ORIGINAL ARTICLE

# TLR4 pathway is hypersensitive to lipopolysaccharide in patients with chronic suppurative otitis media

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**Abstract**

*Background:* Chronic Suppurative Otitis Media (CSOM) is an uncontrolled inflammation in the middle ear due to bacterial infections and has been linked to the overactivation of the Toll-Like Receptor 4 (TLR4) pathway. The pathway is activated by Lipopolysaccharide (LPS) released from the gram-negative bacterial cell wall. Currently, there is limited information about the factors responsible for overactivation. One possible factor could be the hypersensitivity of the TLR4 pathway in CSOM patients. *Aim and Objectives:* To evaluate the effect of LPS on the expression of key markers of the TLR4 pathway viz.,TLR4, Nuclear Factor kappa B (NFkB), and Tumor Necrosis Factor α (TNFα) in CSOM. *Material and Methods:* A case-control study was carried out in patients with CSOM and healthy participants (n = 63). Peripheral blood mononuclear cells from the participants were cultured for 4h in the presence of LPS. *TLR4* and *NFkB* genes expression was measured in the cell pellet by using qPCR. TNFα cytokine levels were measured in the conditioned media by using ELISA. Fold change expression of genes between LPS-treated and untreated samples was calculated and compared using statistical methods. *Results:* LPS-induced fold change in the expression of *TLR4* (2.8 vs. 1.6; $p < 0.001$) and *NFkB* genes (3.8 vs. 1.4; $p < 0.001$) were higher in the CSOM group compared to the control group. Furthermore, LPS-induced fold change in TNFα production was higher in the CSOM group compared to the control group (3.2 vs. 1.1; $p < 0.001$). *Conclusion:* Overall results indicate that the LPS treatment resulted in comparatively higher expression of the selected genes, indicating hypersensitivity of the TLR4 pathway in CSOM patients.

**Keywords:** Chronic Suppurative Otitis Media, Lipopolysaccharide, Toll-Like Receptor 4, Tumor Necrosis Factor, Nuclear factor kappa B.

## Introduction

Chronic Suppurative Otitis Media (CSOM) is an inflammatory condition of the middle ear characterized by frequent ear discharges through the tympanic perforation [1]. According to the World Health Organization (WHO), The highest prevalence of CSOM was observed in Western Pacific countries 2.5- 43%, followed by 0.9 -7.8% in South East Asia, 0.4 - 4.2% in Africa South, 3% in Central America, 1.4% in the Eastern Mediterranean and 0.4% in Europe [2]. A recent study from India also showed the prevalence of CSOM as 5.2% [3]. CSOM may lead to hearing loss, facial nerve paralysis, meningitis, and brain abscess. CSOM is the major cause of hearing impairment among

children in developing countries. In children, hearing loss can eventually result in delayed speech and learning difficulties [4]. Due to antibiotic resistance and ongoing inflammation, there is currently no viable treatment for CSOM. Additionally, some patients who underwent surgery for the condition experienced postoperative problems like hearing loss and advanced disease presentation [5]. Therefore, a better understanding of the pathophysiological changes and the effective management of CSOM is necessary to uncover the novel drug targets.

CSOM involves inflammatory damage to the middle ear such as mucosal ulceration, breakdown of the epithelial lining, and granuloma formation [6]. Middle ear inflammation arises as a part of the innate immune response to bacterial infection. Common bacterial species seen in the middle ear effusion of CSOM patients are *Proteus mirabilis, Escherichia coli, Klebsiella,* and *Pseudomonas aeruginosa* [7-8]. Bacterial infection initially causes acute otitis media which when unresolved progresses into CSOM. However, excessive activation of the innate immune response, particularly *via* the Toll-like receptor 4 (TLR4) pathway appears to be responsible for the chronic inflammation in CSOM. This view is supported by the observation of elevated markers of this pathway [9-10].

Middle ear infection results in the activation of inflammation to clear the infectious agents. This results in a condition called acute otitis media which subsides on its own. However, in some patients, the acute otitis media does not resolve after the clearance of infectious agents and becomes chronic. Chronic inflammation then results in damage to the middle ear tissue [11]. Currently, the factors responsible for the progression of acute otitis media to CSOM are not clear.

