Published in final edited form as:

Infect Genet Evol. 2015 October ; 35: 194–203. doi:10.1016/j.meegid.2015.08.019.

Otitis media induced by peptidoglycan-polysaccharide (PGPS) in TLR2-deficient (*Tlr2*^{-/-}) mice for developing drug therapy

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Abstract

Background—Toll like receptor 2 (TLR2) signaling can regulate the pathogenesis of otitis media (OM). However, the precise role of TLR2 signaling in OM has not been clarified due to the lack of an optimal animal model. Peptidoglycan-polysaccharide (PGPS) of the bacterial cell wall can induce inflammation by activating the TLR2 signaling. This study aimed at examining the pathogenic characteristics of OM induced by PGPS in *Tlr2*^{-/-} mice, and the potential therapeutic effect of Sodium aescinate (SA) in this model.

Methods—Wild-type (WT) and *Tlr2*^{-/-} mice were inoculated with streptococcal PGPS into their middle ears (MEs) and treated intravenously with vehicle or SA daily beginning at 3 days prior to PGPS for 6 consecutive days. The pathologic changes of individual mice were evaluated longitudinally.

Results—In comparison with WT mice, *Tlr2*^{-/-} mice were susceptible to PGPS-induced OM. *Tlr2*^{-/-} mice displayed greater hearing loss, tympanic membrane damage, ME mucosal thickening, longer inflammation state, cilia and goblet cell loss. SA-treatment decreased

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Conflict of interest

The authors have declared that no conflict of interest exists.

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neutrophil infiltration, modulated TLR2-related gene expression and improved ciliary organization.

Conclusions—PGPS induced a relatively stable OM in *Tlr2*^{-/-} mice, providing a new model for OM research. Treatment with SA mitigated the pathogenic damage in the ME and may be valuable for intervention of OM.

Keywords

Sodium aescinate; streptococcal peptidoglycan polysaccharide; mouse models of otitis media

1. Introduction

Otitis media (OM) is one of the most common diseases of early childhood, and is a frequent reason for treatment with antibiotics or surgery in developed countries. The pathogenesis of OM is attributed to many genetic and environmental factors, including the dysfunctional adaptive and native immune system, bacterial infections, and Eustachian-tube dysfunction. However, the molecular pathogenesis of OM is still incompletely understood. Therefore, characterization of OM pathogenesis is of significance for the early intervention and prevention of this disease.

Toll-like receptor 2 (TLR2) recognizes pathogen-associated microbial patterns (PAMPs), such as peptidoglycan (PGN) and lipopeptides, and plays a fundamental role in activation of innate immune response (Kawai and Akira, 2010; Yang et al., 1998). Previous studies have shown that the TLR2 signaling can regulate the pathogenesis of OM (Han et al., 2009a; Xiong et al., 2012). Indeed, the levels of TLR2 expression in the middle-ear mucosa are down-regulated in patients with CSOM (Si et al., 2014). However, the contributions of TLR2 signaling in OM have not been clarified due to the lack of an optimal animal model.

Currently, there are a few mouse models of OM available for the identification of susceptibility genes and the exploration of pathogenesis associated with OM (Hernandez et al., 2008; Ryan et al., 2006). Our previous studies and those of others have infected mice with live bacteria to induce AOM (Han et al., 2009a; Suzukawa et al., 2014; Tong et al., 2014). This protocol causes most *Tlr2*^{-/-} mice death within three days (Han et al., 2009a), making it difficult to observe the long-term effect of OM. Peptidoglycan-polysaccharide (PGPS) of the bacterial cell wall can induce inflammation against bacterial infection by up-regulating TLR2 expression (Komori et al., 2011) and cause acute and chronic arthritis (Esser et al., 1985; Stimpson et al., 1987; Wu et al., 2014). Previous studies have shown that after clearance of Gram+ bacterial infection in the middle ears (MEs) by immune system, bacterial PGPS may be persistent in the MEs, causing COM (Fulghum and Brown, 1998; Komori et al., 2011). However, whether inoculation with PGPS can induce mucosal immune response in the MEs has not been systemically explored. We hypothesized that inoculation with PGPS would induce chronic inflammation in the MEs of *Tlr2*^{-/-} mice, which may provide a unique model to explore the molecular pathogenesis of OM.

