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# Phosphodiesterase 4B Mediates Extracellular Signal-regulated Kinase-dependent Up-regulation of Mucin MUC5AC Protein by *Streptococcus pneumoniae* by Inhibiting cAMP-protein Kinase A-dependent MKP-1 Phosphatase Pathway*[S]

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**Background:** Mucus overproduction is a hallmark of otitis media (OM) induced by *Streptococcus pneumoniae*.
**Results:** PDE4B mediates *S. pneumoniae*-induced MUC5AC up-regulation by inhibiting the expression of a negative regulator MKP-1.
**Conclusion:** PDE4-specific inhibitor rolipram inhibits *S. pneumoniae*-induced MUC5AC up-regulation.
**Significance:** Identifying PDE4B as a molecular target for inhibiting MUC5AC by up-regulating MKP-1 may have significant therapeutic potential for treating OM.

**Otitis media (OM) is the most common childhood bacterial infection and the major cause of conductive hearing loss in children. Mucus overproduction is a hallmark of OM. *Streptococcus pneumoniae* is the most common Gram-positive bacterial pathogen causing OM. Among many mucin genes, MUC5AC has been found to be greatly up-regulated in the middle ear mucosa of human patients with OM. We previously reported that *S. pneumoniae* up-regulates MUC5AC expression in a MAPK ERK-dependent manner. We also found that MAPK phosphatase-1 (MKP-1) negatively regulates *S. pneumoniae*-induced ERK-dependent MUC5AC up-regulation. Therapeutic strategies for up-regulating the expression of negative regulators such as MKP-1 may have significant therapeutic potential for treating mucus overproduction in OM. However, the underlying molecular mechanism by which MKP-1 expression is negatively regulated during *S. pneumoniae* infection is unknown. In this study we show that phosphodiesterase 4B (PDE4B) mediates *S. pneumoniae*-induced MUC5AC up-regulation by inhibiting the expression of a negative regulator MKP-1, which in turn leads to enhanced MAPK ERK activation and subsequent up-regulation of MUC5AC. PDE4B inhibits MKP-1 expression in a cAMP-PKA-dependent manner. PDE4-specific inhibitor rolipram inhibits *S. pneumoniae*-induced MUC5AC up-regulation both *in vitro* and *in vivo*. Moreover, we show that PDE4B plays a critical role in MUC5AC induction. Finally, topical and post-infection administration of rolipram into the middle ear potently inhibited *S. pneumoniae*-induced MUC5AC up-regulation. Collectively, these data demonstrate that PDE4B mediates ERK-dependent up-regulation of mucin MUC5AC by *S. pneumoniae* by inhibiting cAMP-PKA-dependent MKP-1 pathway. This study may lead to novel therapeutic strategy for inhibiting mucus overproduction.**

Otitis media (OM)[4] is the most common childhood bacterial infection (1–6). OM represents the leading cause of the conductive hearing loss in children in the US (1, 2). Because OM causes hearing loss during a crucial time period for speech and language development, children who have had early hearing impairment due to frequent middle ear infections may later suffer from speech and language disabilities (7, 8). Despite need for prophylactic measures, development of highly effective vaccines for preventing OM still remains a great challenge (3, 9–11). *Streptococcus pneumoniae* represents the most common Gram-positive bacterial pathogen causing OM. Although currently available *S. pneumoniae* conjugate childhood vaccine is protective against invasive infections caused by vaccine serotypes, its effect on protecting against *S. pneumoniae* OM is rather modest (12–14). In addition, non-vaccine serotypes and newly emerging serotypes are steadily replacing the vaccine

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[4] The abbreviations used are: OM, Otitis media; TLR, Toll-like receptor; PKA, protein kinase A; PKI, PKA inhibitor; Q-PCR, quantitative RT-PCR; IF, immunofluorescence; PDE4B, phosphodiesterase 4B; MKP-1, MAPK, phosphatase-1; IBMX, isobutylmethylxanthine.

serotypes (15). Moreover, the current treatment of *S. pneumoniae* OM heavily replies on systemic use of antibiotics, which however leads to rapid emergence of multidrug resistant strains (16–18). Therefore, development of alternative novel therapeutic strategies is urgently needed for treating OM.

