

Lactobacillus Protects Against Chronic Suppurative Otitis Media via Modulating RFTN1/ Lipid Raft /TLR4-Mediated Inflammation

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Purpose: Chronic suppurative otitis media (CSOM) is a prominent contributor to preventable hearing loss globally. Probiotic therapy has attracted research interest in human infectious and inflammatory disease. As the most prevalent probiotic, the role of *Lactobacillus* in CSOM remains poorly defined. This study aimed to investigate the antipathogenic effects and underlying mechanism of *Lactobacillus* on CSOM.

Methods: RNA sequencing of granulation of middle ear cavity from CSOM patients and lavage fluid of middle ear from normal volunteer was conducted. Human middle ear epithelial cells (HMEEC) and rats infected with *Bacillus cereus* (*B. cereus*) and *Staphylococcus aureus* (*S. aureus*) were used for CSOM constructing. Western blot, qPCR and Vybrant™ Alexa Fluor™ 488 lipid raft labeling were performed to explore the possible molecular mechanism by which lipid raft linker (RFTN1) regulates lipid raft/toll-like receptor 4 (TLR4). ELISA and HE staining was utilized to evaluate the effect of *Lactobacillus* on the progression of CSOM in vivo.

Results: Based on RNA Sequence analysis, a total of 3646 differentially expressed genes (1620 up-regulated and 2026 down-regulated) were identified in CSOM. RFTN1 was highly expressed in CSOM. Inhibition of RFTN1 not only reduced the inflammatory response of CSOM but also suppressed the formation of lipid rafts. Further investigation revealed that RFTN1 inhibition could reduce the expression of TLR4, which also localizes to the lipid rafts. TLR4 responds to RFTN1-mediated inflammatory responses in CSOM. We treated the CSOM model with *Lactobacillus*, which has great potential for alleviating the inflammatory response, and found that *Lactobacillus* attenuated the development of CSOM by reducing RFTN1 and TLR4 expression.

Conclusion: In conclusion, these findings suggest a crucial role for *Lactobacillus* in alleviating CSOM progression and uncovered the molecular mechanism involving *Lactobacillus*-regulated inhibition of the RFTN1-lipid raft-TLR4 signaling pathway under CSOM conditions.

Keywords: CSOM, *Lactobacillus*, RFTN1, TLR4, *S. aureus*, *B. cereus*

Introduction

Chronic suppurative otitis media (CSOM) is a prominent contributor to preventable hearing loss globally. Once it sets in, patients present with suppurative lesions in the mucosa and submucosa of the middle ear, and in severe cases, may even involve the bone, resulting in tympanic membrane perforation and long-term purulent symptoms in the ear. Characterized by a prolonged disease course and persistence of chronic illness, CSOM exerts a severe impact on patients' daily lives and poses a threat to their health, consistently presenting a challenging issue for otolaryngologists.¹ CSOM is regarded as a multifactorial illness stemming from a series of complex interactions involving the environment, bacteria, hosts, and genetic risk factors. Bacterial infection is the primary cause of CSOM, with coagulase-negative *Staphylococcus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* (*S. aureus*), and *Proteus mirabilis* being the most prevalent pathogens

in CSOM.^{2,3} Thus, animals or cells inoculation with bacterial were used for CSOM model conduction.^{4,5} It is well established that *S. aureus* and *Bacillus cereus* (*B. cereus*) are critical foodborne pathogens in terms of frequency and seriousness of the disease. *S. aureus* is the most predominant pathogens that cause CSOM⁶ and *B. cereus* is a major cause of post-traumatic endophthalmitis⁷ and could be observed in calves with otitis media (OM).⁸ Currently, first-line treatment for uncomplicated CSOM, encompassing antibiotics and anti-inflammatory drugs, is the most effective approach, with minimal side effects.⁹ Nevertheless, the abuse of antibiotics in recent years, particularly their non-standard utilization, may give rise to certain variations in pathogenic bacteria and their susceptibility to antibiotics.³ Therefore, the discovery of novel therapies for CSOM is crucial.

Probiotics are defined as follows “live microorganisms that, when administered in adequate amounts, serve a health benefit on the host” according to the World Health Organization (WHO). Probiotics participate in regulating the microbial balance and ameliorate diseases stemming from microbial community imbalance, especially in the intestinal mucosa.¹⁰ For over a decade, probiotics have been successfully used to treat common infectious diseases of the digestive tract.¹¹ It is indicated that probiotics showed strong antibacterial activity against gram-positive bacterial including *S. aureus* and *B. cereus*.^{12,13} In studies on OM, Scott and Coleman discovered that probiotic therapy holds considerable promise for alleviating OM. They demonstrated its ability to inhibit pathogen growth in the ear canal in vitro, suggesting its potential to offer a more effective alternative to antibiotics in the future and warranting further exploration.^{14,15} *Lactobacillus* have become one of the commonly used probiotics.¹⁶ Accumulating evidence suggested that *Lactobacillus* displayed antibacterial activity against *S. aureus* and *B. cereus*.^{17,18} In addition, the protective role of *Lactobacillus* in CSOM has been characterized.¹⁹ However, the underlying mechanism by which *Lactobacillus* improve *S. aureus* and *B. cereus*-induced CSOM remains unknown.

At present, investigations into the pathogenic biology of OM not only focus on the disparity of pathogenic bacteria among distinct types of OM but also on the inflammatory mechanisms of bacteria, such as the different toxins produced by various pathogenic bacteria or the release of specific inflammatory factors. Lipid rafts are microstructural domains rich in cholesterol and sphingomyelin on the plasma membrane with a size of approximately 70 nm. They are dynamic structures localized on the outer leaflets of the plasma membrane. Lipid rafts function as platforms anchored to proteins and are closely related to messenger formation, synaptic transmission, transmembrane transport, membrane signal transduction, protein sorting, cell proliferation, and apoptosis. Lipid rafts have been reported to play a crucial role in regulating the anti-inflammatory signal of cells and are regarded as the preferred site for the assembly of multimolecular complexes that mediate endocytosis of activated toll-like receptor 4 (TLR4).²⁰

RFTN1 encodes a lipid raft linker that may play a key role in the formation and maintenance of lipid rafts.²¹ In addition, under the stimulation of bacterial lipopolysaccharide, RFTN1 mediates the internalization of TLR4 into endosomes in dendritic cells and macrophages, and poly (I: C) into TLR3-positive endosomes in myeloid dendritic cells and epithelial cells, leading to the activation of TICAM1-mediated signals and subsequent IFN β .^{22,23} It been implicated in T cell antigen receptor-mediated signal transduction by regulating tyrosine kinase LCK localization, T cell-dependent antibody production, and cytokine secretion. It may regulate signal transduction mediated by B cell antigen receptors.²¹ The concept of lipid rafts remains an emerging field of study and further research is required to elucidate its role in bacterial inflammation.

In this study, we sequenced the granulation of middle ear cavity from CSOM patients and lavage fluid of middle ear from normal volunteer and found that RFTN1 expression was enhanced compared to that in the normal group. Further mechanistic research using *S. aureus* and *B. cereus* to induce CSOM models demonstrated that RFTN1 promotes lipid raft formation and stability on the surface of middle ear epithelial cells, leading to TLR4 activation and an inflammatory response. Additionally, this mechanism was verified in a rat model of CSOM, and the probiotic *Lactobacillus* was shown to improve CSOM induced by *S. aureus* and *B. cereus*. In conclusion, our results suggest that *Lactobacillus* may inhibit inflammation in CSOM induced by *S. aureus* and *B. cereus* through the RFTN1-lipid raft-TLR4 pathway, providing novel insights into the pathogenesis of CSOM and probiotic treatments.

