Silencing Nrf2 attenuates chronic suppurative otitis media by inhibiting pro-inflammatory cytokine secretion through up-regulating TLR4

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Abstract
Compromised TLR-mediated chronic inflammation contributes to bacterial infection-caused chronic suppurative otitis media, but the mechanisms are unclear. The present study examined the expression status of nuclear erythroid 2-related factor 2 (Nrf2) and TLRs in human middle-ear mucosae tissues collected from patients with chronic suppurative otitis media, chronic otitis media and non-otitis media, and found that Nrf2 was high-expressed, whereas TLR4, instead of other TLRs, was low expressed in chronic suppurative otitis media compared to chronic otitis media and non-chronic otitis media groups. Consistently, inflammatory cytokines were significantly up-regulated in the chronic suppurative otitis media group, instead of the chronic otitis media and non-chronic otitis media groups. Next, LPS-induced acute otitis media and chronic suppurative otitis media models in mice were established, and high levels of inflammatory cytokines were sustained in the mucosae tissues of chronic suppurative otitis media mice compared to the non-otitis media and acute otitis media groups. Interestingly, continuous low-dose LPS stimulation promoted Nrf2 expression, but decreased TLR4 levels in chronic suppurative otitis media mice mucosae. In addition, knock-down of Nrf2 increased TLR4 expression levels in chronic suppurative otitis media mice, and both Nrf2 ablation and TLR4 overexpression inhibited the pro-inflammatory cytokine expression in chronic suppurative otitis media. Finally, we found that both Nrf2 overexpression and TLR4 deficiency promoted chronic inflammation in LPS-induced acute otitis media mice models. Taken together, knock-down of Nrf2 reversed chronic inflammation to attenuate chronic suppurative otitis media by up-regulating TLR4.

Keywords
Chronic suppurative otitis media, Toll-like receptors, lipopolysaccharide, nuclear erythroid 2-related factor 2

Introduction
Acute bacterial infection-caused otitis media (AOM) accounts for about 80% of 1–6 yr old children with OM worldwide, leading to otalgia, otorrhea, deafness and dizziness.1,2 During AOM development, the innate immune response is triggered by bacterial infections and the immune cells (leukocytes) are recruited to the middle-ear mucosae,3,4 accompanied by acute inflammatory reactions to eliminate bacteria in the middle-ear mucosae.3,5 Of note, in some cases acute inflammation turned into chronic inflammation under bacterial pressure, which was the result of the interactions between...
bacteria and the human innate immune system. Current data suggest that sustaining chronic inflammation caused physiological and pathological changes in the AOM middle-ear mucosa and resulted in chronic suppurrative OM (CSOM), which features persistent otorrhea and is even accompanied by intracranial infection. Although the aetiology and pathogenesis of AOM had been well delineated, the underlying mechanisms of the transition from AOM to CSOM are still largely unknown.

The innate immune system recognizes PAMPs to neutralize bacteria by PRR in immune cells, by which the intrinsic inflammation-associated pathways are activated in response to bacterial infection. TLRs are a family of PRRs, including TLRs 1–9, which recognize various PAMPs, such as bacterial LPS and viral nucleic acids. Recent data indicated that alterations of TLRs (TLR2, TLR4, TLR5 and TLR9) in middle-ear mucosa contributed to susceptibility of CSOM, and TLRs were closely associated with chronic inflammation. Our preliminary experiments (data not shown) identified that merely TLR4, instead of other TLRs, was down-regulated in the middle-ear mucosa of patients with CSOM, in contrast with AOM and non-OM groups, suggesting that TLR4 might be the hub gene to regulate chronic inflammation and AOM-CSOM transition. In addition, Gram-negative bacterial infection caused CSOM pathogenesis through LPS, hence, LPS was used to establish OM models in mice to simulate Gram-negative bacterial infection. Additionally, LPS could be recognized by TLR4 to trigger pro-inflammatory reactions. Based on the above information, LPS was employed to induct mice models for AOM and CSOM in this study.