Mucus overproduction is a hallmark of OM. It has been shown that overproduction of mucin in middle ear plays an important role in the development of conductive hearing loss (7, 9, 19–22). Mucins are high molecular weight glycoproteins that constitute the major component of mucus secretions in the middle ear, trachea, digestive, and reproductive tracts. They normally protect and lubricate the epithelial surface and trap particulates, including bacteria and viruses, for mucociliary clearance, at least in part because of the extraordinary diversity of their carbohydrate side chains (23, 24). However, in patients with OM, whose mucociliary clearance mechanisms become defective, excessive production of mucin occurs, overloading the mucociliary escalator. As the mucus effusion increases, the eardrum and middle ear bones no longer move freely, thus resulting in hearing difficulties. Indeed, it has been shown that a higher concentration of mucin in mucoid effusions is closely associated with more severe hearing impairment (7, 22). Thus, although up-regulation of mucins in infectious disease represents an important host innate defense response against invading microbes (25), excess mucin production contributed significantly to the pathogenesis process of OM. Therefore, tight regulation of mucin expression is critical for balancing beneficial and detrimental effects of mucin production. To prevent overactive mucus overproduction, mucin up-regulation must be tightly controlled.

To date, 24 mucin genes have been identified (24–30). Among these, mucin MUC5AC has been shown to play an important role in the pathogenesis of OM (24, 31–36). Recent studies have demonstrated that the mRNAs for human MUC5AC are highly expressed in middle ear mucosa of patients with OM (24, 25, 37). In addition to the direct evidence for the up-regulation of mucin MUC5AC in human middle ear, *in vitro* molecular biology studies also demonstrate that human mucin MUC5AC is up-regulated at both mRNA and protein levels by the major OM bacterial pathogen *S. pneumoniae* in a well established human middle ear epithelial HMEEC cell culture system as well as in primary human bronchial epithelial NHBE cells cultured under both routine and air-liquid interface conditions (32, 34, 38–44). Consistent with the finding of mucin MUC5AC up-regulation in middle ear of human patients and human middle ear epithelial cells, up-regulation of MUC5AC by *S. pneumoniae* was also confirmed in the middle ear of a well established mouse model of OM (38, 45). Although it is evident that mucin MUC5AC is up-regulated in the pathogenesis of OM, both *in vitro* and *in vivo*, the molecular mechanisms underlying the tight regulation of mucin MUC5AC up-regulation, however, still remain unclear.

Mitogen-activated protein kinases (MAPKs) are a superfamily of serine/threonine protein kinases widely conserved among eukaryotes. They transduce a variety of external signals, leading to a variety of cellular responses that include proliferation, differentiation, apoptosis, and host defense response (46–48). To date, three major MAPK pathways have been identified in mammals: extracellular signal-regulated kinase (ERK), stress-activated protein kinase/JNK, and p38 (46). Growth factor-induced ERK activation is relatively well understood, but the signaling mechanisms underlying Toll-like receptor (TLR)-mediated activation of ERK in host mucosal defense response remain largely unknown (49, 50). Previously, we found that *S. pneumoniae* up-regulates mucin MUC5AC transcription via TLR-dependent activation of ERK, but not p38, in human middle ear epithelial HMEEC cell culture system *in vitro* and in a mouse model of OM *in vivo* (38, 39). To prevent detrimental effects of TLR-dependent induction of host mucosal response, host has evolved negative regulatory mechanisms to specifically inhibit overactive mucosal response by targeting the key positive regulators, *e.g.* ERK. Stimulation of TLRs induces the expression of the gene encoding the MAPK phosphatase-1 (MKP-1), a dual-specificity phosphatase that preferentially dephosphorylates ERK or p38 MAPK, resulting in the attenuation of TLR-triggered host mucosal defense response (51–55). Previously, we found that *S. pneumoniae*-induced ERK activation, in addition to positively mediating up-regulation of mucin MUC5AC, also leads to up-regulation of MKP-1 expression, which in turn acts as a negative feedback regulator for ERK-dependent MUC5AC up-regulation (39). This finding was of particular translational interest and significance because increasing the expression of negative regulators has long been thought as attractive and effective therapeutic strategy for treating overactive host response, *e.g.* mucus overproduction or overactive inflammatory response, without causing serious adverse effects (52, 54, 55). Therefore, investigating the molecular mechanisms by which MKP-1 is up-regulated may not only bring novel insights into the tight regulation of mucin production but also lead to the identification of novel therapeutic targets for controlling mucus overproduction in OM.