Materials and Methods

Ethics Statement

The clinical study was reviewed and approved by the ethics committee of The First Affiliated Hospital of Kunming Medical University (No. 2022-L-171). The study was conducted according to the standards set by the Declaration of Helsinki. All animal procedures were approved by the Institutional Animal Ethics Committee Kunming Medical University (No. KMMU2020225).

RNA Sequence

The clinical samples were part of the routine hospital laboratory procedure. RNA sequencing of granulation of middle ear cavity from CSOM patients (n=15) and lavage fluid of middle ear from normal volunteer (n=9) was conducted. Total RNA from was extracted with Trizol reagent (Invitrogen) and quantity was determined on Nanodrop (Thermo Fisher Scientific). The extracted total RNA samples were stored at a low temperature and sent to the Beijing Cnkingbio Biotechnology Corporation for the determination of total RNA concentration, integrity, purity, and total amount. After passing the test, the samples were used to construct the mRNA bank and sequenced.

Cell Culture

Human middle ear epithelial cells (HMEEC, HX-0344) was obtained from Shanghai Hexu Biotechnology Co., Ltd. Cells were cultured in a mixture of Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) containing 10% fetal bovine serum (Thermo Fisher Scientific), 100 u/mL penicillin (Thermo Fisher Scientific) and 100 µg/mL streptomycin (Thermo Fisher Scientific) and bronchial epithelial basal medium (BEBM)(1:1) containing a bag of BEGM Singlequots (Lonza Bioscience) per 500 mL of medium, the cells were cultured in 37 °C and 5% CO₂ cell incubators. Fresh medium was changed every 2–3 days, and the culture was fused to 80% or more, digested with 0.25% trypsin (Yeasen) and 0.05% EDTA, digested with complete medium, and centrifuged for 5 min (1000r/min). The cells were resuspended in fresh medium.

Cell Transfection

Scrambled siRNA of RFTN1 (siRFTN1) or siRNA control (siCtrl), RFTN1 overexpressed plasmid (oeRFTN1), and vector plasmid (oeNC) were purchased from GeneChem (Shanghai, China). Cells were transfected with siRNA or plasmids using Lipofectamine 3000 (Thermo Fisher Scientific).

Preparation of Bacterial

B. cereus, *S. aureus* and were *Lactobacillus* were cultured at 37°C on plates of Luria-Bertani broth (LB, Becton Dickinson (BD)) with 1% agar (w/v, Sigma). Overnight cultures and subcultures were routinely cultured in LB broth with shaking at 37°C (250 rpm). The strains were dissolved and diluted to the desired concentration of sterile saline.

Inflammatory Model Induced by Bacterial-Cell Co-Culture

The flow chamber was precoated with 2 mg/mL bovine collagen, and 1×10^5 HMEEC cells were inoculated with RPMI1640 medium containing 20% serum at 37 °C, 5% CO₂ incubator for 24 h. The bottom of the chamber was used for all experiments. *S. aureus* and *B. cereus* were inoculated at 2.5×10^7 CFU/mL in an outer dish and co-cultured for 2–3 day.

ELISA

The ELISA kit was employed to determine the levels of TNF- α , IL1- β , IL-6, IL-8, IL-10 in the supernatant of indicated HMEEC cells and lavage fluid of indicated rats.

Quantitative Polymerase Chain Reaction Assay (qPCR)

Total RNAs was isolated from the cell lines using Trizol reagent. The mRNA levels were measured using SYBR Premix Ex Taq (TaKaRa) on an ABI-7500 Fast system (Applied Biosystems, CA). Relative mRNA levels were normalized to those of GAPDH

using the $2^{-\Delta\Delta Ct}$ method. The sequences used were as follows: RFTN1, ATGGGTTGCGGATTGAACAAG/AGCGGTATTCATAGGACACATCT; FLOT1, GCCCTGCATCCAACAGATCC/AATGCCAGTGAAGTGAAGG, FL OT2, TTGCTGACTCTAAGCGAGCC/TCCACGGCAATCTGTTTCTTG; TNF- α , CCTCTCTCTCTAATCAGCCCTCTG/GAGGACCTGGGAGTAGATGAG; IL-1 β , ATGATGGCTTATTACAGTGGCAA/GTCGGAGATTTCGTAGCTGGA; IL-6, ACTCACCTCTTCAGAACGAATTG/CCATCTTTGGAAGGTTTCAGGTTG; IL-8, TTTTGCCAAGGAGTGCTAAAGA/AACCCTCTGCACCCAGTTTTTC; IL-10, GACTTTAAGGGTTACCTGGGTTG/TCACATGCGCCTTGATGTCTG; GAPDH, GGAGCGAGATCCCTCCAAAAT/GGCTGTTGTCATACTTCTCATGG.

Membrane Protein Extraction

Cell membrane proteins were extracted using the Mem-PERTM Plus membrane protein extraction kit. The cells were scraped off the surface of the plate with a cell scraper, and 5×10^6 cells were resuspended in culture medium. The resulting cell suspension was centrifuged at 300 g for 5 min. The cell pellet was washed with 3 mL of cell wash solution and centrifuged at $300 \times g$ for 5 min. The supernatant was carefully removed and discarded. Cells were resuspended in 1.5 mL of cell wash solution and transferred to a 2 mL centrifuge tube. Centrifugation ($300 \times g$) was performed for 5 min and the supernatant was discarded. 0.75 mL permeabilization buffer was added to the cell pellet. Short vortices used to obtain a homogeneous cell suspension. It was incubated at 4 °C for 10 min with continuous mixing condition. The permeabilized cells were centrifuged at $16000 \times g$ for 15 mins. The supernatant containing cytosolic proteins was carefully removed and transferred to a new tube. Solubilization buffer (0.5 mL) was added to the precipitate, which was then resuspended using pipette suction. It was incubated at 4 °C for 30 min with continuous mixing conditions. At 4 °C, the tube was centrifuged for 15 min at a speed of $16000 \times g$. Supernatants containing soluble membrane proteins and membrane-associated proteins were transferred to a new tube. Proceed with downstream applications. Cytosolic and membrane components on ice can be used immediately or stored at -80 °C after packaging for future use.

Western Blot

Proteins in the culture medium of cell lysates were separated using 12% SDS-PAGE and transferred to immobile transfer membranes (Millipore, Bedford, MA). The transferred blots were incubated with a blocking solution containing 5% dry milk in TBST (0.15% Tween 20, 200 mm NaCl, and 25 mm Tris-HCl [pH 7.6]). The blots were washed and incubated for 1 h with secondary peroxidase-conjugated antibodies (Santa Cruz Biotechnology). Immunoreactive bands were detected using ECL detection reagents (Amersham, Buckinghamshire) according to the manufacturer's instructions.