Nuclear erythroid 2-related factor 2 (Nrf2) mainly participates in the regulation of oxidative damage by up-regulating the antioxidant genes. Aside from that, Nrf2 also regulates inflammatory reactions during bacterial infection and is involved in LPS-induced OM progression. Interestingly, we noted that the crosstalk between Nrf2 and TLR pathways existed in the regulation of inflammation in an anti-infective process. Especially, Nrf2 protected against acute lung injury and inflammation by modulating TLR4 signaling and regulated LPS-induced AOM by suppressing inflammation and oxidative stress by targeting TLR4. The above publications rendered the possibility that Nrf2/TLR4 pathway might regulate the pathogenesis of CSOM. By analysing clinical samples, our preliminary work indicated that Nrf2 was highly expressed in CSOM compared to COM and non-OM groups, and the mRNA levels of Nrf2 and TLR4 showed negative correlations to CSOM mucosal tissues.

The previous publications and our preliminary work showed us that Nrf2/TLR4 pathway might be involved in the regulation of chronic inflammation in CSOM and the transition from AOM to CSOM. To verify this, the clinical CSOM samples were collected and LPS-induced CSOM mice models were established in this study, and further gain- and loss-function experiments were conducted to validate our hypothesis. Overall, this study will give some insights into the molecular mechanisms of CSOM progression.

Materials and methods

Clinical samples collection and ethics approval

A total of 93 participants, including patients with CSOM (n = 30), COM (n = 25) and non-OM (n = 18) were involved in this study. All the patients were subjected to surgery from 2015 to 2017 in the First Affiliated Hospital of Xinjiang Medical University. The criteria for distinguishing patients with CSOM, COM and non-OM were recorded in the previous publication, and patient types were judged by three independent experienced doctors in our hospital. The middle-ear specimens were collected for further analysis and the patients with systemic diseases or immunodeficiencies were excluded. All the clinical experiments involved in this study were approved by the ethics committee of the First Affiliated Hospital of Xinjiang Medical University. In addition, written informed consent was obtained from all the participants.

Establishment of LPS-induced AOM and CSOM mice models

The C57BL/6 mice weighing from 18 to 22 g were purchased from Laboratory Animal Research Center of Xinjiang Medical University and were fed under the standard conditions at 22 ± 2°C, humidity 60 ± 10% and under 12 h light/dark cycle at Xinjiang Medical University. All the procedures for animals were in line with the guidelines for Care and Use of Laboratory Animals approved by the National Institutes of Health (United States of America, USA) in 1996, all the experiments were performed to minimize animal suffering. For the AOM models, the mice were injected with high-dose LPS (1.0 mg/ml) (Sigma, USA) in the middle ear via tympanic membrane in right ear for 24 h based on the procedures provided by previous work. To establish CSOM mice models, low-dose LPS was injected into mouse ears in a step-wise manner. Specifically, the mice were sequentially injected with
0.1 mg/ml LPS for 24 h, 0.3 mg/ml LPS for 24 h and 0.5 mg/ml LPS for 24 h. The middle-ear mucosae tissues were collected and pro-inflammatory cytokines were examined to validate that we had successfully established CSOM mice models.

**Vector transfection**

The overexpression vectors for Nrf2 (OE-Nrf2) and TLR4 (OE-TLR4) were designed and synthesized by Sangon Biotech (Shanghai, China), and the small interfering RNAs (siRNAs) for Nrf2 (KD-Nrf2) and TLR4 (KD-TLR4) were obtained from RiboBio (Guangzhou, China). The above vectors were transfected into the mice middle-ear mucosae tissues by using the commercial Invivofectamine™ 3.0 reagent (ThermoFisher Scientific, USA) according to the protocol provided by the manufacturer. The transfection efficiency was evaluated by using the following real-time quantitative PCR (qPCR) analysis.