Currently, patients with OM depend on antibiotic treatment. However, many patients do not well respond to the antibiotic treatment due to the development of drug resistance and

PGPS-related inflammation. Sodium aescinate (SA) is a clinically active anti-inflammatory, anti-edematous, and anti-oxidative drug, and has been widely used in China. Previous studies have shown that SA can inhibit inflammation by elevating the concentration of blood glucocorticoid and increasing the expression of glucocorticoid receptor (Montopoli et al., 2007; Wang et al., 2009; Xin et al., 2011). However, whether SA can control the PGPS-induced inflammation in the MEs and improve their function has not been clarified.

To address these questions, we inoculated with PGPS into the MEs of *Tlr2*^{-/-} mice to induce OM and characterized the pathogenic characteristics of OM in mice. Furthermore, we investigated the potential therapeutic effect of SA on the pathogenesis of experimental OM and the function of the MEs in *Tlr2*^{-/-} mice.

2. Materials and Methods

2.1 Mice

Both male and female *Tlr2tm1Kir* (*Tlr2*^{-/-}) and wild-type (WT) C57BL/6J mice at 6–8 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in a specific pathogen-free facility. A total of 64 *TLR2*^{-/-} mice (31 male and 33 female) and 55 WT mice (25 male and 30 female) were used in this study. The experimental protocol was approved by the Animal Use and Care Committee of Binzhou Medical University.

2.2 Mouse model of OM

Individual mice were anesthetized intraperitoneally with 4% chloral hydrate (0.01 ml/g) and injected with different doses (10, 20, 30, 60, or 100 µg) of PGPS (100P, BD Bioscience, San Jose, USA) prepared freshly in 5 µl of saline through the tympanic membrane into the middle ear cavity using a Hamilton syringe. Their tympanic membranes were examined on day 1, 2, 3, 7 and 15 post injection using an otoscopic digital imaging system, MedRx VetScope System® (MedRx, Largo, USA). After inflammation was detected, the mice were examined every 12 h.

2.3 The auditory brainstem response (ABR) and tympanometry procedure

The ABR and tympanometry of individual experimental (*Tlr2*^{-/-} mice) and control mice (WT mice) were assessed before and at 3 days post-injection with PGPS, as described previously (Zheng et al., 2007). Briefly, individual mice were anesthetized intraperitoneally with 4% chloral hydrate (0.01 ml/g) and their body temperatures were maintained at 37–38°C with environmental noise < 50 dB SPL. The stimulus amplitudes for the ABR test were dB SPL (re. 20 mPa) and their responses to broadband click and pure-tone 8 kHz, 16 kHz, or 32 kHz auditory stimuli were recorded (Zheng et al., 1999). Averaging thresholds (± 5dB) were determined because the minimum stimulus amplitude produced an ABR wave pattern similar to that of the highest intensity stimulus (110 dB). Tympanometry measurement was performed using an MT 10 tympanometer (Interacoustics, Assens, Denmark).

2.4 Histological analysis of middle ear

On day 3, 7 and 15 post PGPS injection, the experimental *Tlr2*^{-/-} and WT control mice were sacrificed (n=6 per group per time point) and their bullae (including both the middle and inner ear) were dissected, followed by pathological examination (Johnson, 2003). Briefly, the bulla tissues were fixed with 10% paraformaldehyde for 24 h, decalcified with 10% EDTA solution for 5 days, and embedded in paraffin. The tissue sections (5 µm) were stained with hematoxylin-eosin (H&E) and examined under a light microscope. To identify goblet cells, the bulla tissue sections were stained with AB-PAS using an alcian blue and periodic acid schiff (AB-PAS) staining kit (Nanjing Jiancheng Technology, Nanjing, China), according to the manufacturers' instruction.