Detection of Lipid Raft Content in HMEEC Cells by Vybrant™ Alexa Fluor™ 488 Lipid Raft Labeling Kit

The cells were labeled with a fluorescent CtxB conjugate. The cells were centrifuged, and the cell particles were gently resuspended in complete medium on ice. The cells were centrifuged again, and the cell particles were suspended in 2 mL of the fluorescent CtxB coupling working solution. The cells were incubated at 4 °C for 10 min. After incubation, cells were gently washed several times with $1 \times$ ice PBS. CtxB-labelled lipid rafts were cross-linked with anti-CtxB antibodies. The cells were centrifuged, and the cell particles were gently resuspended in 2 mL of ice anti-CtxB antibody working solution. The cells were incubated at 4 °C for 15 min. After incubation, cells were gently washed several times with $1 \times$ ice PBS. The cells were placed in $1 \times$ ice PBS and observed under a fluorescence microscope with the corresponding filter groups.

CSOM Rat Model Establishment and Treatment

Animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals. For the *S. aureus* and *B. cereus* infection experiment, Sprague-Dawley (SD) rats weighing 200–250 g were anesthetized by intramuscular injection of ketamine-toluene thiazide mixture at a dose of 0.25 ~ 3 mL/100 g body weight. Animals were placed in the supine position, and a transverse incision was made at a position approximately 0.5 cm below the hyoid level to separate and expose the anterior cervical muscles. The hyoid scapula and hyoid sternum muscles were separated

near the hyoid bone, so that the bottom wall of the ear bleb could be seen. Sterile saline was injected into the left ear of the rats as a control, and 200 μL of the bacterial suspension (containing 3×10^8 CFU) was injected into the right ear. The injection hole in the ear wall was sealed with bone wax and the incision was sutured layer-by-layer.

For *Lactobacillus* treatment, *Lactobacillus* suspension (200 μL , 1×10^9 CFU) was applied to the right ears of the rats.

HE Staining

After the administration ended, all the rats in each group were sacrificed through cervical vertebra dislocation. The inner ear was dissected, fixed with 4% paraformaldehyde, decalcified, dehydrated, paraffin-embedded for sectioning, and subjected to HE staining. The pathological morphological changes of the middle ear mucosa were observed under a microscope.

Statistical Analysis

Statistical analyses were performed using Statistical Program for Social Science version 26.0 (SPSS 26.0), R software (version 4.0.3), and GraphPad Prism (version 9). Quantitative data with a normal distribution is presented as the mean \pm SD and were analyzed using Student's *t*-test or one-way analysis of variance. To estimate the significance of gene expression differences between the patient subgroups, we performed a two-sample *t* test for each gene. $P \leq 0.05$ indicates a statistically significant difference between measured values. The significance was set as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Differentially expressed genes were screened using P -value < 0.05 and $|\log\text{FC}| > 1.5$ as cut-off criteria, respectively.

Results

The Role of RFTN1 and Lipid Rafts in CSOM

Variable differentially expressed genes (DEGs) between middle ear cavity from CSOM patients and lavage fluid of middle ear from normal volunteer were identified. A total of 3646 DEGs (1620 up-regulated and 2026 down-regulated) in CSOM were identified. Among them, we found that RFTN1 was highly expressed in the CSOM ($P=0.045$; Figure 1A and B). To explore the role of RFTN1 in CSOM, we co-cultured *S. aureus* and *B. cereus* with the HMEEC cell and constructed a CSOM cell model. The mRNA and protein expression levels of RFTN1 and lipid raft proteins FLOT1 and FLOT2 were increased in the CSOM cell model (Figure 1C–E). We observed changes in the expression of lipid rafts under inflammatory conditions using a lipid raft labeling kit. The results showed that the lipid raft content of the cell membrane increased significantly in the CSOM group (Figure 1F). These results indicate that CSOM induced by *S. aureus* and *B. cereus* can induce the expression of RFTN1 in HMEEC and the formation of lipid rafts on the cell membrane.

RFTN1 Regulates Lipid Raft Formation and Inflammation in CSOM

To determine the function of RFTN1 in CSOM, we knocked down RFTN1 in HMEEC cells (Figure 2A and B). RFTN1 inhibition reduced TNF- α , IL-1 β , IL-6, IL-8, and IL-10 levels (Figure 2C and D). In addition, lipid rafts on the cell membrane were reduced in RFTN1 knockdown cells (Figure 2E). These results indicated that RFTN1 is a key regulatory factor in the formation of lipid rafts and inflammatory responses.

RFTN1 Modulates the Inflammation Response in CSOM via Lipid Raft Formation

We further determined the mechanism underlying RFTN1 expression in CSOM. We firstly conducted the RFTN1 overexpressed cells and introduced methyl- β -cyclodextrin (M β CD), an inhibitor of lipid raft²⁴ to treat cells. The expression of RFTN1 in RFTN1 overexpressed cells was also determined (Figure 3A). Administration of M β CD counteracted the promoting effect of RFTN1 overexpression on the inflammatory response (Figure 3B and C) and the formation of lipid rafts (Figure 3D).