Bacterial infection activates inflammation through specialized pathways connected to pattern recognition receptors. These receptors identify unique molecules released from the infectious agents and trigger intracellular signal transduction pathways. TLR4 is an important pattern recognition receptor that activates inflammation via the Nuclear Factor kappa B (NF-kB) pathway upon sensing Lipopolysaccharide (LPS). The molecule is released from the cell wall of Gram negative bacteria. Activation of the TLR4 pathway has been observed in the middle ear mucosa of CSOM patients [12]. Our hypothesis was that the TLR4 pathway is intrinsically hypersensitive to LPS in CSOM patients. As a result, exposure to the cognate bacterial ligand viz., LPS, results in disproportionate overactivation of the inflammatory response. The aim of this study was to test this hypothesis. To do this, Peripheral Blood Mononuclear Cells (PBMC) from CSOM patients and healthy controls were activated with LPS, and the expression of the selected markers of the TLR4 pathway was compared. Blood cells were used as a surrogate for testing the hypothesis since intrinsic predisposition to overactivation is the object of measure.

## Material and Methods

### Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional ethics committee and with the 1964 Helsinki declaration and its later amendments. The study protocol was approved by the institutional ethics committee vide letter number SDUAHER/KLR/CEC/01/2017-18 dated 6 October 2017.

**Informed consent:** Informed consent was obtained in writing from all the individual participants included in the study.

**Participant selection and study design**

A case-control investigation was established by including cases (63 CSOM patients) and controls (63 healthy subjects) who were gender and age-matched. The study was approved by the Central Ethics Committee (CEC) with reference No. (SDUAHER/KLR/CEC/01/2017-18). For patients attending the Dept. of Otorhinolaryngology, R L Jalappa Hospital and Research Centre, Tamaka, Kolar, Karnataka, India, written informed consent was obtained before participant recruitment for the study. CSOM patients diagnosed with: (i) hard of hearing (ii) chronic ear infection, and (iii) perforation involving the tympanic membrane were selected [13]. Criteria for inclusion in CSOM patients were (i) patients diagnosed with CSOM and (ii) both genders (male:female) with age ranges between 18-60 years. The exclusion criteria were (i) post-traumatic CSOM, (ii) otomycosis, (iii) Down's syndrome (iv) cleft lip/palate, and (v) acute otitis media without effusion. Inclusion criteria for control participants were (i) Age- and gender-matched individuals and (ii) without a history of any chronic infections. Exclusion criteria for controls were individuals with a known genetic disorder.

**Isolation of PBMCs**

PBMCs were isolated from 2 ml of peripheral blood samples collected in a heparinized vacutainer (Beckton Dickinson, USA) from all the study subjects using Ficoll-Histopaque (Himedia, India) [14]. Briefly, 2 ml of anticoagulated blood was layered on an equal volume of Ficoll-histopaque and centrifuged at 3000 rpm for 30 min. After centrifugation, four layers formed with different cell types. The top layer contained plasma, which was discarded by pipetting. The second layer contained PBMCs and was white. A 15 ml Falcon tube was used to collect the PBMC layer, which was then twice cleaned with 10 ml of 1X Phosphate-Buffered Saline (PBS).

**LPS treatment**

*In vitro* culture was set up in a 15 ml falcon tube by mixing the following components: PBMCs cultured using 5 ml of working RPMI 1640 medium (Gibco, USA), Fetal bovine serum (20% final; Invitrogen, USA), antibiotics solution (1% final; Himedia, India) and LPS (10 ng/ml; Sigma, USA). For every sample, two culture tubes were prepared. To treat the first culture tube, LPS was applied. Phosphate-buffered saline was used to treat the second culture (vehicle control). The culture tubes were kept at $37^0$C in 5% $CO_2$ for 4 hours. After 4 hours of incubation, the culture tubes were centrifuged for 10 minutes at room temperature at 1500 rpm the supernatant (conditioned medium) was collected for cytokine profile assessment, and the pellet was collected for RNA isolation followed by cDNA and qRT-PCR and stored at -$80^0$C until further use [15].

**Gene expression quantification**

A quantitative reverse transcription polymerase chain reaction (qRT-PCR) (CFX96 touch system, Bio-Rad, USA) was used to measure the expression of the *TLR4* and *NFkB* genes. Trizol reagent (GeNei, Bangalore) was used to separate total RNA from PBMCs cultured pellet in accordance with the manufacturer's instructions [16].