The cyclic nucleotide phosphodiesterase (PDE) superfamily comprises a group of structurally and functionally related enzymes that degrade the phosphodiester bond in the important second messenger molecules cAMP and cGMP (51, 56–58). They play critical roles in regulating cellular response by controlling the intracellular levels of cAMP and cGMP (59–63). To date, 11 families, namely PDE1-PDE11, have been identified in mammals based on their distinct kinetic properties, regulatory mechanisms, and sensitivity to selective inhibitors (51, 64–66). PDEs have long been thought as attractive and excellent therapeutic targets due to their unique tissue distribution, structural properties, and functional properties as well as sensitivity to selective inhibitors (67–73). Indeed, a number of PDE inhibitors have been already successfully developed as effective therapeutic agents used in clinic (51, 65, 71). One of the most well known PDE inhibitors is Viagra, an inhibitor of cGMP-specific PDE5, which enhances the vasodilator effects of cGMP and has been used to successfully treat erectile dysfunction (74). Despite their known roles in regulating the pathogenesis processes of certain cardiovascular and neurological disorders (75, 76), the role of PDE in host defense, in particular the overactive host mucosal defense response, *e.g.* mucus overproduction, remains largely unknown.

In this study we report that PDE acts as a positive regulator for *S. pneumoniae*-induced mucin MUC5AC expression both

in the middle ear epithelial cells *in vitro* and in the middle ear of mice *in vivo*. PDE up-regulates MUC5AC expression by inhibiting MKP-1 expression, which in turn enhances ERK-mediated up-regulation of MUC5AC expression. Moreover, PDE-mediated inhibition of MKP-1 expression is dependent on cAMP-protein kinase A (PKA) signaling pathway. Finally, we found that among many PDE superfamilies, PDE4B is specifically involved in regulating MUC5AC up-regulation by *S. pneumoniae*. Of particular interest in this study is that topical and post-infection administration of rolipram, a PDE4-specific inhibitor, through tympanic membrane to the middle ear inhibits its MUC5AC expression in the mouse model of OM by *S. pneumoniae*. These results suggest that inhibition of PDE4B may represent a potential therapeutic strategy for treating mucus overproduction in OM.

## EXPERIMENTAL PROCEDURES

*Reagents and Antibodies*—IBMX, rolipram, Ro-20-1724, 8-bromo-cAMP, forskolin, and cAMP "PLUS" EIA kit were from Biomol (Farmingdale, NY). PKA inhibitor (PKI) was purchased from Calbiochem. Antibodies against phospho-ERK1/2 and total ERK1/2 are from Cell Signaling. Antibodies for MKP-1, PDE4B, and actin were purchased from Santa Cruz.

*Bacteria Strains and Culture*—Clinical OM isolates of *S. pneumoniae* strains 6B, 19F, and 23F as well as the well characterized D39 were used in this study (38, 39, 77–79). Cells were treated with *S. pneumoniae* at a concentration of 10–100 multiplicity of infection. For *in vivo* animal experiments, mid-log phase of *S. pneumoniae* obtained 6 h after incubation were prepared at the concentration of $1 \times 10^9$ colony forming unit (cfu) per ml in saline by centrifugation followed by washing with sterile saline.

*Cell Culture*—Human middle ear epithelial HMEEC cells and primary human bronchial epithelial NHBE cells were cultured as described previously (38, 40, 80). Primary normal human bronchial epithelial NHBE cells were purchased from Lonza (Walkersville, MD) and were maintained in bronchial epithelial cell growth medium. Air-liquid culture of human primary NHBE cells was conduced as described previously (32, 81). All cells were cultured in a humidified atmosphere of 5% $CO_2$ at 37 °C.

*Plasmids, Transfections, and Luciferase Reporter Assay*—MUC5AC luciferase reporter gene construct was described previously (32, 34, 38). Cells were transfected with MUC5AC luciferase reporter gene in triplicate with TransIL-LT1 reagent (Mirus, Madison, WI) following the manufacturer's instructions. For experiments with inhibitors, the transfected cells were pretreated with inhibitors including IBMX, rolipram, Ro-20-1724, 8-bromo-cAMP, forskolin, or PKI for 1 h before *S. pneumoniae* treatment. Transcriptional activity of MUC5AC was measured by using luciferase assay as described previously (32, 34, 38).

*Small Interfering RNA (siRNA)*—siRNA for PDE4B was purchased from Dharmacon (Lafayette, CO). The siRNA was transfected into epithelial cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions and as described previously (38, 82–84).