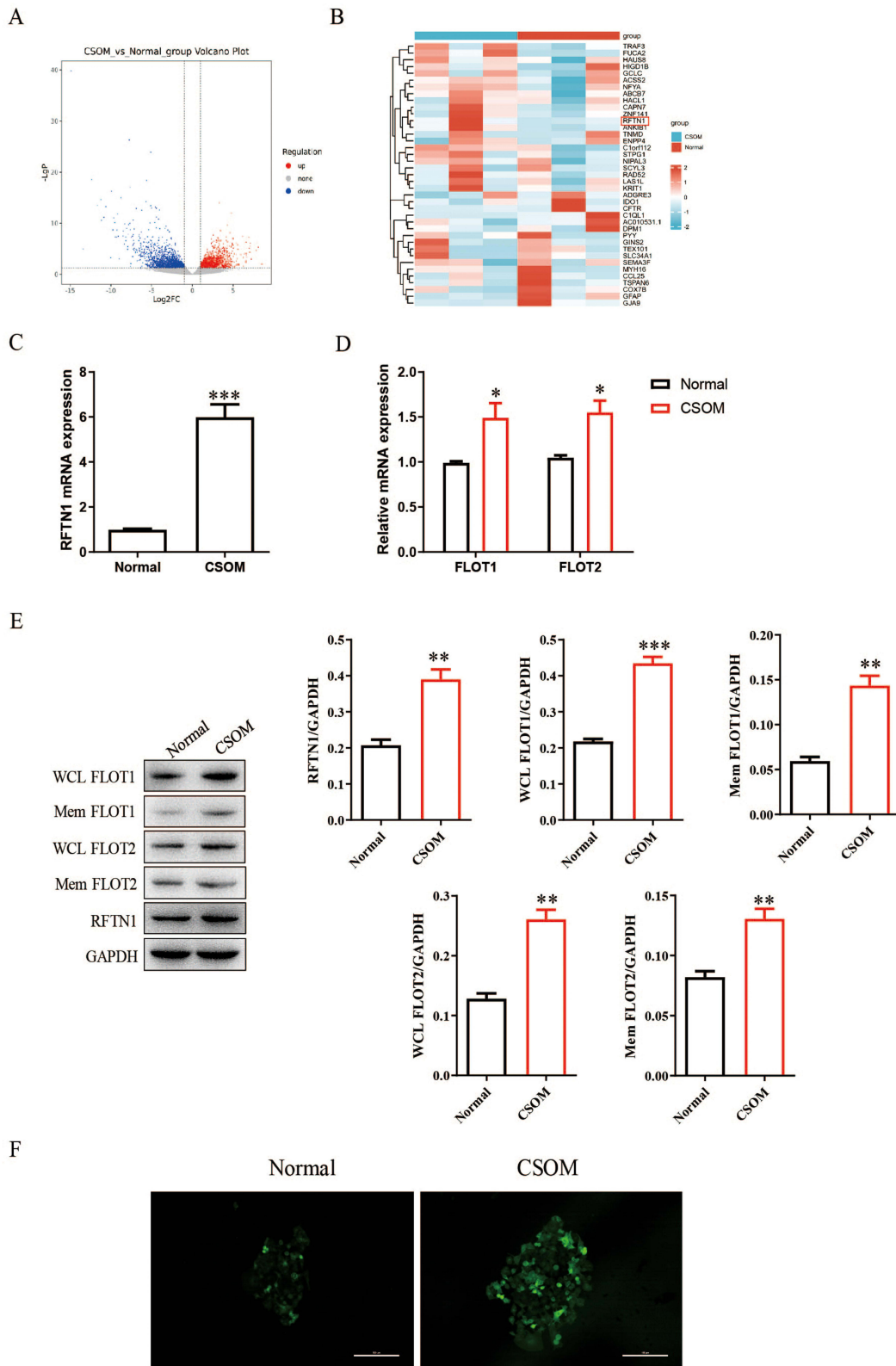


Figure 1 The role of RFTN1 and lipid rafts in CSOM. **(A)** Volcano plot and **(B)** A hierarchical clustering heatmap were used to display the differentially expressed mRNA patterns between normal and CSOM group. The cell model of CSOM was induced by co-culture of *S. aureus* and *B. cereus* with HMEEC cells. The same number of cells were collected, and RNA, whole cell lysate (WCL) and membrane protein (Mem) were extracted. **(C and D)** The expression of RFTN1 and raft protein FLOT1/2 mRNA was detected by qPCR. **(E)** Western blot was used to detect the expression of RFTN1 and FLOT1/2 proteins in cells and cell membranes. **(F)** The lipid raft content of HMEEC cells was detected by Vybrant™ Alexa Fluor™ 488 lipid raft labeling kit. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

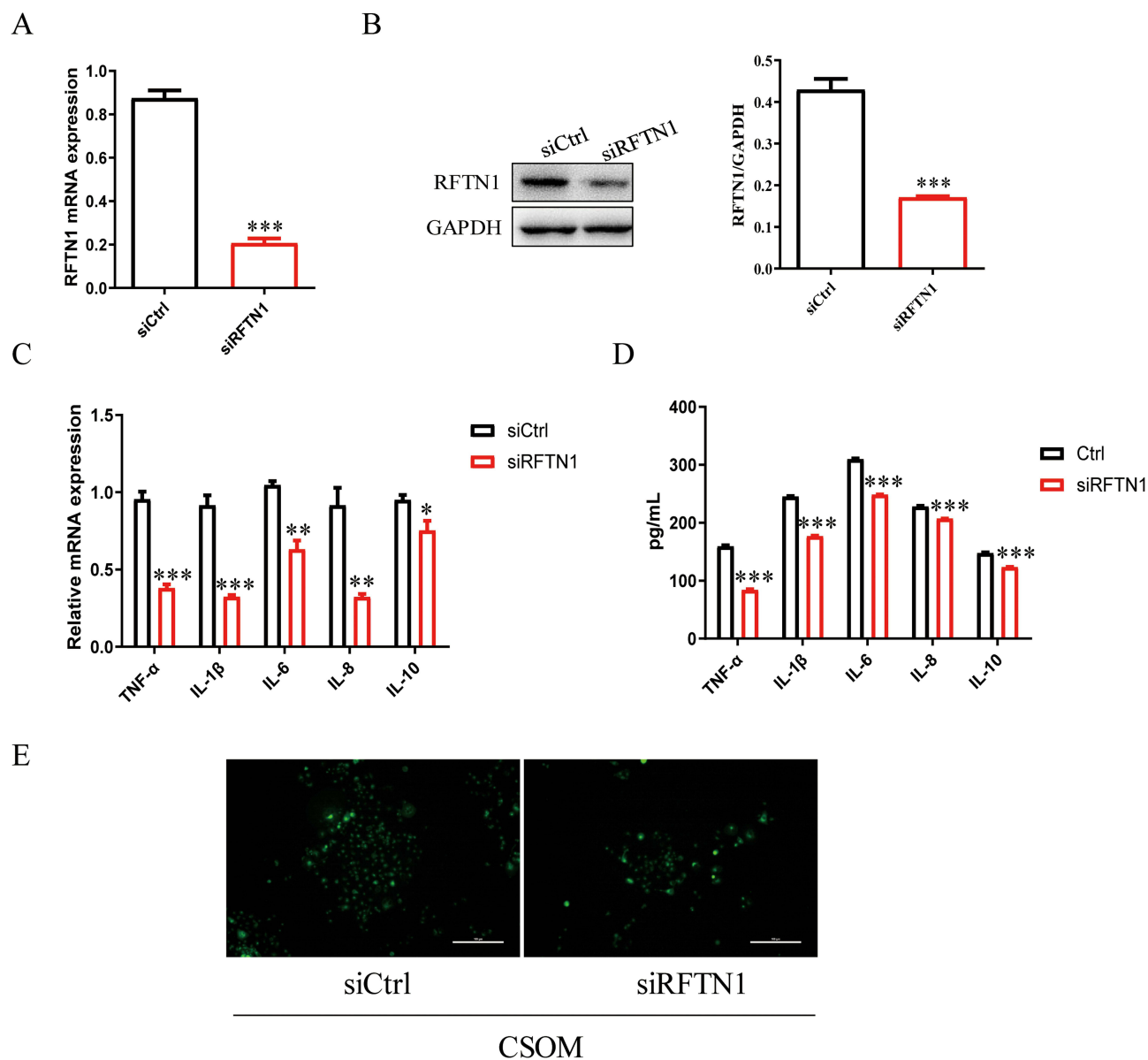


Figure 2 RFTN1 regulates lipid raft formation and inflammation in CSOM. siRFTN1 were transfected into HMEEC cells knock down RFTN1, and the transfected cells were cultured for 48 hours. (A and B) The mRNA and protein expression level of RFTN1 were detected using qPCR and Western blot. (C and D) qPCR and ELISA were used to detect the levels of inflammatory cytokines TNF- α , IL1- β , IL-6, IL-8, IL-10. (E) Vybrant™ Alexa Fluor™ 488 lipid raft labeling kit was used to detect the content of cell lipid rafts. * P < 0.05, ** P < 0.01, *** P < 0.001.