A PerkinElmer UV-VIS (Lambda 35) spectrophotometer (USA) was used to determine the

concentration and purity of the total RNA. Until they were used, RNA samples were kept at -20$^0$C. Total RNA samples were transcribed to cDNA using the iScript cDNA conversion kit (Bio-Rad, USA) from high-quality RNA samples (260/280 ratio of >2). The SYBR green technique (Bio-Rad, USA) was used to quantify the *TLR4, NFkB*, and *GAPDH* genes in real-time.The following primers were used to quantify the expression of *TLR4, NFkB*, and *GAPDH* (Sigma, USA): *TLR4* gene*:* 5'- *GAACCTGGACCTGAGCTTTAAT* 3' and 5'-*GTCTGGATTTCACACCTGGATAA*-3', for the sequence NM_138554.5 and *NFkB* gene*:*5' -*5'-TACCGACAGACAACCTCACC-3'* and *3'-CAGCTTGTCTCGGGTTTCTG-5',* for the sequence NM_001077494.3 And For the sequence NM_001289745.3*,* the *GAPDH* gene was *5'-GATCATCAGCAATGCCTCCT-3' and 3'-GACTGTGGTCATGAGTCCTTC-5'*. The following components comprised the qRT-PCR reaction mixture: 5 µl of SYBR green, 2 µl of nuclease-free water, 0.5 µl of forward and reverse primers, and 2 µl of cDNA (1:10 dilution). Hold at 50$^0$C for 2 minutes, 40 cycles of hold at 95$^0$C for 10 minutes, denature at 95$^0$C for 15 seconds, and anneal/extension at 57.0$^0$C for 60 seconds were the reaction conditions utilized for qRT-PCR. The analytical program provided the Cycle Threshold (Ct) values at the conclusion of the reaction. GAPDH was used as the endogenous control for normalizing the expression of the TLR4 and NFkB genes, and by estimating 2-^ΔΔCT, where ΔCT = Ct (TLR4) - Ct (GAPDH) and ΔΔCT = ΔCt (treated) - ΔCt (untreated), the fold change in the expression of the *TLR4* and *NF-kB* genes was determined. For the statistical comparisons between treated and untreated samples within each study group, the ΔCt values were used [17].

**Cytokine estimation**

The Enzyme-Linked Immuno Sorbent Assay (ELISA) technique was used to measure the levels of TNFα cytokine in the conditioned medium. Kits that are commercially available were obtained from Krishgen Biosystem in India.

**Statistical analysis**

The data were processed using SPSS software (version 22.0, IBM, USA) and the diagrams were drawn using GraphPad software (GraphPad Software, USA). The Shapiro-Wilk test was performed to check the normal distribution for LPS treated and untreated cases and control data. For data with a normal distribution, a parametric test (t-test) was employed, while for data without a normal distribution, a non-parametric test (Mann-Whitney U) was utilized. Statistical significance was described as a *p*-value of less than 0.05.

**Results**

**Demographic details**

The demographic information of the study subjects is summarised in Table 1. The majority of CSOM patients presented with bilateral involvement (58.7%), central perforation (of the tympanic membrane) 69.9%, the duration of the disease was 5 years, and cholesteatoma was less commonly seen in the patients (15.9%).

**Table 1: Sociodemographic characteristics of patients**

| Parameter | CSOM (n = 63) | Control (n = 63) |
|---|---|---|
| **Age (Years; Mean ± SD)** | 38.0 ± 13.8 | 38.1 ± 13.7 |
| **Gender** | | |
| **Male** | 28 (44.5%) | 28 (44.5%) |
| **Female** | 35 (55.5%) | 35 (55.5%) |
| **Ear affected** | | |
| **Unilateral** | 26 (41.3 %) | NA |
| **Bilateral** | 37 (58.7 %) | |
| **Duration of disease** | | |
| **⩽ 1 year** | 10 (15.9%) | NA |
| **> 1 to ⩽ 5 years** | 37 (58.7%) | |
| **> 5 to ⩽ 10 years** | 16 (25.4%) | |
| **Type TM perforation** | | |
| **Central** | 44 (69.9%) | NA |
| **Anterior** | 07 (11.1%) | |
| **Posterior** | 12 (19.0%) | |
| **Presence of cholesteatoma** | 10 (15.9%) | NA |
| **Presenting symptoms** | | |
| **Ear discharge** | 55 (87.3%) | NA |
| **Hearing loss** | 56 (88.8%) | |
| **Earache** | 59 (93.6%) | |