*RNA Isolation and Real-time Quantitative RT-PCR (Q-PCR)*—Q-PCR analysis of human and mouse MUC5AC, MKP-1, and PDEs was conducted as follows. Total RNA was isolated with TRIzol reagent (Invitrogen) by following the manufacturer's instructions. The reverse transcription reaction was performed using TaqMan reverse transcription reagents (Applied Biosystems). PCR amplification was performed with SYBR Green Universal Master Mix (Applied Biosystems). Reactions were amplified and quantified by using an ABI 7500 sequence detector and the manufacturer's software (Applied Biosystems). Relative quantities of mRNAs were obtained by using the comparative threshold cycle (Ct) method and were normalized using human or mouse glyceraldehyde-3-phosphate dehydrogenase as an endogenous control. The primers for human and mouse MUC5AC and MKP-1 were described previously (33, 34, 38, 39, 44, 81). The primer sequences for human and mouse PDE4A & B are as follows: human PDE4A forward primer, 5′-TCTCCTCCATCCGTACCTTG-3′; human PDE4A reverse primer, 5′-TGGCTTGGAGAAAAATGGTC-3′; human PDE4B forward primer, 5′-TGATGCTCAGGACATTCTCG-3′; human PDE4B reverse primer, 5′-AGTGGTGGTGAGGGACTTTG-3′; mouse PDE4A forward primer, 5′-GCCATGGAACAGTCAAAGGT-3′; mouse PDE4A reverse primer, 5′-ATGTGCTGAGGCTGTCTCCT-3′; mouse PDE4B forward primer, 5′-GTAGAGGCCAGTTCCCATCA-3′; mouse PDE4B reverse primer, 5′-CCAACACCTAGTGCAGAGCA-3′.

*Enzyme-linked Immunosorbent Assay (ELISA)*—Direct ELISA assay was used to measure the MUC5AC protein production as described previously (38, 39). Cells were treated with *S. pneumoniae* for 12 h with inhibitors IBMX or rolipram or vehicle pretreatment. MUC5AC protein production was measured in the cell culture supernatant as described previously (38, 39). The intracellular level of cAMP was measured as described previously (85, 86). Cells were treated with *S. pneumoniae* in the presence of rolipram or vehicle, and intracellular cAMP concentration was measured by using cAMP "PLUS" EIA kit (Biomol, NY) by following the manufacturer's instruction.

*PDE4 Activity*—cAMP-specific PDE activity was measured in the cell lysate in the presence of vehicle or rolipram with 0.3 $\mu$M [$^3$H]cAMP as substrate. PDE4-specific activity was determined by subtracting PDE specific inhibited activity from total PDE activity as described previously (85–87).

*Western Blot Analysis*—Western blot analyses were performed as described previously (40, 77, 79, 82, 88). Cells were treated with *S. pneumoniae* in the presence of rolipram or vehicle. Cell lysate was separated in sodium dodecyl sulfate polyacrylamide gel, transferred to polyvinylidene fluoride membrane, and probed with antibodies against phospho-ERK1/2, total ERK1/2, MKP-1, PDE4B, or actin.

*Mice and Animal Experiments*—C57BL/6 mice were purchased from the National Cancer Institute, National Institutes of Health. Middle ears of mice were inoculated with *S. pneumoniae* through tympanic membrane, and mice were then sacrificed by intraperitoneal inoculation of sodium pentobarbital (100 mg/kg in 100 $\mu$l of saline) at the times indicated in the figures (38, 84). Eardrums of mice were inspected for signs of OM and the direct visualization of opaque fluid behind the tympanic membrane and photographed for recording patho-