2.5 Mucociliary clearance in vivo

On day 3 post PGPS inoculation, the mice from individual groups were sacrificed (n= 6 per group). Their right bullae were dissected immediately and placed in 10% FBS RPMI 1640 medium in a plastic culture dish. After removing all non-middle ear-associated tissues, the mucosa in the middle ear was gently held with fine forceps, and moved into the center of a 35 mm glass bottom culture dish containing 100–150 µl RPMI 1640 medium. The ciliary motility along the edge of the ciliated epithelia of individual samples was examined on an inverted microscope with a 100X objective, and DIC optics and recorded using a high-speed camera at 200+ frames per second using a movie-acquisition software. The cilia beat frequency (CBF) was also determined as described previously (Francis and Lo, 2013).

2.6 Real time quantitative PCR

Tlr2^{-/-} and WT mice were injected with 60 µg PGPS in their right MEs and three days after inoculation, the mice were sacrificed (n=6 per group) and their right bullae were dissected. Total RNA was isolated from individual right bullae using TRIzol® reagent (Invitrogen, Carlsbad, USA), according to the manufacturers' protocol. The concentrations of RNA were measured using a Biophotometer (Eppendorf, Hamburg, Germany). Each RNA sample (1 µg) was reversely transcribed into cDNA using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Quantitative real-time PCR was performed using the FastStart Universal SYBR Green Master Kit (Roche, Mannheim, Germany) and specific primers (Supplementary Table 1) in a Bio-Rad iCycler iQ5 peltier thermal cycler. The relative levels of target gene mRNA transcripts to the control of GAPDH were analyzed by the 2^{-Ct} method (Livak and Schmittgen, 2001).

2.7 SA treatment

SA was purchased from Shandong Luye Pharmaceutical (Yantai, China). *Tlr2*^{-/-} mice were randomly divided into three groups of PGPS control, saline or SA (n=6 per group). The mice in the saline or SA group were injected intravenously with saline or 3.6 mg/kg SA daily for six consecutive days, respectively. Three days after initial saline or SA injection, the mice in the PGPS, saline and SA groups were injected with 60 µg PGPS. The effects of drug treatment were evaluated by histology, ABR, tympanometry, cilia beat frequency (CBF), and relative levels of gene expression.

3. Statistical analysis

Data are expressed as mean $\pm$ SEM. The difference between groups was determined by one-way or two-way ANOVA or Unpaired Student T test when applicable using GraphPad Prism 5 software. The animal survival was estimated by Kaplan Meier method and analyzed by the log rank test. A P value of <0.05 was considered statistically significant.

4. Results

4.1 PGPS induces severe OM in *Tlr2*^{-/-} mice at day 3 post-inoculation

Infection with live bacteria to induce OM usually cause acute death in *Tlr2*^{-/-} mice, which is difficult to investigate the long-term effect of OM (Han et al., 2009a). PGPS can induce acute and chronic inflammatory arthritis (Esser et al., 1985; Stimpson et al., 1987) and CSOM, secondary to acute OM (Clark et al., 2000; Fulghum and Brown, 1998). However, little is known on the early pathogenic process of OM in *Tlr2*^{-/-} mice following inoculation with PGPS. To address it, *Tlr2*^{-/-} and wide-type (WT) C57BL/6 mice were inoculated with different doses of PGPS in the right tympanic cavity and the early pathogenic process of OM in mice was determined. Measurements of the auditory brainstem response (ABR) and tympanometry indicated that *Tlr2*^{-/-} mice developed severe hearing impairment and were more susceptible to PGPS-induced OM, as compared with WT mice (Figure 1, A and B). Histological examination revealed obviously more numbers of inflammatory infiltrates in the tympanic cavity and severer tissue damages in the *Tlr2*^{-/-} mice than the WT mice at 3 days post inoculation with 30 or 60 μ g PGPS (Figure 2A and Supplementary Table 2). Interestingly, neutrophil-related inflammation was detected in the left MEs of some mice with severe OM at 3 days post inoculation. Given that inoculation with 60 μ g PGPS induced relatively stable OM in both WT and *Tlr2*^{-/-} mice, we chose inoculation with 60 μ g PGPS for the following experiments. Further histological analysis indicated neutrophil-related mucosal inflammation in both strains of mice, many proliferative fibroblasts in the sub-epithelial connective tissue layers of the *Tlr2*^{-/-} mice, and hyperplastic epithelium in the WT mice (Figure 2B). While the inflammation was almost subsided in the MEs of WT mice at 7 days post inoculation it remained in the MEs of *Tlr2*^{-/-} mice up to 15 days after inoculation with PGPS (Figure 2C). The areas covering neutrophil infiltrates and epithelial thickness in the *Tlr2*^{-/-} mice were significantly larger than that in the WT mice (Figure 2, D and E). Semi-quantitative analysis revealed severer inflammation in the MEs of *Tlr2*^{-/-} mice at 3 days post inoculation (Supplementary Fig. S1). Hence, inoculation with PGPS induced a severe and long-lasting inflammation as well as tissue damages in the early process of OM in *Tlr2*^{-/-} mice, and suggesting that TLR2-related signaling may be crucial for the development of anti-inflammatory responses during the early process of OM in mice.