**Real-time qPCR**

The total RNA was extracted from human and mice middle-ear mucosae tissues by using the commercial TRIzol reagent purchased from Invitrogen (USA) based on the protocol provided by the manufacturer. A Taqman Reverse Transcription reagent (Applied Biosystems, CA, USA) was bought to transcribe the total RNA into cDNA. The primers for Nrf2, TLR2, TLR4, TLR5, TLR9, β-actin, TNF-α, IL-1β, IFN-γ and IL-6 were designed and synthesized by Sangon Biotech (Shanghai, China), the detailed information is listed in Table 1. After that, a PCR reaction reagent containing SYBR Green Supermix (Applied Biosystems, CA, USA) was employed to examine the expression levels of the above genes, which were all normalized to β-actin. After that, the Applied Biosystems 7300 Real-Time PCR system (ThermoFisher Scientific, USA) was used to quantify gene expressions.

**Western blot**

Total proteins were extracted and purified by using the commercial RIPA lysis buffer purchased from Beyotime Biotechnology (Shanghai, China). Next, a Bicinchoninic Acid Kit (Sigma, USA) was employed to determine total protein concentrations. The Western blot analysis was conducted according to the procedures provided by previous work. The primary Abs against Nrf2 (1:1000, Abcam, UK), β-actin (1:2000, Abcam, USA), TLR2 (1:1000, Abcam, UK), TLR4 (1:1000, Abcam, UK), TLR5 (1:1000, Abcam, UK), TLR9 (1:1500, Abcam, United Kingdom (UK)), Cyclin D1 (1:1000, Abcam, UK), CDK2 (1:1000, Abcam, UK), cleaved Caspase-3 (1:1500, Abcam, UK) and Bax (1:1500, Abcam, UK). The HRP-conjugated secondary Ab (1:2000) was also purchased from Abcam (UK). Finally, an ECL Western blot detection kit (GE Healthcare Bio-Sciences, USA) was used to visualize the protein bands, which were next quantified by detecting the grey values through using Image J software.

**ELISA**

The protein levels for the pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ and IL-6) were determined using their corresponding ELISA kit purchased from Cell Signaling Technology (USA) according to the manufacturer’s protocol. In brief, the mucosae tissues collected from human and mice were homogenized by using PBS buffer containing 0.1% Tween 20.

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**Table 1. The primer sequences for real-time quantitative PCR.**

| Genes   | Sequences                                      |
|---------|------------------------------------------------|
| Nrf2    | Forward: 5'-TGGAATCGCCATCTGCGAAAC-3'          |
|         | Reverse: 5'-AGATCTCGAGTCATCCACATCA C-3'       |
| TLR2    | Forward: 5'-CCGTAGATGAAACATGTCAGCTACCC    |
|         | Reverse: 5'-CCTCCGAGAGTCCCAACAGTGAAGATG TAC-3' |
| TLR4    | Forward: 5'-GAGGACCGCAGCAAAATGATG TAC-3' |
|         | Reverse: 5'-GAACGAATGGAGTGCAACACAC C-3'       |
| TLR5    | Forward: 5'-TGCTCAACACCTGGATGCTAC    |
|         | Reverse: 5'-ACAGCAGGCTTAGTGGAGATG TAC-3' |
| TLR9    | Forward: 5'-ACCTTCCATCACCTGACCCACTC    |
|         | Reverse: 5'-GCCTGCTAGTCAAGAACCTC C-3'       |
| β-Actin | Forward: 5'-GCTCCTCTGGAGCAAG-3'              |
|         | Reverse: 5'-CATCGCTGGAGGTAAGCAAC-3'          |
| TNF-α   | Forward: 5'-CCAGGACCATCAGTGCACCTCTGC-3'      |
|         | Reverse: 5'-GTTATCTCTCAGCTCCAGCC-3'          |
| IL-1β   | Forward: 5'-CGACAGCACAGCAAAACGAC GC-3'      |
|         | Reverse: 5'-TGCTCCTGCCAGGTTTGCCAC G-3'       |
| IFN-γ   | Forward: 5'-GTATTGCCAGCCAGCAAGTGTCATT G-3'   |
|         | Reverse: 5'-ACCATCCTTTTGGCAGTTCTCCA G-3'     |
| IL-6    | Forward: 5'-TTCCCTCTGAAAGACTCCTCC ATC-3'     |
|         | Reverse: 5'-GCCTCCAGCTGTGAAGTGTA TAG-3'      |

Nrf2: Nuclear erythroid 2-related factor 2.
After that, centrifugation was conducted at 13,000 g for 5 min and the supernatants were collected to analyse the expression levels of TNF-α, IL-1β, IFN-γ and IL-6.