*SD = Standard deviation, NA = Not applicable, TM = Tympanic membrane, CSOM = Chronic Supportive Otitis Media*

**Effect of LPS on *TLR4, NFkB,* and TNFα expressions**

The normalized expression (ΔCt) of the *TLR4*, and *NFkB* gene except for TNF*α* cytokine showed normal distribution in both CSOM and control groups. So Mean ± Standard Deviation (SD) was calculated in the case of *TLR4*, *NFkB*, while the median (IQR) was calculated in case of TNF*α*. In the CSOM group, the average ΔCt of the *TLR4* gene was 4.95 ± 2.14 for the LPS-treated group and 6.45 ± 2.02 for the Untreated group, while for the *NFkB* gene, it was 2.23 ± 2.84 in the LPS treated group and 4.17 ± 2.54 for the in the untreated groups. The average LPS-induced fold change (log $2^{-\Delta\Delta Ct}$) in the *TLR4* gene expression was 2.8 times and the *NFkB* gene was 3.8 times.In the control group, the average ΔCt of the *TLR4* gene was 4.06 ± 2.07 for the LPS-treated group and 4.75 ± 1.95 for the untreated group, while for the *NFkB* gene, it was 1.54 ± 2.63 for the LPS-treated group and 2.02 ± 2.49 for the untreated group. The higher normalized expression of the *TLR4* and *NFkB* genes observed in the LPS-treated samples was found to be statistically significant ($p < 0.001$; t-test). The average LPS-induced fold change in the *TLR4* gene expression was 1.6 times, and for NFkB gene expression, it was 1.4 times. LPS-induced fold change of the *TLR4,* and *NFkB* genes in CSOM and control groups was compared by statistical methods. The difference between the groups was found to be significant ($p < 0.001$; t-test). The results are depicted in Figures 1 (A-C) and 2 (A-C). The average TNFα levels of CSOM were 466.0 (137.2-778.8) pg/ml in the LPS treated and 142.3 (97.1-313.1) pg/ml in the untreated group. Average LPS-induced fold change in the TNFα expression levels was 3.2 times. In the control group, the average TNFα expression levels were 243.9 (149.1-371.9) pg/ml in the LPS treated and 140.3 (82.3-271.1) pg/mlin the untreated group. The higher expression levels of TNFα observed in the LPS-treated samples were found to be statistically significant ($p < 0.001$; Mann-Whitney test). Average LPS-induced fold change in the TNFα expression levels was 1.1 times. The LPS-induced fold change of the TNFα expression levels in the CSOM and control groups was compared by statistical methods. A significant difference was observed between the groups ($p < 0.001$; Mann-Whitney test). The results are depicted in Figures 3 (A-C). Furthermore, the *TLR4, NFkB,* and TNFα expressions were also analyzed after subgrouping the CSOM patients based on clinical profiles. However, there was no difference in the LPS-induced fold change between CSOM with bilateral and unilateral involvement, the duration of disease between less than 4 years and above 4 years, and no differences between males and females.

**Correlation analysis**

The relationship between the three markers in the CSOM group was evaluated by using appropriate correlation tests. There was a positive correlation between TLR4 and Nf-kB levels ($r = 0.77$, $p = 0.01$, Pearson's correlation). Furthermore, a positive correlation between Nf-kB and TNFα was observed after removing outliers (final $n = 50$) in the data ($r = 0.37$, $p = 0.01$, spearman's rho correlation).