4.2 PGPS causes significant destruction of ciliary structure and function in *Tlr2*^{-/-} mice

The function of motile cilia in the epithelia is critical for mucociliary clearance of inflammatory mucus in the MEs. To investigate the function of mucociliary system, the epithelium of the MEs of individual groups of mice was dissected (Supplementary Fig. S2 A and Supplementary video 1), and the mucociliary movement of individual samples was evaluated for its clearance activities (Figure 3A). Firstly, the direction of cilia-generated

flow was from the tympanic membrane (TE) toward the Eustachian tube (ET) (Supplementary Fig. S2 B). It suggested that cilia regular swing followed by the Eustachian tube opening was essential for draining any accumulated secretions, infectious microbia, or debris from the ME space. Secondly, while there was no significant difference in the cilia beat frequency (CBF) between WT and mutant mice (data not shown), the cilia from the *Tlr2*^{-/-} mice survived significantly longer than that of the WT mice without PGPS inoculation (Figure 3B). Furthermore, there was no significant difference in the survival periods of cilia between the WT and mutant mice, but the CBF from the *Tlr2*^{-/-} mice was significantly lower than that from the WT mice following PGPS inoculation (Figure 3C and Supplementary video 2 &3). In addition, severer ciliary loss and mucosal hyperplasia as well as broken and bent cilia were observed in the mutant mice, as compared with that in the WT mice (Figure 3D, Supplementary Fig. S2 C & Supplementary Video 3). To the best of our knowledge, this was the first time to report the motile cilia of the MEs in mice. Our novel data indicated that TLR2-related signaling deteriorated the survival of ciliated cells and inoculation with PGPS impaired the mucociliary clearance and promoted the ciliated cell death and ciliary destruction in the *Tlr2*^{-/-} mice. The impairment in ciliary structure and function may hinder the recovery of OM in *Tlr2*^{-/-} mice.

4.3 *Tlr2*^{-/-} mice display goblet cell absence, accompanied by lower levels of NF- κ B, TNF- α , MIP1 α , MUC5ac, MUC5b, but higher levels of SOD, MPO, iNOS, caspase3

It is well known that PGPS can up-regulate TLR2 expression, which enhances inflammatory responses against infection (Komori et al., 2011; Wu et al., 2014). The enhanced inflammatory responses are involved in many factors, including inflammatory regulators and mucin during the development of OM (Han et al., 2009a; Han et al., 2009b; Roy et al., 2014; Trune and Zheng, 2009). To understand the pathogenic process of OM in the absence of TLR2, the relative levels of relevant gene mRNA transcripts in the MEs from both WT and mutant mice were analyzed (Figure 4A). There was no significant difference in the relative levels of MyD88, NOD2, IL-6, EGF, MEK1/2 and p38MAPK mRNA transcripts between two groups. In comparison with that in the WT mice, significantly lower levels of NF- κ B, TNF- α , MIP1 α , MUC5AC, and MUC5B, but higher levels of SOD, MPO, iNOS and caspase3 expression were detected in the MEs of *Tlr2*^{-/-} mice. The altered expression levels of these genes may be associated with impairment of macrophage recruitment and mucin protection, inducing oxidative stress and ciliated cell apoptosis, supporting the notion that oxidative stress is involved in the pathogenic process of OM (Garca et al., 2013; Testa et al., 2012). It is possible that PGPS in the absence of TLR2 stimulates inflammation and neutrophil accumulation that induce oxidative stress and tissue damage, and limit the recovery of OM and promote the chronic progression of OM in mice.