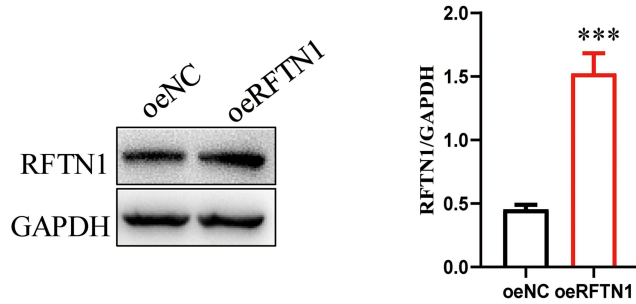
RFTN1 Modulated the Expression of TLR4 in CSOM Cells

Previous studies have suggested that raft proteins are involved in the activation of TLR4²⁰ and that the recruitment of TLR4 to lipid rafts promotes a proinflammatory response.²⁵ However, the function of TLR4 in RFTN1-induced inflammatory response in CSOM remains unknown. RFTN1 inhibition reduce the expression of TLR4 (Figure 4A). M β CD administration alleviated the promoting effect of RFTN1 overexpression on TLR4 expression (Figure 4B), indicating that RFTN1 modulated TLR4 expression via lipid rafts. In addition, immunofluorescence analysis confirmed that TLR4 expression was suppressed by RFTN1 inhibition (Figure 4C).

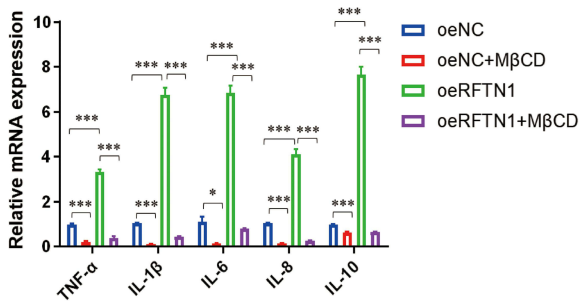
RFTN1 Positively Regulates *S. aureus* and *B. cereus* -Induced Inflammation in vivo

To further confirm the key role of RFTN1 in the pathogenesis of CSOM, we injected *S. aureus* and *B. cereus* suspensions into the right ears of rats to induce the CSOM model. After five days, M β CD was injected subcutaneously into rats in the

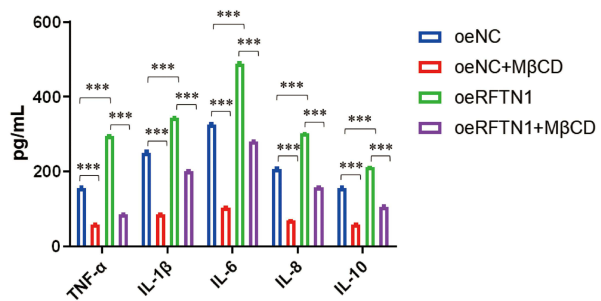
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B



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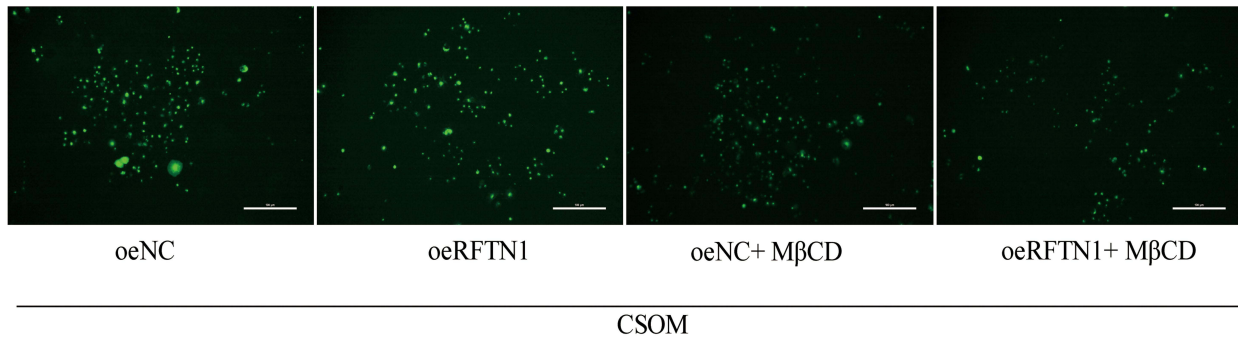


Figure 3 RFTN1 modulates the inflammation response in CSOM via lipid raft formation. The cells transfected with oeRFTN1/control plasmid (oeNC) were treated with MβCD for 8 hours, and the cells were collected. **(A)** Expression of RFTN1 was determined by western blot. **(B and C)** qPCR and ELISA were used to detect the expression level of inflammatory cytokines. **(D)** The content of lipid rafts in cells was detected by Vybrant™ Alexa Fluor™ 488 lipid raft labeling kit. * $P < 0.05$, *** $P < 0.001$.

control and CSOM groups. The middle ear was perfused with 1 mL of PBS thrice, and the levels of TNF- α , IL-1 β , IL-6, IL-8, and IL-10 in the lavage fluid were measured. The rats were killed, pathological changes were observed by HE staining, and the expression of RFTN1 and TLR4 was detected by immunofluorescence. The results showed that In CSOM rats, the middle ear mucosa and mucosal layer were thickened, and tissue edema and gland dilatation were observed. Eosinophils of plasma cells were aggregated, goblet cells were increased, and nuclei were deeply stained, indicating that *S. aureus* and *B. cereus* can effectively induce CSOM development (Figure 5A). Consistent with CSOM cells, upregulated expression of RFTN1 and TLR4 induced by *S. aureus* and *B. cereus* was observed in middle ear tissue (Figure 5B). The colocalization of RFTN1 and TLR4 also increased in CSOM rats, and the expression of TNF- α , IL-1 β , IL-6, IL-8, and IL-10 was higher in CSOM rats than in normal rats (Figure 5B and C). Additionally, MβCD treatment partially eliminated the effects of *S. aureus*- and *B. cereus*-induced pathological changes and inflammatory responses (Figure 5A and C). These results further prove that RFTN1 regulates the expression of TLR4, subsequently promoting the progression of CSOM *in vivo*.