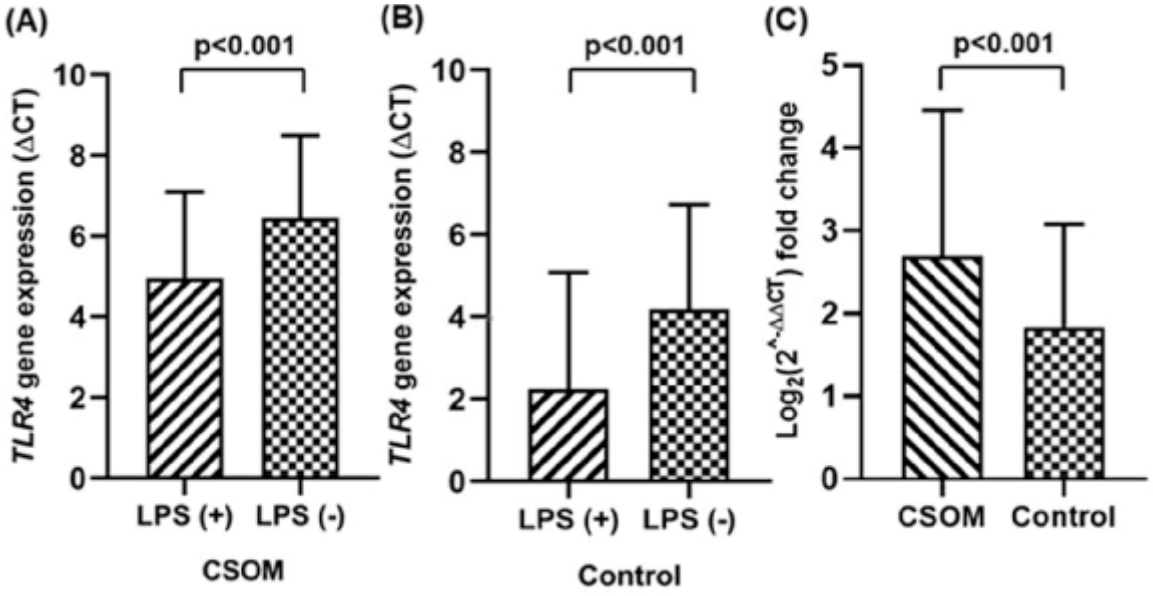


**Figure 1: Effect of LPS on *TLR4* gene expression in the study groups: (A)** Normalized gene expression in the CSOM group, **(B)** Normalized gene expression in the control group, and **(C)** Comparison of LPS-treated fold changes in *TLR4* gene expression between the study groups ($n = 63$ per group).