Mucins are important structural components of the mucociliary transport system that protects the epithelium against microorganisms invaded. Goblet cells can secrete mucus, which functions as a protective barrier upon infection. To further understand the pathogenic process of OM, the goblet cells in the different groups of mice were characterized by staining with alcian blue and periodic acid schiff (AB-PAS) and H&E. The goblet cells were detected in the mucosa of the MEs and the epithelium of the Eustachian tube from the OM WT mice, but not from the OM *Tlr2*^{-/-} mice although both groups of mice developed

similar degrees of inflammation in the MEs (Figure 4B). We are interested in further investigating whether PGPS in the absence of TLR2 can induce the goblet cell apoptosis in mice (Wang et al., 1998).

4.4 Sodium aescinate (SA) treatment mitigates the severity of OM induced by PGPS in *Tlr2*^{-/-} mice

Sodium aescinate (SA) is an anti-inflammation drug and has been widely used for treatment of post-operative inflammation in the clinic. SA has potent anti-inflammatory, anti-edematous and anti-oxidative activity by enhancing glucocorticoid and its receptor expression (Du et al., 2011; Montopoli et al., 2007; Wang et al., 2009; Wang et al., 2012; Wei et al., 2011; Xin et al., 2011; Yang et al., 2011). Accordingly, the effect of SA treatment on the PGPS-induced inflammation was investigated in *Tlr2*^{-/-} mice. While there was no significant difference in the values of all measures tested between the saline-injected and un-injected *Tlr2*^{-/-} mice, treatment with SA (3.6 mg/Kg/day) for 6 consecutive days significantly inhibited the PGPS-induced inflammation and improved the hearing function. In comparison with the PGPS control group, the mean ABR thresholds and the gradient values were significantly reduced in the SA-treated mice, reflecting the structural intact in the MEs (Figure 5A). Furthermore, significantly less neutrophil infiltration, but more macrophages (Figure 5B), less severity in epithelial thickness and less inflammatory areas were detected in the MEs of SA-treated mice (Figure 5, C and D). In addition, the CBF and survival periods were significantly greater (Figure 6, A and B) and the degrees of ciliary damage were significantly reduced in the MEs of SA-treated mice (Figure 6C and Supplementary video 4). Finally, significantly higher levels of MUC5ac, MUC5b, and MIP1 α expression and lower levels of IL-6, SOD, iNOS, MPO, and caspase 3 expression were detected in the MEs of SA-treated mice (Figure 7A). Such novel data clearly demonstrated that treatment with SA inhibited the PGPS-related inflammation and improved the hearing function in *Tlr2*^{-/-} mice.

5. Discussion

A previous study has shown that the levels of TLR2 expression are down-regulated in the MEs of patients with OM. In this study, we inoculated with PGPS into the ME of *Tlr2*^{-/-} mice to induce OM and to explore the molecular pathogenesis of OM and potential therapies. *Tlr2*^{-/-} mice were susceptible to PGPS-induced OM. PGPS inoculation induced severer OM in *Tlr2*^{-/-} mice with a relative lower mortality (data not shown). The low mortality of OM induced by PGPS may stem from the lack of bacteremia in the mice. Inoculation with PGPS may be a new strategy to induce OM for exploring the molecular pathogenesis of OM. Previous studies have indicated that PGPS up-regulates TLR2 expression and the NF- κ B activation (Dziarski et al., 2001; Kielian et al., 2005; Yoshimura et al., 1999), promoting the recruitment of neutrophils and macrophages (Arroyo et al., 2013; Krysko et al., 2011). We found that neutrophils were present in the MEs of *Tlr2*^{-/-} mice following PGPS inoculation. Apparently, neutrophil infiltration was TLR2-independent. Previous studies have suggested that PGPS stimulates LTB4 production by human neutrophils and LTB4 can enhance neutrophil infiltration (Lammermann et al., 2013;

Valera et al., 2007). It is possible that PGPS may also induce LTB₄ production by mouse neutrophils and promote neutrophil infiltration into the MEs of mice (Fig. 7b).