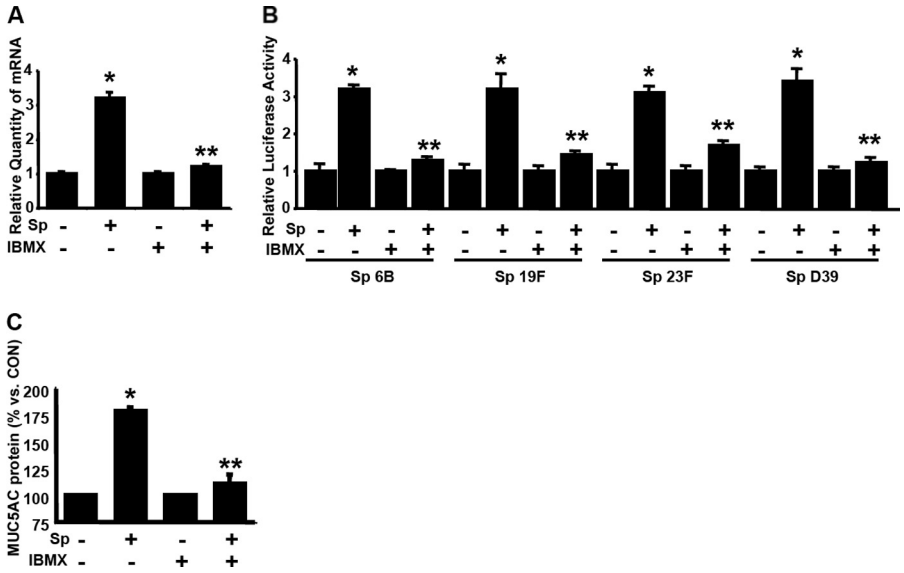


FIGURE 1. **PDE acts as a positive regulator for *S. pneumoniae*-induced mucin MUC5AC expression in human middle ear epithelial cells *in vitro*.** *A*, human middle ear epithelial HMEEC cells were pretreated with IBMX, a general inhibitor for PDEs, for 1 h and treated with *S. pneumoniae* for 5 h. Relative quantity of human MUC5AC mRNA expression was measured by real-time Q-PCR analysis. *B*, cells transfected with MUC5AC-luciferase reporter gene were treated with *S. pneumoniae* strains 6B, 19F, 23F, or D39 for 5 h with or without IBMX pretreatment. MUC5AC transcriptional activity was measured by luciferase assay. *C*, cells were treated with *S. pneumoniae* for 12 h with or without IBMX pretreatment, and expression level of MUC5AC protein was measured in the cell culture supernatant by direct ELISA assay against MUC5AC. Data represent the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* control; **, $p < 0.05$ *versus S. pneumoniae* (*Sp*) alone. Data are representative of three or more independent experiments.

logical changes of OM under the otoscope. Ears were dissected from the skull, snap-frozen in the liquid nitrogen for RNA extraction, or fixed with 10% buffered neutral formalin for histological analysis. For inhibitor experiments, animals were intraperitoneally inoculated with IBMX or rolipram before trans-tympanic inoculation of *S. pneumoniae*. For the post-treatment experiments, *S. pneumoniae*-inoculated mice were topically treated with rolipram through tympanic membrane 3, 6, or 9 h after *S. pneumoniae* inoculation. All animal experiments were approved by the Institutional Animal Care and Use Committee at Georgia State University.

*Histological Analysis and Immunofluorescence (IF) Staining*—For histological analysis, dissected whole bullae were fixed with 10% buffered neutral formalin overnight followed by overnight decalcification. Tissues were then processed for paraffin embedding, and whole bullae were serial-sectioned at 4-$\mu$m thickness and mounted into 12 slides. Sections were then stained with hematoxylin and eosin stain to visualize, evaluated using Axiovert 40 CFL (Carl Zeiss), and recorded with an AxioCam MRC (Carl Zeiss). Mucosal thickness in the middle ear cavity was measured from 10 sections per each experimental group using AsioVison LE Image system software (Carl Zeiss) to measure and quantify the degree of inflammation such as mucosal hyperplasia and inflammatory cell infiltration. The average of mucosal thickness from 10 sections was presented in a *bar graph* as the mean ± S.D. along with representative tissue sections. IF detection of MUC5AC protein was performed using mouse anti-MUC5AC (clone 45M1, NeoMarker) and FITC-conjugated goat anti-mouse IgG (Santa Cruz) in the middle ear tissues of WT mice inoculated with *S. pneumoniae* with PDE inhibitors IBMX or rolipram or vehicle control. Before MUC5AC stain, endogenous mouse IgG was masked using unconjugated Fab fragments of affinity-purified anti-mouse IgG (Rockland) to remove nonspecific background staining. MUC5AC protein expression in the mouse middle ear tissues was then quantified by measuring the percentages of MUC5AC positively stained areas from whole middle ear mucosa by using quantitative image analysis software Image-Pro Plus (Image Pro 6.2, Media Cybenetics Inc.) (89) and are presented in a bar graph as the mean ± S.D. along with representative staining sections.