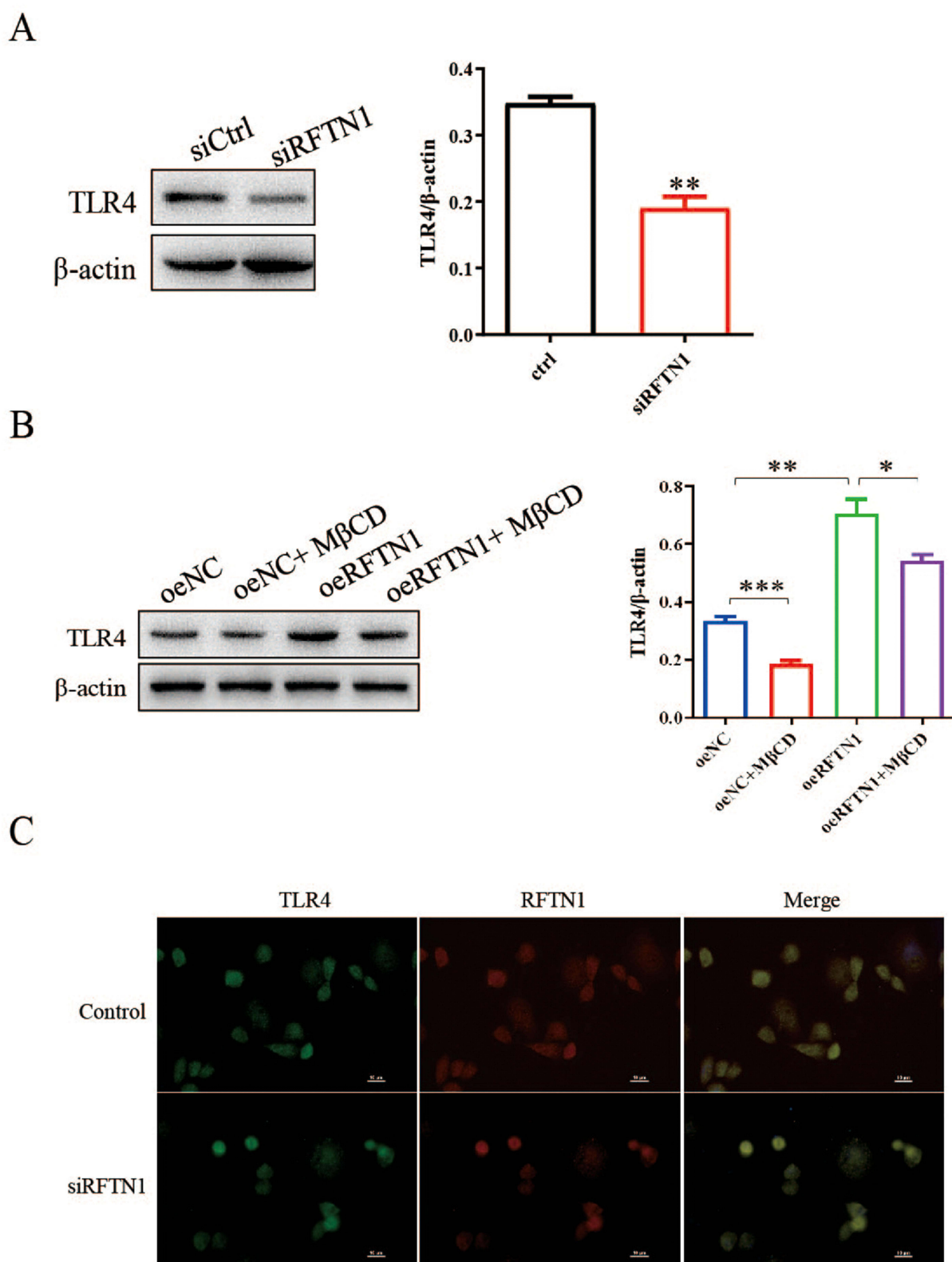


Figure 4 RFTN1 modulated the expression of TLR4 in CSOM cells. **(A)** Expression of TLR4 in RFTN1 knocked down cells was detected by Western blot. **(B)** Expression of TLR4 in RFTN1 overexpressed cells with MβCD administration was detected by Western blot. **(C)** Immunofluorescence of TLR4 and RFTN1 in RFTN1 knocked down cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

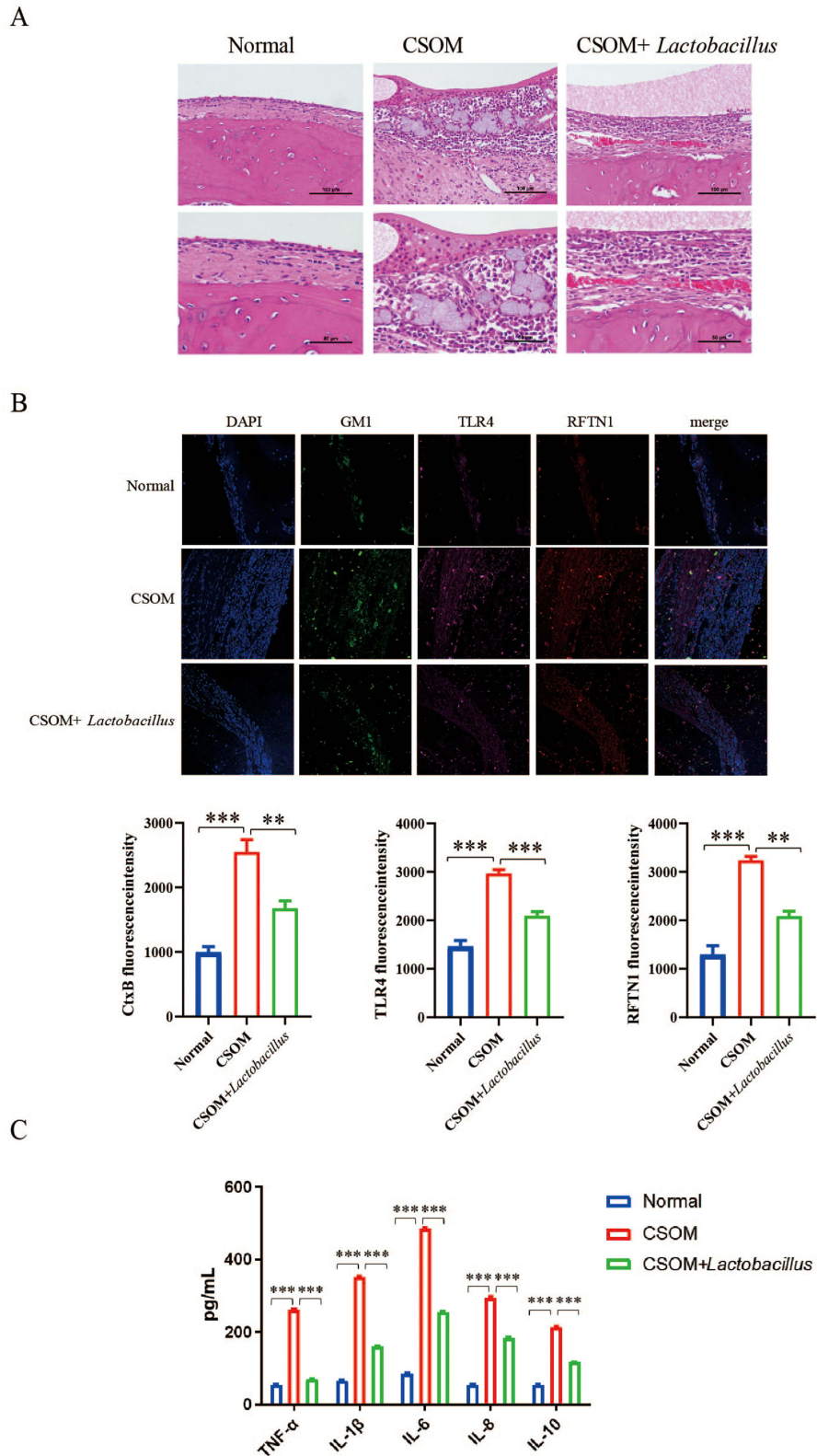


Figure 5 RFTN1 positively regulates *S. aureus* and *B. cereus*-induced inflammation in vivo. After 5 days establishing an CSOM model, M β CD is administered subcutaneously to rats in the control group and CSOM group. After 48 hours, the middle ear lavage fluid was collected, and the middle ear tissues are separated. **(A)** HE staining was used to observe the pathological changes of middle ear tissue; **(B)** GM1, RFTN1 and TLR4 were detected by immunofluorescence; **(C)** Determination of inflammatory factors in the middle ear lavage fluid. *** $P < 0.001$.