In the model of PGPS-induced OM, we found that mice displayed severe OM and neutrophil infiltrates not only in the inoculated right ears, but also in the uninoculated left ears. This observation suggests that PGPS-induced systemic inflammation may cause a reactive inflammation in the uninoculated left ears of mice. Furthermore, we did not detect goblet cells in the mucosa of the MEs in *Tlr2*^{-/-} mice, supporting the notion that the TLR2 gene regulates the development and function of goblet cells (Harb et al., 2013; Liu et al., 2014). It is possible that PGPS-induced inflammation in the absence of TLR2-related signaling may trigger the goblet cell apoptosis, because the alternated NF-κB activation can modulate the caspase activation (Wang et al., 1998). Alternatively, the mucin-producing and ciliated cells can transdifferentiate into mucous or goblet cells in the ME of OM animals (Lin et al., 2012). PGPS through the unknown pathway may hinder the transdifferentiation of cells. We are interested in further investigating the molecular mechanisms by which PGPS regulates the development and function of goblet cells in the absence of TLR2-related signaling.

Oxidative-stress contributes to the inflammatory process of OM (Aydogan et al., 2013; Balıkcı et al., 2014; Harrison et al., 2012). Activated neutrophils can generate high levels of reactive oxygen species (ROS), including superoxide (O₂^{•-}) and its derivatives (hydrogen peroxide (H₂O₂), singlet oxygen (¹O₂), and the hydroxyl radical (•OH)) via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex and hypochlorite (OCl⁻) via myeloperoxidase (MPO)-dependent reaction between chloride and H₂O₂ (Witko-Sarsat et al., 2000). We found many neutrophil infiltrates in the MEs, which might produce ROS, further exacerbating mucosal barrier damage and cell death. Furthermore, PGPS can also stimulate NADPH oxidase expression and generate superoxide (Li et al., 2001). Apparently, PGPS induced oxidative stress and inflammation in the ME in a TLR2-independent manner. Thus, our findings may provide new insights into the pathogenesis of OM and oxidative stress may be a new target for treatment of OM.

It is well known that the tissue integrity, the host immune response and regression of inflammation are crucial for the recovery of OM. Our results showed the degradation of ME mucosa, the absence of goblet cells and the loss of cilia in *Tlr2*^{-/-} mice at day 3. The difficulty to restore normal structure and function of cilia and goblet cells in a short period may promote the process of chronic inflammation. A recent study has suggested that oxygen radical-mediated damage to the mucosa may delay recovery and lead to recurrent infections and chronic ear disease (Aydogan et al., 2013). Oxidative-stress mediated mucosal immunity plays a role in the OM recovery, which may contribute to the incidence of COM. The longer period of severe inflammation induced by PGPS in *Tlr2*^{-/-} mice may stem from stronger oxidative stress and severer tissue damage, limiting the recovery in *Tlr2*^{-/-} mice. Therefore, treatment with anti-oxidant at the early stage of the disease process may be valuable in management of patients with COM.

SA has potent anti-inflammatory, anti-edematous, and anti-oxidative activity (Du et al., 2011; Montopoli et al., 2007; Wang et al., 2012; Wei et al., 2011; Xin et al., 2011; Yang et al., 2011). In this study, we found that treatment with SA mitigated the severity of OM and

improved the function of MES in *TLR2*^{-/-} mice. It is possible that SA elevated the concentrations of blood glucocorticoid and up-regulated the expression of glucocorticoid receptor, leading to inhibition of inflammation in *TLR2*^{-/-} mice. Given that SA is a relatively safe and has long-lasting effects, but does not cause immunosuppression (Wang et al., 2009) our novel data suggest that SA may be valuable for treatment of OM.

