Yufen Li, Dongqing Wang and Zhipeng Chen wrote the paper and conceived and designed the experiments; Peizhen Yang and Peizhen Yang analyzed the data; Dongqing Wang and Zhipeng Chen collected and provided the sample for this study. All authors have read and approved the final submitted manuscript.

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Availability of data and materials

The datasets generated/analysed during the current study are available

Ethics approval and consent to participate

Written informed consent was obtained from all patients and their guardians prior to the study. Study protocols according to the ethical principles for medical research involving human subjects of the Helsinki Declaration were approved by Ethic Committee of Linyi People's Hospital. The animal experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol of animal experiments was approved by the Institutional Animal Care and Use Committee of Linyi People's Hospital. The animal experiments were conducted based on minimized animal number and the least pains on experimental animals.

Consent for publication

All authors consent for publication.

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Hernandez M, Leichtle A, Pak K, Webster NJ, Wasserman SI, Ryan AF. The transcriptome of a complete episode of acute otitis media. BMC Genomics. 2015;16:259.
2. Nitsche MP, Carreno M. Antibiotics for acute otitis media in children. Medwave. 2015;15 Suppl 2:e6295.

3. Lieberthal AS, Carroll AE, Chonmaitree T, Ganiats TG, Hoberman A, Jackson MA, Joffe MD, Miller DT, Rosenfeld RM, Sevilla XD, Schwartz RH, Thomas PA, Tunkel DE. The diagnosis and management of acute otitis media. Pediatrics. 2013;131:e964-99.
4. Nesbit CE, Powers MC. An evidence-based approach to managing acute otitis media. Pediatr Emerg Med Pract. 2013;10:1-26; quiz 26-7.
5. Pettigrew MM, Gent JF, Pyles RB, Miller AL, Nokso-Koivisto J, Chonmaitree T. Viral-bacterial interactions and risk of acute otitis media complicating upper respiratory tract infection. J Clin Microbiol. 2011;49:3750-5.
6. Zhao C, Li Z, Zhang F, Zhang X, Ji P, Zeng J, Hu B, Hu Z, Liao K, Sun H, Zhang R, Cao B, Zhuo C, Jia W, Mei Y, Chu Y, Xu X, Yang Q, Jin Y, Fu Q, Xu X, Li H, Wang L, Ni Y, Liang H, Wang H. Serotype distribution and antibiotic resistance of Streptococcus pneumoniae isolates from 17 Chinese cities from 2011 to 2016. BMC Infect Dis. 2017;17:804.
7. Morris MC, Pichichero ME. Streptococcus pneumoniae burden and nasopharyngeal inflammation during acute otitis media. Innate Immun. 2017;23:667-77.
8. Cohen HB, Mosser DM. Extrinsic and intrinsic control of macrophage inflammatory responses. J Leukoc Biol. 2013;94:913-9.
9. Liu X, Wu XP, Zhu XL, Li T, Liu Y. IRG1 increases MHC class I level in macrophages through STAT-TAP1 axis depending on NADPH oxidase mediated reactive oxygen species. Int Immunopharmacol. 2017;48:76-83.
10. Michelucci A, Cordes T, Ghelfi J, Pailot A, Reiling N, Goldmann O, Binz T, Wegner A, Tallam A, Rausell A, Buttini M, Linster CL, Medina E, Balling R, Hiller K. Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. Proc Natl Acad Sci U S A. 2013;110:7820-5.
11. Jamal Uddin M, Joe Y, Kim SK, Oh Jeong S, Ryter SW, Pae HO, Chung HT. IRG1 induced by heme oxygenase-1/carbon monoxide inhibits LPS-mediated sepsis and pro-inflammatory cytokine production. Cell Mol Immunol. 2016;13:170-9.
12. Ren K, Lv Y, Zhuo Y, Chen C, Shi H, Guo L, Yang G, Hou Y, Tan RX, Li E. Suppression of IRG-1 Reduces Inflammatory Cell Infiltration and Lung Injury in Respiratory Syncytial Virus Infection by Reducing Production of Reactive Oxygen Species. J Virol. 2016;90:7313-22.
13. Kobayashi EH, Suzuki T, Funayama R, Nagashima T, Hayashi M, Sekine H, Tanaka N, Moriguchi T, Motohashi H, Nakayama K, Yamamoto M. Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. Nat Commun. 2016;7:11624.
14. Gomez JC, Dang H, Martin JR, Doerschuk CM. Nrf2 Modulates Host Defense during Streptococcus pneumoniae Pneumonia in Mice. J Immunol. 2016;197:2864-79.
15. Nair S, Huynh JP, Lampropoulou V, Loginicheva E, Esaulova E, Gounder AP, Boon ACM, Schwarzkopf EA, Bradstreet TR, Edelson BT, Artyomov MN, Stallings CL, Diamond MS. Irg1 expression in myeloid cells prevents immunopathology during M. tuberculosis infection. J Exp Med. 2018;215:1035-45.