Lactobacillus Improves CSOM Induced by *S. aureus* and *B. cereus* Through Lipid Raft-Dependent TLR4 Inflammatory Signal

Microbiological research on healthy children shows that *Lactobacillus* have a potential protective effect on OM,²⁶ and *Lactobacillus* can be used as probiotics to treat diseases caused by other pathogenic bacteria, such as *α hemolytic streptococcus*.²⁷ To study the therapeutic effect of *Lactobacillus* on CSOM caused by *S. aureus* and *B. cereus*, we treated a rat model with *Lactobacillus*. The results showed that *Lactobacillus* administration significantly reduced the inflammatory reaction of the middle ear epithelium and swelling of the tympanic membrane and pus (Figure 6A). The expression and colocalization of RFTN1 and TLR4 decreased, as did the expression of cytokines (Figure 6B and C). In summary, *Lactobacillus* therapy may effectively improve CSOM induced by *S. aureus* and *B. cereus* by inhibiting the RFTN1-TLR4 axis.

Discussion

In the present study, we performed RNA sequence analysis and the DEGs consisting of 1620 up-regulated genes and 2026 down-regulated genes in CSOM were identified. The expression of RFTN1 was significantly increased in CSOM patients. We treated HMEEC cell and rats with *S. aureus* and *B. cereus* to induce CSOM and confirmed the key regulatory role of RFTN1 in the progression of CSOM. RFTN1 can affect the formation of lipid rafts on the cell membrane and the localization of TLR4 on lipid rafts, subsequently promoting the development of CSOM. We treated a rat model of CSOM with *Lactobacillus* and found that *Lactobacillus* treatment alleviated CSOM progression by inhibiting RFTN1 and TLR4 expression. Our findings suggest a crucial role for *Lactobacillus* in alleviating CSOM progression and uncovered the molecular mechanism involving *Lactobacillus*-regulated inhibition of the RFTN1-lipid raft-TLR4 signaling pathway under CSOM conditions.

OM refers to inflammation and/or infection of the middle ear, including a series of acute and chronic diseases clinically characterized by middle ear effusion.²⁸ CSOM is characterized by a persistent and threatening pattern of severe ear discharge, tympanic perforation, and varying degrees of hearing loss over a period of more than six weeks. CSOM is a multi-etiological disease associated with irreversible pathological changes in middle ear tissue.²⁹ It is estimated that 65–300 million patients, especially children, are affected. Currently, the most common risk factors for CSOM include persistent local gram-positive or gram-negative bacterial infections, recurrent acute OM, recurrent upper respiratory tract infections, poor sanitation, middle ear trauma, internal tympanic canals, and nutritional deficiencies.³⁰ At present, although there are a variety of options for the standard treatment of CSOM, no significant breakthrough has been made in either surgical or conservative treatment, or in solving otorrhea or hearing loss. Therefore, probiotic therapy may be a potential target for the treatment and prevention of CSOM in the future.¹⁹ Many randomized controlled studies have reported that *Lactobacillus rhamnosus* GG,^{31–33} *Bifidobacterium lactis* BB-12,^{32,34} *Streptococcus alpha hemolyticus*,³⁵ *Streptococcus salivarius* 24SMB,³⁶ *Streptococcus salivarius* K12³⁷ have good effects on the treatment and prevention of OM.

Lipid rafts are microstructure domains rich in cholesterol and sphingomyelin in the plasma membrane that play important roles in cell signal transduction. The structure of lipid rafts is dynamic, and lipid and protein contents are constantly changing. The types of lipids and proteins in the lipid rafts determine their size, stability, structure, and function. Most pathological conditions associated with lipid rafts are caused by an increase in their abundance or stability. Using lipid rafts as therapeutic targets has a significant effect on slowing the disease process. Studies have shown that lipid rafts play an important role in the regulation of diseases, such as inflammation, cancer, systemic lupus erythematosus, neurodegenerative diseases, and atherosclerosis. Considering the important role of inflammation in various pathological processes, targeting lipid rafts can alleviate various inflammation-induced diseases.³⁸ Based on their different contents, lipid rafts can be divided into inflammatory, apoptosis signal molecule enrichment, and immune lipid rafts. Inflammatory rafts contain activated receptors, which mediate cellular inflammatory reactions.

TLR4 forms dimer in the ligand-activated reaction, which requires the microenvironment of lipid rafts, and the integrity of lipid rafts is very important for normal TLR4 signal transduction.³⁹ A decrease in cellular cholesterol reduces the number of lipid rafts and destroys their structure of lipid rafts, thus inhibiting receptor dimerization and TLR4