Accordingly, the therapeutic potential and molecular mechanisms of SA-treatment in OM deserve us to further elucidate.

In summary, our data indicated that inoculation with PGPS effectively induced OM in *TLR2*^{-/-} mice, which provided a novel model for exploring the molecular pathogenesis of OM and screening anti-OM drugs. Furthermore, our findings demonstrated that treatment with SA significantly mitigated the severity of OM and improved the function of MEs in *TLR2*^{-/-} mice. Therefore, our findings may provide new insights in the pathogenesis for OM and aid in design of new therapies for OM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Fengchan Han for experiment guidance; Jing Yuan for comments on the manuscript. This work was supported by the National Institutes of Health, National Institute on Deafness and Other Communication Disorders [R01-DC009246, R01-DC007392, R21-DC005846]; Foundation of Taishan Scholar, China [tshw20110515]; National Natural Science Foundation of China [81271085, 30772760], and Natural Science Foundation [ZR2012HZ004, ZR2012HL30] of Shandong Province.

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Highlights

- We provided a novel model for exploring the molecular pathogenesis of OM.
- *Tlr2*^{-/-} mice were susceptible to PGPS-induced OM.
- We successfully used an *ex vivo* technique to quantitatively analyze motile cilia function in mice.
- Treatment with SA mitigated the pathogenic damage in the ME.

Figure 1. Inoculation with PGPS induces a severer OM in *Tlr2*^{-/-} mice

WT and *Tlr2*^{-/-} mice were inoculated with the indicated doses of PGPS and their average ABR thresholds and tympanometry were measured longitudinally after inoculation. Data are representative images and expressed as the mean $\pm$ SEM of individual groups (n=6 per group per time point) of mice from separate experiments. (A) The average ABR thresholds and tympanometry. Dose-dependent effects of PGPS-induced OM on hearing function. V: the mean values of volume; C: compliance in tympanometry parameters; G: the gradient; P: the pressure. (B) Quantitative analysis of average ABR thresholds and tympanometry values. As the indicated doses of PGPS, the ABR thresholds increased in both groups, but particularly in the mutant mice. The mean ABR thresholds in the mutant mice were significantly higher than that of the wild group at each stimulus frequency, and at each indicated dosage, except at 16 KHz. There were significant differences in compliance between the wild type and mutant mice at the dosage of 30 μ g and 60 μ g. (* P < 0.05, ** P < 0.01 vs. the pre-inoculation; two-tailed unpaired Student's t -test)

Figure 2. H&E histology shows developments of middle ear structures and pathology after inoculation with PGPS

(A) Histopathological analysis (H&E staining). As the dose increased, inflammation became increasingly obvious in both mice, particularly in the mutant mice progressed to a much greater severity. (B) Inflammation areas in the MEs. Enlarged image corresponding to the rectangular region in the upper left corner. It indicated neutrophil-related mucosal inflammation in both strains of mice (right panel), and many proliferative fibroblasts in the sub-epithelial connective tissue layers of the *Tlr2*^{-/-} mice, and hyperplastic epithelium in the WT mice (left panel). (C) Histological examination of the MEs at 7 and 15 days post inoculation. (D and E) The epithelial thickness and inflammatory areas in the MEs. The areas covering neutrophil infiltrates and epithelial thickness in the *Tlr2*^{-/-} mice were significantly larger than that in the WT mice. * $P < 0.05$, ** $P < 0.01$ (two-tailed unpaired Student's *t*-test); Scale bars, 100 μ m (A, C), 10 μ m (B).