16. Kelly B, O'Neill LA. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. Cell Res. 2015;25:771-84.
17. Mills EL, Ryan DG, Prag HA, Dikovskaya D, Menon D, Zaslona Z, Jedrychowski MP, Costa ASH, Higgins M, Hams E, Szpyt J, Runtsch MC, King MS, McGouran JF, Fischer R, Kessler BM, McGettrick AF, Hughes MM, Carroll RG, Booty LM, Knatko EV, Meakin PJ, Ashford MLJ, Modis LK, Brunori G, Sevin DC, Fallon PG, Caldwell ST, Kunji ERS, Chouchani ET, Frezza C, Dinkova-Kostova AT, Hartley RC, Murphy MP, O'Neill LA. Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1. Nature. 2018;556:113-17.
18. Guo H, Li M, Xu LJ. Apigetrin treatment attenuates LPS-induced acute otitis media though suppressing inflammation and oxidative stress. Biomed Pharmacother. 2019;109:1978-87.
19. Wu J, Zhu Y, Li F, Zhang G, Shi J, Ou R, Tong Y, Liu Y, Liu L, Lu L, Liu Z. Spica prunellae and its marker compound rosmarinic acid induced the expression of efflux transporters through activation of Nrf2-mediated signaling pathway in HepG2 cells. J Ethnopharmacol. 2016;193:1-11.
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402-8.
21. Marom T, Nokso-Koivisto J, Chonmaitree T. Viral-bacterial interactions in acute otitis media. Curr Allergy Asthma Rep. 2012;12:551-8.
22. Pan J, Zhao X, Lin C, Xu H, Yin Z, Liu T, Zhang S. Immune responsive gene 1, a novel oncogene, increases the growth and tumorigenicity of glioma. Oncol Rep. 2014;32:1957-66.
23. Lefrancois C, Casadevall I, Betremieux P, Donnio PY, Jouan H, Laisney N, Le Marec B. [Fatal legionellosis in an infant treated with ACTH]. Arch Fr Pediatr. 1989;46:591-3.
24. Kaur R, Casey J, Pichichero M. Cytokine, chemokine, and Toll-like receptor expression in middle ear fluids of children with acute otitis media. Laryngoscope. 2015;125:E39-44.
25. Tallam A, Perumal TM, Antony PM, Jager C, Fritz JV, Vallar L, Balling R, Del Sol A, Michelucci A. Gene Regulatory Network Inference of Immunoresponsive Gene 1 (IRG1) Identifies Interferon Regulatory Factor 1 (IRF1) as Its Transcriptional Regulator in Mammalian Macrophages. PLoS One. 2016;11:e0149050.
26. Zakkar M, Van der Heiden K, Luong le A, Chaudhury H, Cuhlmann S, Hamdulay SS, Krams R, Edirisinghe I, Rahman I, Carlsen H, Haskard DO, Mason JC, Evans PC. Activation of Nrf2 in endothelial cells protects arteries from exhibiting a proinflammatory state. Arterioscler Thromb Vasc Biol. 2009;29:1851-7.
27. Li W, Khor TO, Xu C, Shen G, Jeong WS, Yu S, Kong AN. Activation of Nrf2-antioxidant signaling attenuates NFkappaB-inflammatory response and elicits apoptosis. Biochem Pharmacol. 2008;76:1485-9.
28. Liu K, Kaur R, Almudevar A, Pichichero ME. Higher serum levels of interleukin 10 occur at onset of acute otitis media caused by Streptococcus pneumoniae compared to Haemophilus influenzae and Moraxella catarrhalis. Laryngoscope. 2013;123:1500-5.