*Statistical Analysis*—Data are shown as the mean ± S.D. Statistical evaluation was done by unpaired Student's *t* test, and $p < 0.05$ was taken as a significant difference.

## RESULTS

*PDE Acts as a Positive Regulator for S. pneumoniae-induced Mucin MUC5AC Expression in Human Middle Ear Epithelial Cells in Vitro*—The PDE superfamily comprises 11 subfamilies, namely PDE1-PDE11 in mammals (51, 56–58). They act as important positive and negative regulators of cellular response via second messenger cAMP and cGMP (59–63). PDEs have long been thought as excellent therapeutic targets due to their unique tissue distribution, structural, and functional properties (51, 64–66). However, their roles in pathogenesis of OM, especially in regulating mucin overproduction, are totally unknown. We, thus, sought to determine whether PDEs are also critically involved in mediating *S. pneumoniae*-induced up-regulation of mucin MUC5AC expression.

We first assessed the effect of IBMX, a general inhibitor for PDEs, on MUC5AC induction by *S. pneumoniae* at the mRNA steady-state level using a well established human middle ear epithelial HMEEC cell culture system *in vitro* by performing real-time Q-PCR analysis. As shown in Fig. 1*A*, IBMX inhibited up-regulation of MUC5AC mRNA expression induced by *S. pneumoniae*. Because transcriptional regulation plays an important role in regulating gene expression at the mRNA

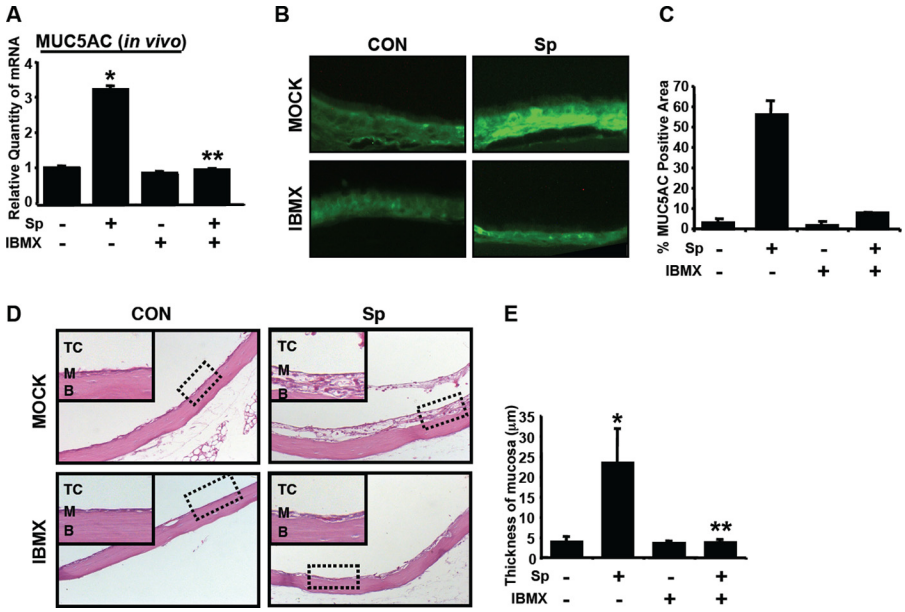


FIGURE 2. **PDE acts as a positive regulator for *S. pneumoniae*-induced mucin MUC5AC expression in the middle ear of mice *in vivo*.** *A*, C57BL/6 mice were intraperitoneally inoculated with IBMX (10 mg/kg body weight). Two hours after IBMX inoculation, *S. pneumoniae* ($1 \times 10^6$ cfu per ear) was inoculated into the middle ear of mice via tympanic membrane. Total RNA was extracted from the bullae 9 h after *S. pneumoniae* inoculation, and relative quantity of mouse MUC5AC mRNA expression was measured by real-time Q-PCR analysis. *B* and *C*, middle ear of the mice inoculated with *S. pneumoniae* ($1 \times 10^6$ cfu per ear) with or without IBMX inoculation was isolated, fixed with formaldehyde, decalcified, and embedded in paraffin. Tissues sections were stained with antibody against MUC5AC, probed with FITC-conjugated goat anti-mouse IgG, and imaged with AxioVert (magnification ×400). *B*, expression of MUC5AC in IF-stained middle ear tissues was quantitated using Image-Pro Plus system (Image-Pro 6.2) (*C*). *D* and *E*, middle ear tissue sections of mice inoculated with *S. pneumoniae* with IBMX or vehicle were stained with hematoxylin and eosin stain (magnification ×200 in large frame; ×400 in inserted frame) and visualized with AxioVert (*D*). Thickness of middle ear mucosa was measured from 10 middle ear tissue sections per experimental group (*E*). Data represented in *A*, *C*, and *E* are the mean ± S.D. ($n = 3$ in *A*, $n = 10$ in *C* and *E*). *, $p < 0.05$ *versus* control inoculation; **, $p < 0.05$ *versus S. pneumoniae* (*Sp*) alone. *CON*, control; *TC*, tympanic cavity; *B*, bone; *M*, mucosa. Data are representative of three or more independent experiments.