Statistical analysis

The data involved in this study were collected and represented as means ± SD, and analysed using the SPSS 18.0 and GraphPad Prism 9 software. The comparisons between two groups were analysed by the unpaired Student’s t-test, and one-way ANOVA was used for multiple group comparisons. The Pearson correlation analysis was conducted to analyse the correlations of mRNA levels of Nrf2 and TLR4 in clinical specimens. Each experiment repeated at least three times. A $P$ value < 0.05 was regarded as statistically significant.

Results

Aberrant expressions of Nrf2 and TLRs in CSOM clinical samples

Previous data indicated that Nrf2 was involved in LPS-induced OM progression, and aberrant expressions of TLRs in middle-ear mucosa were closely associated with susceptibility of CSOM. Hence, we first investigated the expression status of Nrf2 and TLRs (TLR2, TLR4, TLR5 and TLR9) in the middle-ear mucosa tissues collected from patients with CSOM, COM and non-OM, respectively. As expected, Nrf2 was highly expressed in the CSOM group compared to the COM and non-OM groups ($P < 0.05$, Figure 1a, b), suggesting that Nrf2 was associated with CSOM progression. In addition, by screening the TLRs in the above clinical tissues, we found that merely TLR4, instead of other TLRs (TLR2, TLR5 and TLR9), was significantly down-regulated in the middle-ear mucosa tissues collected from patients with CSOM, COM and non-OM, respectively. This result suggested that TLR4 might contribute to CSOM pathogenesis.

Patients with CSOM suffered from chronic inflammation

Because chronic inflammation was pivotal for the transition of COM to CSOM, we next investigated whether the pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ and IL-6) could be examined in patients with CSOM. The real-time qPCR results showed that the mRNA levels of TNF-α, IL-1β, IFN-γ and IL-6 in CSOM tissues were significantly higher than in COM and non-OM groups ($P < 0.05$, Figure 2a-d). Consistently, the ELISA assay results validated the above findings, and showed the protein levels of the above pro-inflammatory cytokines were all up-regulated in CSOM groups, instead of COM or non-OM groups ($P < 0.05$, Figure 2e-h), suggesting that chronic inflammation existed in the middle-ear mucosa tissues of patients with CSOM.

Establishment of mice models for AOM and CSOM

The mice models for acute OM (AOM) were induced by high-dose LPS (1 mg/ml, 24 h) according to the previous study and the CSOM mice models were established by low-dose LPS in a step-wise manner. The results showed the expression levels of pro-proliferation proteins (cyclin D1 and CDK2) were down-regulated ($P < 0.05$, Figure 4a, b), whereas pro-apoptosis proteins (cleaved caspase-3 and Bax) were up-regulated by LPS treatment in mice ear tissues ($P < 0.05$, Figure 4c, d), indicating that LPS successfully induced mice middle-ear mucosa injury in AOM and CSOM mice. In addition, the inflammatory cytokines (TNF-α, IL-1β, IFN-γ and IL-6) were examined to evaluate chronic inflammation in mice models with AOM and CSOM. The results showed that the above cytokines sustained in high levels in mice middle-ear mucosa tissues after continuous low-dose LPS stimulation in CSOM mice, instead of AOM mice ($P < 0.05$, Figure 3a, b, c, d), indicating that chronic inflammation existed in the middle-ear mucosa tissues of CSOM mice.