29. Kurabi A, Pak K, Ryan AF, Wasserman SI. Innate Immunity: Orchestrating Inflammation and Resolution of Otitis Media. Curr Allergy Asthma Rep. 2016;16:6.
30. Gao H, Liu L, Zhao Y, Hara H, Chen P, Xu J, Tang J, Wei L, Li Z, Cooper DKC, Cai Z, Mou L. Human IL-6, IL-17, IL-1beta, and TNF-alpha differently regulate the expression of pro-inflammatory related genes, tissue factor, and swine leukocyte antigen class I in porcine aortic endothelial cells. Xenotransplantation. 2017;24:
31. Emonts M, Veenhoven RH, Wiertsema SP, Houwing-Duistermaat JJ, Walraven V, de Groot R, Hermans PW, Sanders EA. Genetic polymorphisms in immunoresponse genes TNFA, IL6, IL10, and TLR4 are associated with recurrent acute otitis media. Pediatrics. 2007;120:814-23.
32. Li Y, Zhang P, Wang C, Han C, Meng J, Liu X, Xu S, Li N, Wang Q, Shi X, Cao X. Immune responsive gene 1 (IRG1) promotes endotoxin tolerance by increasing A20 expression in macrophages through reactive oxygen species. J Biol Chem. 2013;288:16225-34.
33. 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.
34. Yalcinkaya E, Cakiroglu Y, Doger E, Budak O, Cekmen M, Caliskan E. Effect of follicular fluid NO, MDA and GSH levels on in vitro fertilization outcomes. J Turk Ger Gynecol Assoc. 2013;14:136-41.
35. McCord JM, Edeas MA. SOD, oxidative stress and human pathologies: a brief history and a future vision. Biomed Pharmacother. 2005;59:139-42.
36. Jiraskova A, Novotny J, Novotny L, Vodicka P, Pardini B, Naccarati A, Schwertner HA, Hubacek JA, Puncocharova L, Smerhovsky Z, Vitek L. Association of serum bilirubin and promoter variations in HMOX1 and UGT1A1 genes with sporadic colorectal cancer. Int J Cancer. 2012;131:1549-55.
37. Dhar P, Ng GZ, Dunne EM, Sutton P. Mucin 1 protects against severe Streptococcus pneumoniae infection. Virulence. 2017;8:1631-42.
38. Yao H, Yang SR, Kode A, Rajendrasozhan S, Caito S, Adenuga D, Henry R, Edirisinghe I, Rahman I. Redox regulation of lung inflammation: role of NADPH oxidase and NF-kappaB signalling. Biochem Soc Trans. 2007;35:1151-5.

Tables

Table 1 Primer sequences for RT-qPCR

Gene	Primer sequence
Nrf2	F: 5'-CCGCTCGAGATGATGGACTTGGAGTTGCC-3'
	R: 5'-CGGGGTACCCTAGTTTTTCTTTGTATCTGGCTTCTT--3'
Irg1	F: 5'-GGT ATC ATT CGG AGG AGC AA-3'
	R: 5'-ACA GAG GGA GGG TGG AAT CT-3'
Hmox1	F: 5'-TTCAGAAGGGTCAGGTCC-3'
	R: 5'-CAGTGAGGCCCATACCAGAA-3'
GAPDH	F: 5'-TCA ACA GCA ACT CCC ACT CTT CCA-3'
	R: 5'-ACC CTG TTG CTG TAG CCG TAT TCA-3'

Notes: RT-qPCR, reverse transcription quantitative polymerase chain reaction; F, forward; R, reverse; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Nrf2, NF-E2-related factor 2; Irg1, immunoresponsive gene 1; Hmox1, heme oxygenase-1.