Figure 3. Inoculation with PGPS impairs the ciliary structure and function in *Tlr2*^{-/-} mice
 The mucosa in the MEs of individual mice was carefully removed at 3 days post inoculation and the ciliary motility, cilia beat frequency (CBF) and survival periods of individual mucosal samples were recorded. The ciliary structure and mucosal hyperplasia were imaged. Data are representative images and expressed as the mean $\pm$ SEM of individual groups (n=5-8 per group per time point) of mice from three separate experiments. (A) Cilia swings and generated the waveforms. The red arrows indicate the ciliary swing. (B) The survival periods of the cilia (*, #p < 0.05 vs. the unmanipulated *Tlr2*^{-/-} mice). (C) The quantitative analysis of the CBF. (D) The ciliary structure and mucosal hyperplasia (MC: mucosal cells). *, #P < 0.05, **P < 0.01; two-tailed unpaired Student's *t*-test (C); Kaplan-Meier and log-rank test (B); Scale bar, 10 μ m (A, D)

Figure 4. Goblet cell absence and dysregulated gene expression in $Tlr2^{-/-}$ mice following PGPS inoculation

The relative levels of the indicated gene mRNA transcripts in the MEs were determined by quantitative RT-PCR and the ME tissue sections of WT and $Tlr2^{-/-}$ mice at 3 days post inoculation were stained with H&E and AB-PAS. Data are representative images and expressed as the mean $\pm$ SEM of individual groups (n=6 per group per time point) of mice from three separate experiments. (A) The relative levels of gene expression in the MEs. (B) H&E and AB-PAS staining of the mucosa and epithelium of the MEs (GC: goblet cells). Scale bars, 20 μ m. * P < 0.05, ** P < 0.01.

Figure 5. SA treatment inhibits the PGPS-induced inflammation and tissue damage and improves hearing function in *Tlr2*^{-/-} mice

Tlr2^{-/-} mice were treated with saline vehicle or SA for six consecutive days beginning at three days before PGPS inoculation and the hearing function and tissue damage in individual mice were analyzed. Data are representative images and expressed as the mean $\pm$ SEM of individual groups (n=6 per group per time point) of mice from three separate experiments. (A) The average ABR and tympanometry values. ABR thresholds were lower in the SA-treated group than in the untreated group (PGPS control group) and saline group. There was no significant difference in ABR thresholds between the control and saline group. The gradient (G) in SA-treated group was significantly lower than those of control group. There was no significant difference in tympanometry values between the control and saline group. Accordingly, we combined the control and saline groups as the control group for the following experiments. (B) Histological examination of the MEs (top panel; Scale bar, 100 μm). The arrows indicate the infiltrated macrophages and asterisk for degraded macrophages (below panel; Scale bar, 10 μm). (C) The epithelial thickness. (D) The inflammatory areas. * $P < 0.05$; ** $P < 0.01$; one-way ANOVA (A); two-tailed unpaired Student's *t*-test (C, D)

Figure 6. SA-treated group reduces cilia degeneration

Cilia in the middle ear mucosa from SA-treated group and the untreated group (control group). (A, B) The CBF and cilia survival. (C) SA treatment decreased the ciliary loss and damage (Scale bar, 10 μ m). * $P < 0.05$; ** $P < 0.01$; two-tailed unpaired Student's t -test (A); Kaplan-Meier and log-rank test (B)

Figure 7. Potential pathways and SA-treatment regulates the gene expression

(A) Quantitative RT-PCR analysis of the relative levels of selected gene expression. In comparison with that in the control group, significantly higher levels of MUC5ac, MUC5b, and MIP1α expression and lower levels of IL-6, SOD, iNOS, MPO, and caspase 3 expression were detected in the MEs of SA-treated group. (B) Illustration of the potential pathways. Traditionally, PGPS activates the TLR2-related signaling, which is absent in TLR2-deficient mice (indicated by the inset purple images). Therefore, a deficiency in TLR2 expression may hinder the expression of its related cytokines/chemokines/mucin in response to PGPS-infection, thus resulting in the changes in relevant gene expression (showed by Figure 7A). However, PGPS may induce LTB4 secretion via recognizing peptidoglycan recognition proteins (PGRPs) on neutrophils, thus recruiting neutrophil into the MEs in a TLR2-independent manner. SA increases the expression of glucocorticoid and glucocorticoid-receptor (GR), thus activating the NF-κB signaling, which regulates the expression of its related cytokines/chemokines/mucin (showed by Figure 7A), inhibiting the activity of phospholipase A2 (PLA2), decreasing the secretion of LTB4 to achieve anti-inflammatory effect. * $P < 0.05$; ** $P < 0.01$