The effects of LPS stimulation on Nrf2 and TLR4 expression in mice middle-ear mucosa tissues

We next investigated whether LPS stimulation influenced the expression status of Nrf2 and TLR4 in AOM and CSOM mice. The results showed that high-dose LPS had little effect on the expression levels of Nrf2 ($P > 0.05$, Figure 4a, b) and TLR4 ($P > 0.05$, Figure 4c, d) in AOM mouse tissues. Besides, continuous low-dose LPS stimulation increased Nrf2 ($P < 0.05$, Figure 4a, b), whereas decreased TLR4 ($P < 0.05$, Figure 4c, d) expression levels in CSOM mice. In addition, a previous publication indicated that TLR4 could be negatively regulated by Nrf2, and we identified that Nrf2 was negatively correlated with TLR4 in clinical CSOM tissues ($P < 0.05$, Figure 4e). Besides, by co-injecting the vectors for Nrf2 down-regulation (Figure 4f) and TLR4 overexpression (Figure 4g) with low-dose LPS into mice ear tissues, we found that knock-down of Nrf2 reversed the inhibiting effects of low-dose LPS on TLR4 in CSOM mice models ($P < 0.05$, Figure 4h-j), whereas TLR4 overexpression did not influence Nrf2 expression ($P < 0.05$, Figure 4j, k).
Figure 1. The expression status of Nrf2 and TLRs (TLR2, TLR4, TLR5 and TLR9) was examined in the human middle-ear mucosae tissues collected from 30 patients with CSOM, 25 patients with COM and 18 patients without OM (non-OM). (a) Nrf2 mRNA was up-regulated in CSOM tissues, but not in non-OM and COM, as detected by real-time qPCR. (b) Up-regulated Nrf2 protein levels were observed in CSOM tissues, as determined by Western blot. Real-time qPCR was conducted to screen the mRNA levels of (c) TLR2, (d) TLR4, (e) TLR5 and (f) TLR9 in clinical tissues collected from patients with non-OM, COM and CSOM, respectively. Western blot was performed to validate the protein levels of (g) TLR2, (h) TLR4, (i) TLR5 and (j) TLR9 in the involved clinical tissues. Each experiment had at least three repetitions. **p < 0.01, NS indicates no statistical significance.
Nrf2/TLR4 pathway participated in the regulation of LPS-induced chronic inflammation in mice models

Based on the above work, the pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ and IL-6) were then measured to evaluate whether the Nrf2/TLR4 pathway was involved in the regulation of LPS-induced CSOM in mice. As expected, both down-regulated Nrf2 and up-regulated TLR4 decreased the expression levels of the above cytokines in the middle-ear mucosae tissues of CSOM mice ($P < 0.05$, Figure 5a, b), indicating that targeting the Nrf2/TLR4 pathway attenuated chronic inflammation in CSOM. In addition, Nrf2/TLR4 pathway also regulated the transition from AOM to CSOM. Functionally, the vectors for Nrf2 overexpression ($P < 0.05$, Figure 5c) and TLR4 silence ($P < 0.05$, Figure 5d) were successfully transfected into AOM mice stimulated with high-dose LPS, and the results showed that both Nrf2 overexpression and TLR4 ablation sustained pro-inflammatory cytokines in high levels after high-dose LPS treatment ($P < 0.05$, Figure 5e, f), indicating the Nrf2/TLR4 pathway was crucial for AOM-CSOM transition through sustaining chronic inflammation.

Discussion

In some cases, bacterial infections caused AOM developed into COM$^{6,7}$ and even CSOM,$^{8,9}$ which was characterized by persistent otorrhea and even accompanied by intracranial infection,$^{10}$ and seriously degraded the life quality of human beings.$^{8,9}$ However, the underlying mechanisms for AOM-CSOM transition are still largely unknown. Recent data agreed that chronic inflammation pressure induced by bacterial infections caused physiological and pathological changes in human AOM middle-ear mucosae,$^8$ which contributed to AOM-CSOM transition. By analysing the expression status of pro-inflammatory cytokines in the middle-ear mucosae tissues collected from patients with CSOM, COM and non-OM, we found the above cytokines were up-regulated in CSOM tissues, instead of COM and non-COM groups. In addition, different concentrations of LPS were utilized to induce AOM and CSOM mice models according to a previous publication.$^{19}$ As expected, we found that LPS induced mice middle-ear mucosae injury and sustained chronic inflammation was observed in CSOM mice models, instead of normal and AOM mice. The above
information indicated that CSOM was characterized by chronic inflammation.