Table 2 Positive rate of *streptococcus pneumoniae* in the middle ear lavage fluid from AOM patients and healthy subjects

Pathogen	Positive rate of specimens from healthy subjects	Positive rate of specimens from AOM patients	Chi-square value (X^2)	*p* value
	(n = 40)	(n = 85)		
Streptococcus pneumoniae	7	43	12.41	< 0.001
Ratio	17.50%	50.59%		

Note: AOM, acute otitis media.

Table 3 MDA and NO contents and SOD activity in the middle ear lavage fluid of AOM mice

	AOM	Irg1-NC + AOM	sh-Irg1 + AOM	Irg1 + AOM	RA + AOM	Irg1 + RA + AOM
SOD (μ/mg)	79.08 ± 8.21	82.19 ± 13.4	60.15 ± 5.90*	143.42 ± 9.88*	62.38 ± 6.27*	85.00 ± 7.91
NO (μmol/L)	44.89 ± 10.63	48.90 ± 6.84	71.39 ± 11.28*	23.76 ± 8.00*	66.08 ± 8.41*	41.33 ± 5.88
MDA (nmol/mg)	5.11 ± 0.98	4.76 ± 0.67	9.23 ± 1.18*	2.33 ± 0.52*	10.91 ± 1.56*	5.73 ± 1.10

Note: * $p < 0.05$ compared with AOM mice. The MDA and NO contents and SOD activity are expressed as mean ± standard deviation, and comparisons among multiple groups are analyzed by one-way analysis of variance with Tukey's post hoc test. n = 6; AOM, acute otitis media; Irg1, immunoresponsive gene 1; sh, short hairpin; RA, retinoic acid; NC, negative control; SOD, superoxide dismutase; MDA, malondialdehyde; NO, nitric oxide.