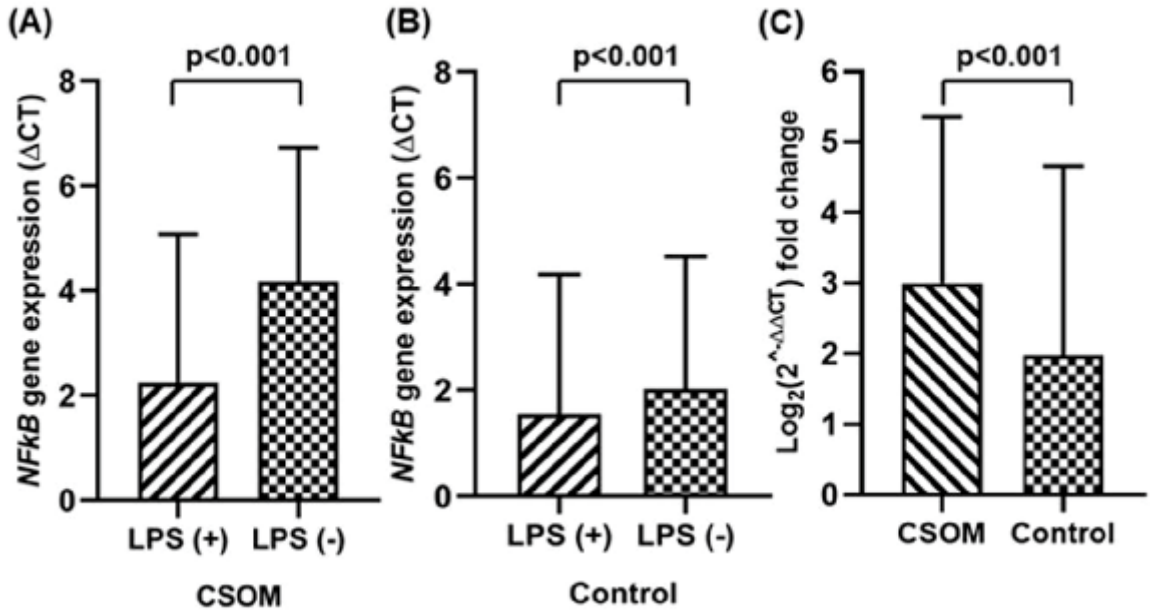


**Figure 2: Effect of LPS on *NF-κB* gene expression in the study groups: (A)** Normalized gene expression in the CSOM group, **(B)** Normalized gene expression in the control group, and **(C)** Comparison of LPS-treated fold changes in *NF-κB* gene expression between the study groups ($n = 63$ per group).

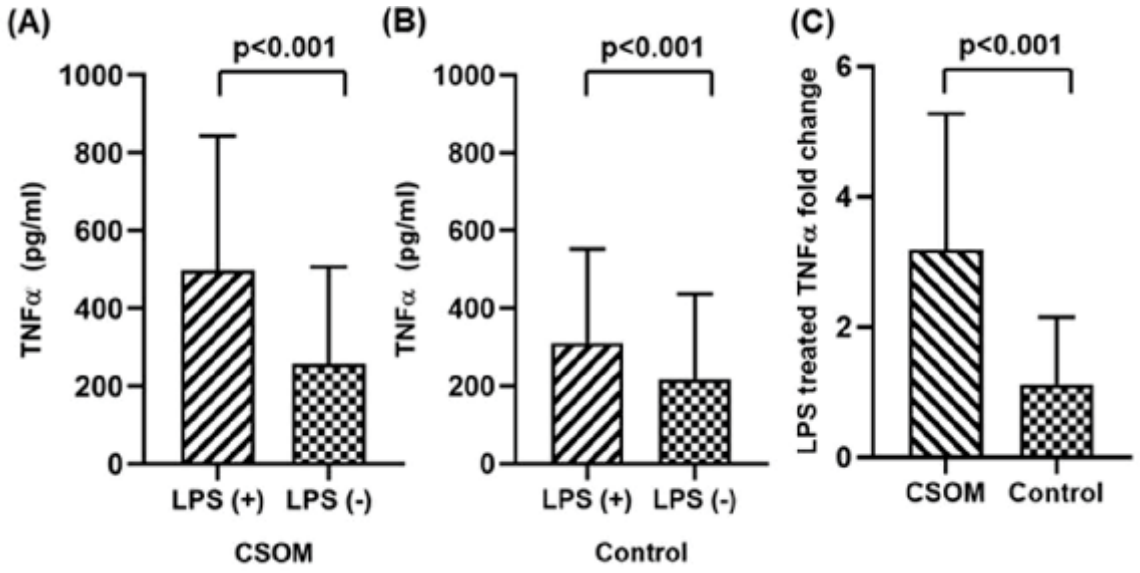


**Figure 3: Effect of LPS on TNFα secretion in the study groups: (A)** TNFα levels in the treated and untreated conditioned media in the CSOM group, **(B)** TNFα levels in the treated and untreated conditioned media in the control group, and **(C)** Comparison of LPS-treated TNFα fold change between CSOM and control groups (n = 63 per group).

## Discussion

The purpose of this study was to examine the responsiveness of the TLR4 pathway to LPS in CSOM patients. TLR4 pathway comprises three components viz., receptor, modulator, and effector. The receptor is specialized for recognizing bacterial ligands and relaying the signal to the nucleus through the cytoplasmic signal transduction pathway. This leads to the activation of Nf-kB, a master transcriptional regulator that modulates inflammatory gene expression. In this study, the key markers of all three components of the pathway viz., TLR4, NF-kB, and TNFα were elevated in response to LPS. Furthermore, the elevation was higher in the samples from CSOM than that of healthy controls. In addition, there was a positive correlation between levels of the markers in the CSOM group. These observations indicate that the TLR4 pathway may be hypersensitive to LPS in CSOM patients. Schematic representation of the study hypothesis is depicted in Figure 4.