TLRs are a family of PRRs, including TLRs 1–9,13,14 which recognize various PAMPs such as bacterial LPS, and viral nucleic acids,13,14 which are crucial for regulating the innate immune response. Previous data indicated that TLRs sustained chronic inflammation during bacterial infection,17,18 and compromised TLRs were recently identified to be pivotal for CSOM progression by promoting chronic inflammation.15,16 Based on the above information, we screened TLR2, TLR4, TLR5 and TLR9 expressions in the middle-ear mucosae tissues of patients with CSOM, COM and non-OM and found that merely TLR4, instead of other TLRs, was significantly down-regulated in CSOM tissues compared to the non-OM and COM groups, which indicated that compromised TLR4 was associated with CSOM development, in line with previous work.15,16 The gain- and loss-function experiments were conducted to validate the biological functions of TLR4 in regulating CSOM progression, and the results showed that TLR4 overexpression abrogated chronic inflammation in CSOM mice, whereas knock-down of TLR4 promoted AOM-CSOM transition, suggesting that down-regulated TLR4 contributed to CSOM pathogenesis.

Nrf2 is involved in the regulation of oxidative stress22–24 and immune response during bacterial infections.25,26 A recent study also identified that aberrant Nrf2 expressions are involved in the regulation of
Figure 4. TLR4 was negatively regulated by Nrf2 in CSOM mice. (a, b) Higher levels of Nrf2 and (c, d) lower levels of TLR4 were observed in CSOM mice, determined by real-time qPCR and Western blot, respectively. (e) Pearson correlation analysis elucidated that the mRNA levels of Nrf2 and TLR4 showed negative correlations in CSOM clinical tissues. (f) Nrf2 was successfully silenced and (g) TLR4 was up-regulated in CSOM mice tissues. Real-time qPCR and Western blot showed that (h, i) knock-down of Nrf2 promoted TLR2 expressions, while (j, k) overexpression of TLR4 had little effects on Nrf2 levels in CSOM mice. Each experiment had at least three repetitions. **P < 0.01, NS indicates no statistical significance.
LPS-induced OM progression, which rendered the possibility that Nrf2 might participate in sustaining chronic inflammation in CSOM. According to the above information, we found that Nrf2 was upregulated in CSOM tissues, but not in non-OM and COM tissues. Consistently, continuous low-dose LPS stimulation increased Nrf2 expression levels in CSOM mice, but not in normal and AOM mice, suggesting that Nrf2 was relevant to CSOM progression. Further functional experiments validated that knockdown of Nrf2 attenuated chronic inflammation in CSOM mice, and Nrf2 overexpression increased the expression levels of pro-inflammatory cytokines in AOM mice, implying that Nrf2 contributed to the transition from AOM to CSOM. Interestingly, previous data suggested that there was a crosstalk between Nrf2 and NLR4 in regulating cell functions, which was validated in this study. Specifically, we found that Nrf2 negatively regulated NLR4 expression in CSOM mice, whereas altered NLR4 had little impact on Nrf2 levels. Additionally, levels of Nrf2 and NLR4 showed negative correlations in CSOM clinical tissues, suggesting that TLR4 could be targeted by Nrf2 during CSOM progression, which was in accordance with previous studies.

Collectively, we identified that knock-down of Nrf2 attenuated CSOM progression by reversing chronic inflammation through up-regulating TLR4.
This study uncovered the underlying mechanisms of AOM-CSOM transition and potentiated the Nrf2/TLR4 pathway as a potential biomarker for CSOM.

**Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Ethics approval**

All the clinical experiments involved in this study were approved by the ethics committee of the First Affiliated Hospital of Xinjiang Medical University. Written informed consent was obtained from all participants. In addition, all the procedures for animals were in line with the guidelines for Care and Use of Laboratory Animals approved by the First Affiliated Hospital of Xinjiang Medical University.

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