Figures

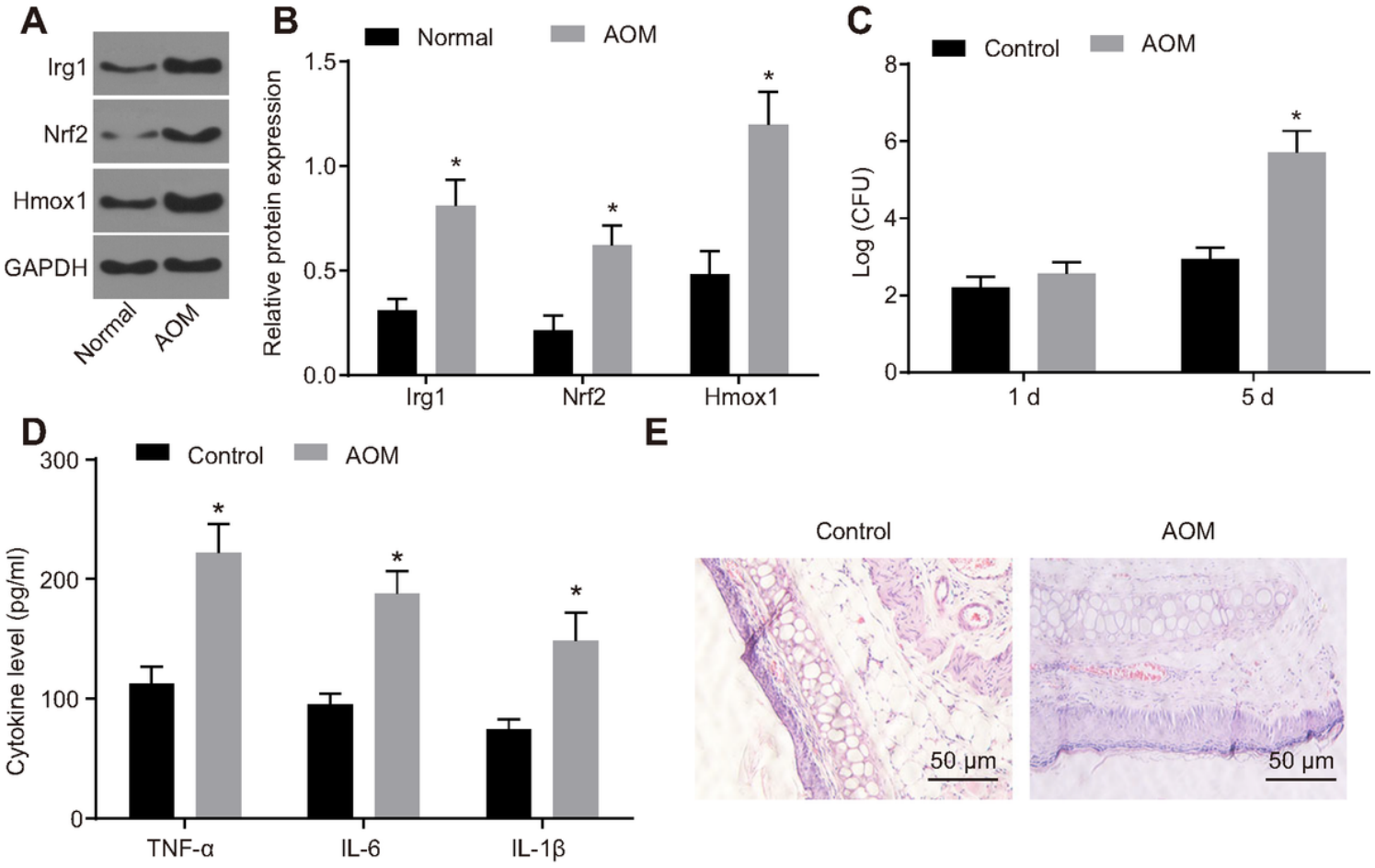

Figure 2

Irg1 was highly expressed and Nrf2 signaling pathway was activated in AOM. A-B, western blot analysis of Irg1, Nrf2 and Hmox1 protein expression in the middle ear lavage fluid from AOM patients and healthy subjects. The band intensity was expressed as mean ± standard deviation and analyzed by non-paired t test. Healthy subjects: n = 40; AOM patients: n = 85. * $p < 0.05$ compared with healthy subjects. C, streptococcus pneumoniae capacity in the middle ear lavage fluid from mice. D, ELISA analysis of inflammatory cytokine (TNF-α, IL-6 and IL-1β) expression. E, HE staining of the histopathology of the middle ear tissues from mice 4 weeks after infection (× 200). * $p < 0.05$ compared with the control mice. Control mice: n = 8; Mice with AOM: n = 48. The data were expressed as mean ± standard deviation and analyzed by non-paired t test. AOM, acute otitis media; Nrf2, NF-E2-related factor 2; Irg1, immunoresponsive gene 1; Hmox1, heme oxygenase-1; TNF-α, tumor necrosis factor-α; IL, interleukin; ELISA, enzyme-linked immunoassay; HE, hematoxylin-eosin.