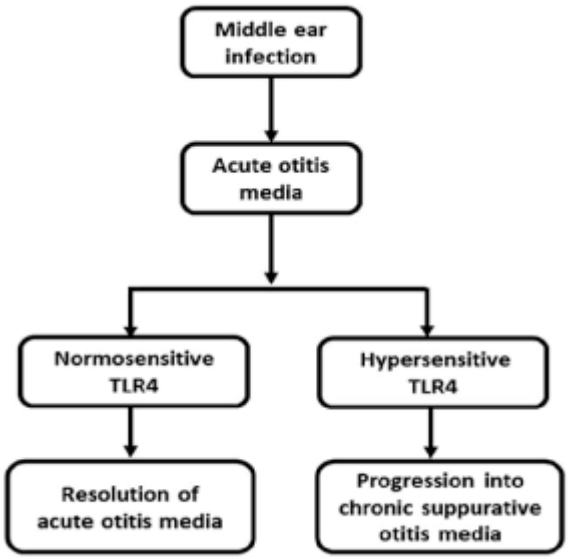


**Figure 4: Schematic representation of the study hypothesis**

TLR4 upregulation observed in this study is in accordance with the previous reports [18-21]. Si *et al.,* (2014) compared *TLR4* gene and protein expression in CSOM and health control by using qRT-PCR and immunohistochemistry respectively. Expression of the *TLR4* gene and protein expression was higher in the middle ear mucosa of CSOM when compared to normal canal skin [18]. A similar elevation of TLR4 expression was observed by Hirai *et al.,* (2013) and also by Jotic *et al.,* (2015) [19, 20]. However, the upregulation of NFKB observed herein is contrary to the previous report. Jesic and co-workers (2014) analyzed TLR4 and NFKB expression in the middle ear mucosa by using immunohistochemistry. Expression of TLR4 but not of NFKB was found to be higher in the middle ear mucosa of CSOM when compared to normal canal skin. Increased TNFα production observed in this study is also in accordance with the previous reports [22-24]. Kuczkowski and co-workers (2011), by using Western blot, reported higher TNFα expression in the middle ear mucosa of the CSOM than controls [22]. Also, Baike and co-workers (2017) reported six-fold higher levels of TNFα in CSOM patients than in controls [23]. Also, a recent study by Edward and co-workers (2019), using qRT-PCR, showed higher TNFα expression levels in the middle ear tissue samples of CSOM patients than in normal canal skin [24].

The focus of the previous reports on the TLR4 pathway in CSOM was mainly on disease-induced changes [18-24]. Whereas, this study attempted to elucidate the intrinsic responsiveness of the TLR4 pathway to LPS in CSOM. This approach encouraged us to hypothesize that CSOM patients are intrinsically predisposed to produce a disproportionate response to LPS. The differential response may arise due to polymorphisms in the TLR4 pathway genes. This view is corroborated by a previous report of a positive association between CSOM and polymorphisms in the *TLR4* gene [25].

Compared to previous studies with smaller sample sizes, *TLR4* gene expression was exclusively measured directly from patients' blood samples [26]. In contrast, the current study employed a more comprehensive approach by assessing *TLR4* gene expression (receptor) in both LPS-induced and uninduced in vitro cultures. Importantly, the sample size in each group was increased, providing a more robust dataset for analysis.

Furthermore, in comparison to earlier research, the present study extended its focus beyond *TLR4* gene expression. Specifically, it included an evaluation of *NFkB* (Modulator in the TLR4 pathway) gene expression and TNFα (effector of the TLR4 pathway) expression levels. This expanded scope allowed for a more thorough investigation into the molecular mechanisms involved, offering a deeper understanding of the regulatory pathways associated with the studied conditions.

The results of this study hold translational promise for therapeutic development. Antagonists of the TLR4 pathway may be explored for the amelioration of CSOM. Several anti-TLR4 molecules are currently under clinical trial for various clinical conditions [27]. Successful molecules may be repurposed and evaluated for the management of CSOM.

However, this study has limitations such as a need for further investigation into specific polymorphisms in the TLR4 pathway genes that may contribute to the differential response to LPS in CSOM patients

## Conclusion

The results suggest that the *TLR4* and *NFkB* genes expression were upregulated and the production of TNFα cytokine was higher in the blood lymphocytes of CSOM patients treated with LPS. Overall, these results conclude that the TLR4 pathway may be hyperactive in CSOM. This new understanding of the CSOM pathomechanism may open new possibilities for developing appropriate drugs.

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