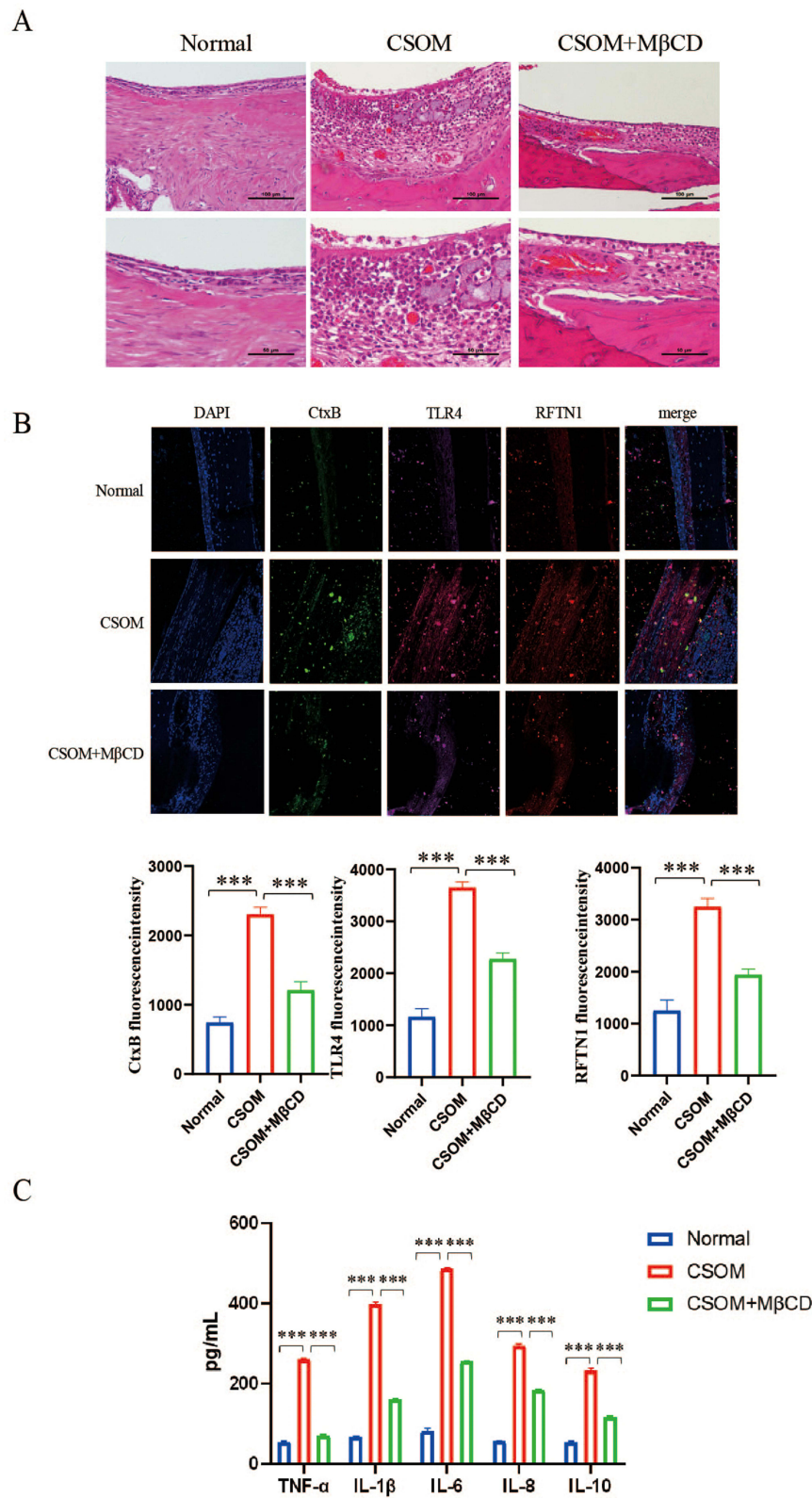


Figure 6 *Lactobacillus* alleviates CSOM induced by *S. aureus* and *B. cereus* in rat. CSOM model was established, and *Lactobacillus* solution was injected at the same time. After 7 days, the middle ear lavage fluid was collected, and the middle ear tissue was separated. (A) HE staining was used to observe the pathological changes of middle ear tissue; (B) GM1, RFTN1 and TLR4 were detected by immunofluorescence; (C) Determination of inflammatory factors in the middle ear lavage fluid. ** $P < 0.01$, *** $P < 0.001$.

recruitment to lipid rafts and weakening TLR4 signal transduction. However, an increase in cellular cholesterol increases the number of lipid rafts and enhance the signal transduction of TLR4.⁴⁰

Saeki et al²¹ knocked out RFTN1 in chicken DT40B cells and observed a decrease in cell proliferation. By reintroducing the gene, raftlin was shown to be required for normal growth rate. In the absence of raftlin, the number of lipid rafts decreased and increased due to the overexpression of raftlin, indicating that raftlin is necessary for the formation or maintenance of lipid rafts. By consuming cholesterol, the lipid raft was destroyed, which led to a decrease in raftlin and a decrease in other components of lipid rafts similar to raftlin gene deletion, which confirmed previous observations. BCR-mediated tyrosine phosphorylation and calcium mobilization were impaired due to the lack of raftlin in DT40 cells, and the overexpression of raftlin was enhanced, indicating that raftlin is necessary for effective BCR signal transduction by forming or maintaining lipid rafts. Tatematsu et al²² studied the role of raftlin in TLR4 signal transduction in raftlin knockdown HEK293 cells and found that raftlin is necessary for activation of the IFN- β promoter mediated by TLR4 induced by lipopolysaccharide (LPS) in the presence or absence of membrane-bound CD14. Raftlin is essential for LPS-induced IFN- β production and the regulation of TLR4 signal transduction in human primary immune cells. Confocal microscopy showed that under the stimulation of endotoxin, the internalization of TLR4 also required raftlin. In response to LPS stimulation, raftlin moves from the cytoplasm to the plasma membrane, colocalizes with TLR4 on the cell surface, and is internalized by TLR4. Immunoprecipitation experiments confirmed that raftlin interacted with the clathrin/AP2 complex and mediated endocytosis of TLR4 by participating in the selection of goods with AP2. Our research, using humans and rats as representatives, confirmed the key role of RFTN1 in the signal transduction of cell membrane lipid rafts and TLR4 inflammation, and expounded the effects of RFTN1 on TLR4 activation either directly (binding with TLR4) or indirectly (affecting the formation of lipid rafts). The main and side effects of these two agents must be proven through further experiments.

Lactic acid bacteria are recognized as safe and non-pathogenic food-grade microorganisms that widely exist in the digestive tract of plants, dairy products, human beings, animals, and female vaginas, and mainly include 43 genera, such as *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc* and *Streptococcus*.⁴¹ Some strains of lactic acid bacteria are considered to be probiotics. According to the definition of probiotics by the World Health Organization (WHO), probiotics refer to a kind of living microorganisms that can exert beneficial effects on host health when ingested in sufficient quantities. The most common types are *Lactobacillus* and *Bifidobacterium*.¹⁶ Recently, probiotic lactic acid bacteria have attracted wide attention because of their important functions in improving intestinal flora balance, promoting host immunity, and preventing and treating diseases, such as reducing inflammatory response, anti-diabetes, and anti-tumor.⁴²⁻⁴⁶ However, at present, most research on probiotic therapy focuses on improving intestinal flora ecology by oral administration, and there is little research on the application of probiotics outside the intestine. Here, we propose a therapeutic strategy for implanting *Lactobacillus* into the skin or mucosa of the middle ear canal and other parts to inhibit the occurrence of inflammation and demonstrate the possible mechanism by which *Lactobacillus* can improve OM through the lipid raft -TLR4 axis. Many studies have shown that lactic acid bacteria play a regulatory role in cholesterol metabolism in vivo. Lactic acid bacteria in the intestine can inhibit cholesterol absorption in vivo by inhibiting the expression of NPC1L1, mediating the upregulation of the cholesterol transporter ABCG5/G8, and producing bile salt hydrolase during metabolism, which is the main regulator of serum cholesterol content.⁴⁷⁻⁵⁰ However, at present, research on the mechanism by which lactic acid bacteria regulate cholesterol degradation lacks systematization, and most studies only analyze and measure the expression and enzyme activity of one or a limited number of metabolic enzymes or proteins. There are few studies on the material basis, signal pathway, and the relationship between these proteins regulated by lactic acid bacteria.^{51,52} There are relatively few reports on whether the substances that lactic acid bacteria regulate cholesterol metabolism are metabolites of strains or cell wall components, and whether they regulate cholesterol metabolism directly or indirectly. Therefore, we speculate that one of the mechanisms by which lactic acid bacteria regulate inflammation may be to affect cholesterol metabolism in epithelial cells and subsequently affect the formation of lipid rafts.

It is important to emphasize the potential limitations of this study. For example, a suspension of *Lactobacillus* was employed to directly treat the infected ear in this study. In other studies, means such as intraduodenal injection,⁵³ intraperitoneal injection⁵⁴ and oral administration⁵⁵ were utilized. Whether there are any differences among these

treatment methods, and which one is the best remain to be explored. And some studies indicated that the bactericidal effect of *Lactobacillus*,⁵⁶ whether *Lactobacillus* can exert a bactericidal effect in CSOM remains to be investigated. Additionally, there have been clinical studies applying *Lactobacillus* to the treatment of diseases.⁵⁷ How to enhance the therapeutic efficacy of *Lactobacillus* is also something that requires exploration. Whether *Lactobacillus* can exert a better effect through certain modifications or packaging remains a subject that we will continue to explore.

To sum up, although we have preliminarily proved the potential of *Lactobacillus* to improve CSOM by inhibiting lipid raft -TLR4 signaling pathway, there is still a lack of sufficient experiments to prove the molecular mechanism of *Lactobacillus* inhibiting lipid raft and affecting cholesterol metabolism in epithelial cells, which may be an issue worth exploring. In the subsequent study, we aim to utilize *Lactobacillus* in the clinical treatment of CSOM.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing interests.

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