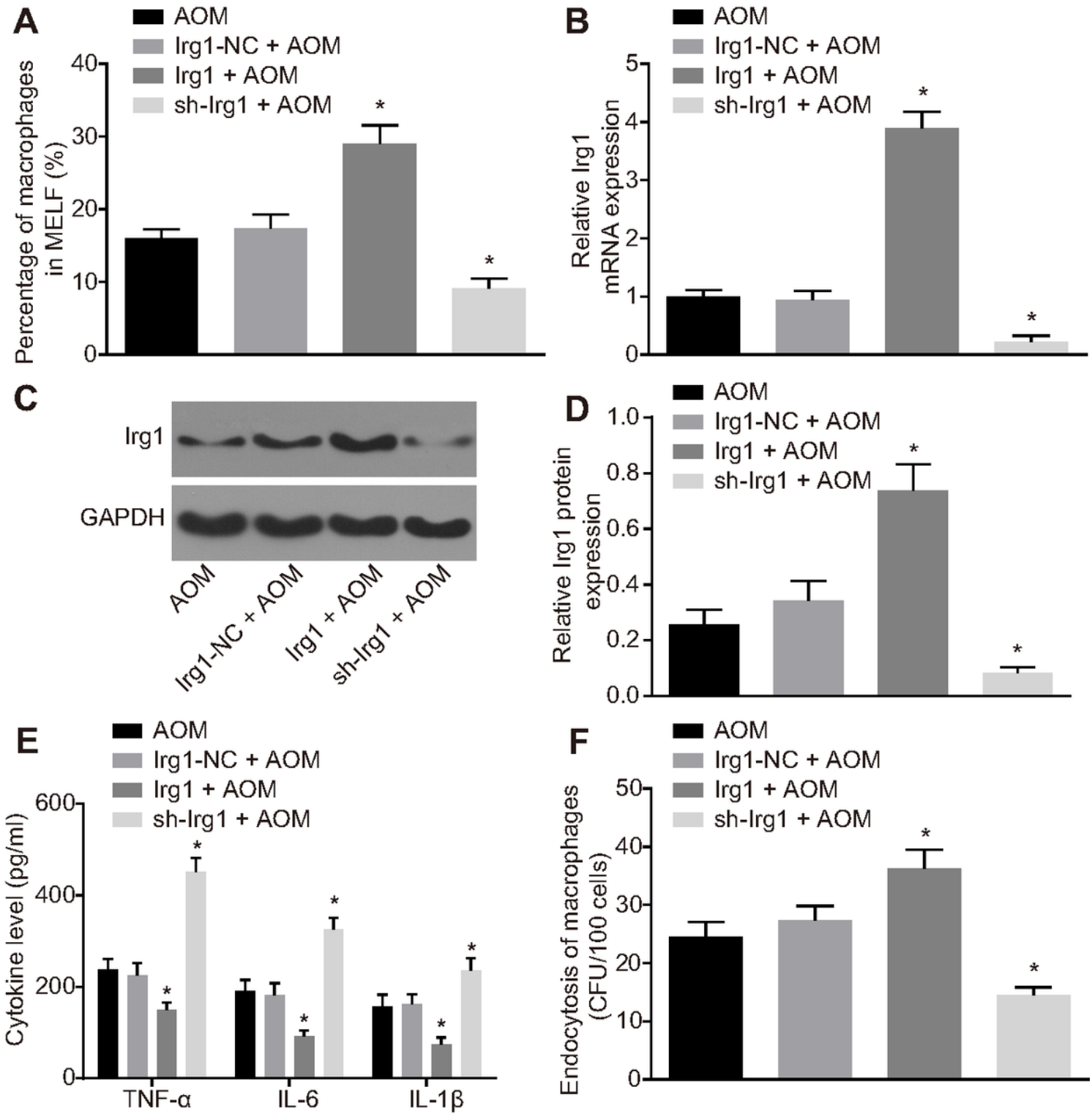

Figure 4

Elevated Irg1 attracted macrophages to remove streptococcus pneumoniae in AOM. AOM mice were treated with Irg1-NC, Irg1 overexpression and sh-Irg1. A, the percentage of macrophages in the middle ear lavage fluid of AOM mice assessed by flow cytometry. B, RT-qPCR analysis of Irg1 mRNA expression in macrophages. C-D, western blot analysis of Irg1 protein level in macrophages. The band intensity is quantified. E, ELISA analysis of expression of TNF-α, IL-6 and IL-1β in macrophages. F, phagocytosis of

macrophages. * p < 0.05 compared with AOM mice. The data were expressed as mean ± standard deviation, and comparisons among multiple groups were analyzed by one-way analysis of variance with Tukey's post hoc test. The experiment was repeated 3 times. AOM, acute otitis media; Nrf2, NF-E2-related factor 2; Irg1, immunoresponsive gene 1; Hmox1, heme oxygenase-1; TNF-α, tumor necrosis factor-α; IL, interleukin; ELISA, enzyme-linked immunoassay; NC, negative control; sh, short hairpin; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

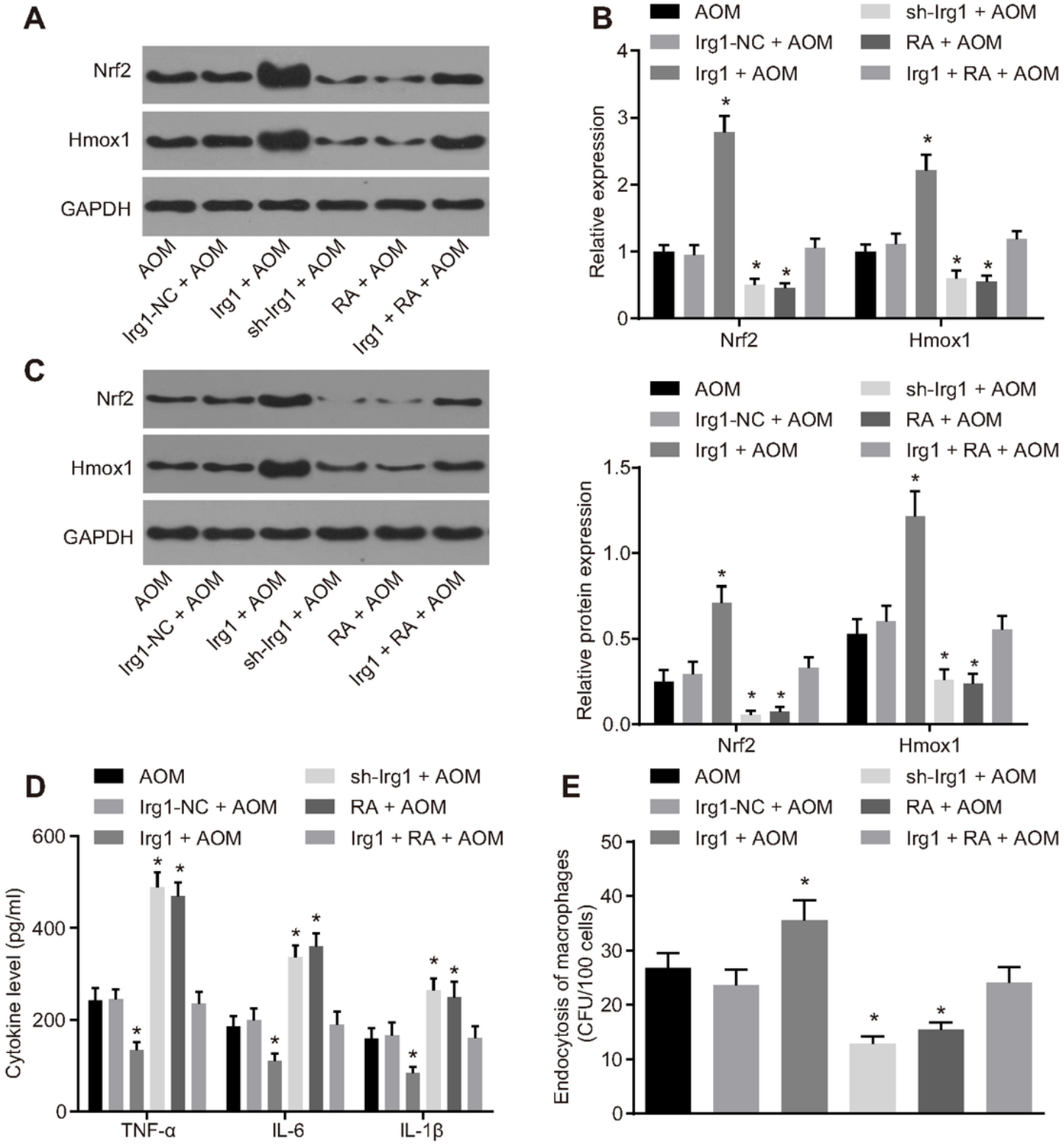

Figure 6

Elevated Irg1 decoyed macrophage to obliterate streptococcus pneumoniae through activation of Nrf2 signaling pathway. AOM mice were treated with Irg1-NC, Irg1, sh-Irg1, RA or combined Irg1 and RA. A, RT-qPCR analysis of Nrf2 and Hmox1 mRNA expression in macrophages. B-C, western blot analysis of Nrf2 and Hmox1 protein expression in macrophages. The band intensity was quantified. D, ELISA analysis of expression of TNF-α, IL-6 and IL-1β in macrophages. E, phagocytosis of macrophages. * $p < 0.05$ compared with AOM mice. The data were expressed as mean ± standard deviation, and comparisons among multiple groups were analyzed by one-way analysis of variance with Tukey's post hoc test. The experiment was repeated 3 times. AOM, acute otitis media; Nrf2, NF-E2-related factor 2; Irg1, immunoresponsive gene 1; Hmox1, heme oxygenase-1; TNF-α, tumor necrosis factor-α; IL, interleukin; ELISA, enzyme-linked immunoassay; NC, negative control; sh, short hairpin; RT-qPCR, reverse transcription quantitative polymerase chain reaction; RA, retinoic acid.

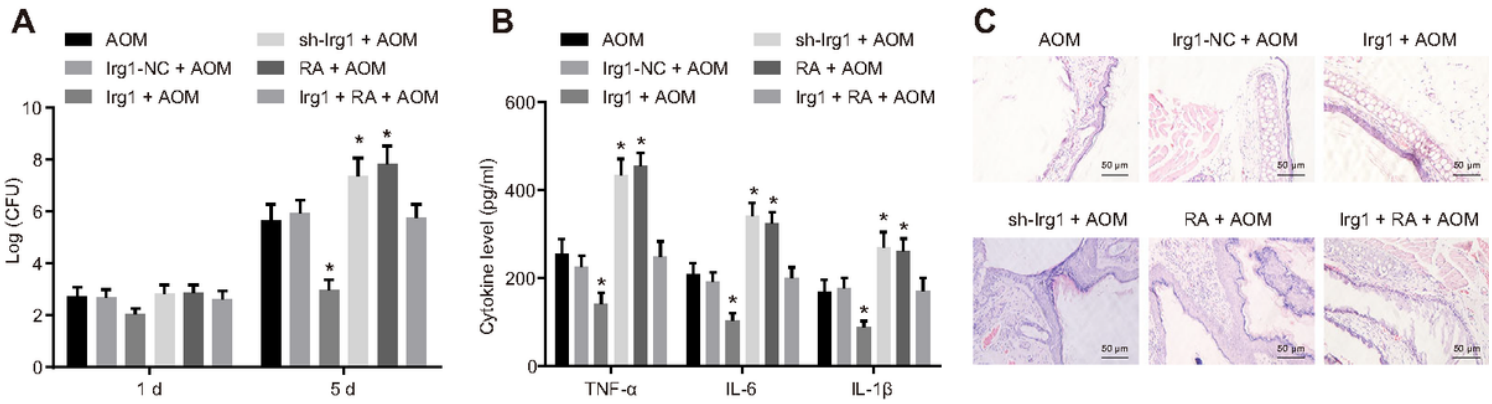

Figure 8

Irg1 repressed infection of streptococcus pneumoniae through activation of Nrf2 signaling pathway. AOM mice were treated with Irg1-NC, Irg1, sh-Irg1, RA or combined Irg1 and RA. A, streptococcus pneumoniae capacity in the middle ear lavage fluid of AOM mice. n = 3. B, ELISA analysis of TNF-α, IL-6 and IL-1β expression in the middle ear lavage fluid of AOM mice. n = 6. C, HE staining result of histopathology of the middle ear epithelial cells from AOM mice (× 200). * $p < 0.05$ compared with AOM mice. The data were expressed as mean ± standard deviation, and comparisons among multiple groups were analyzed by one-way analysis of variance with Tukey's post hoc test. Comparisons at different time points were analyzed by one-way analysis of variance of repeated measurements with bonferroni's post hoc test. AOM, acute otitis media; Nrf2, NF-E2-related factor 2; Irg1, immunoresponsive gene 1; Hmox1, heme oxygenase-1; TNF-α, tumor necrosis factor-α; IL, interleukin; ELISA, enzyme-linked immunoassay; NC, negative control; sh, short hairpin; RA, retinoic acid.