steady-state level, we next sought to determine whether IBMX also inhibits *S. pneumoniae*-induced up-regulation of MUC5AC transcription using a MUC5AC promoter-driven luciferase reporter assay. As shown in Fig. 1*B*, up-regulation of MUC5AC transcription by a variety of *S. pneumoniae* strains including common OM pathogens 6B, 19F, and 23F strains as well as a well characterized strain D39 was also blocked by IBMX, thereby suggesting that the inhibitory effect of IBMX on MUC5AC induction may be generalizable to most OM-causing strains of *S. pneumoniae*.

Moreover, inhibition of MUC5AC induction by IBMX was also observed in a variety of other MUC5AC-expressing human epithelial cells including primary airway epithelial NHBE cells, lung epithelial A549 cells, cervical epithelial HeLa cells, and colon epithelial HM3 cells as well as air-liquid interface culture of human lung epithelial cells (data not shown). These data suggest that the inhibitory effect of IBMX on MUC5AC may be common for most mucin-expressing human epithelial cells. Finally, the inhibitory effect of MUC5AC induction by IBMX was also confirmed at the protein level by performing ELISA assay for mucin MUC5AC (Fig. 1*C*), which was chosen because mucin MUC5AC is a secreted protein. Together, these data demonstrate that IBMX blocks *S. pneumoniae*-induced MUC5AC transcription in a number of human mucin-expressing epithelial cells including middle ear epithelial cells *in vitro*.

*PDE Acts as a Positive Regulator for S. pneumoniae-induced Mucin MUC5AC Expression in the Middle Ear of Mice in Vivo*—Because PDE was found as a positive regulator for *S. pneumoniae*-induced MUC5AC up-regulation in the middle ear epithelial cells *in vitro*, we next determined if PDE also regulates MUC5AC expression in the middle ear of mice *in vivo*. We first assessed the effect of IBMX on MUC5AC induction by *S. pneumoniae* at the mRNA level in the middle ear mucosa of mouse, in which *S. pneumoniae* were inoculated into the middle ear of wild-type strain C57BL/6 via a trans-tympanic membrane route. As shown in Fig. 2*A*, IBMX blocked MUC5AC expression at the mRNA level in the middle ear of mouse inoculated with *S. pneumoniae* as assessed by performing Q-PCR analysis. Pre-inoculation of IBMX also blocked MUC5AC up-regulation at the protein level in the middle ear tissues of mice as assessed by IF staining using MUC5AC-specific antibody (Fig. 2*B*). MUC5AC expression at the protein level was also quantified using a Quantitative Imaging Analysis system Image-Pro Plus (Image-Pro 6.2, Media Cybernetics Inc.) (Fig. 2*C*) (89). Consistent with these results, IBMX also inhibited *S. pneumoniae*-induced middle ear mucosal thickening and polymorphonuclear neutrophil infiltration in the middle ear mucosa, the key characteristic pathological changes of OM (Fig. 2*D*). The inhibitory effects of IBMX on these pathological changes were also quantitatively analyzed using AxioVision LE image system (Carl Zeiss) (Fig. 2*E*). Collectively, our data demonstrated that IBMX, a general inhibitor for PDEs, inhibits *S. pneumoniae*-induced mucin MUC5AC gene expression *in vitro* and *in vivo*, thereby suggesting that PDEs play a critical role in regulating *S. pneumoniae*-induced MUC5AC up-regulation.

*PDE4 Plays a Critical Role in S. pneumoniae-induced Mucin MUC5AC Expression in Human Middle Ear Epithelial Cells in Vitro*—Because we identified PDE as a positive regulator for *S. pneumoniae*-induced mucin MUC5AC expression, next we

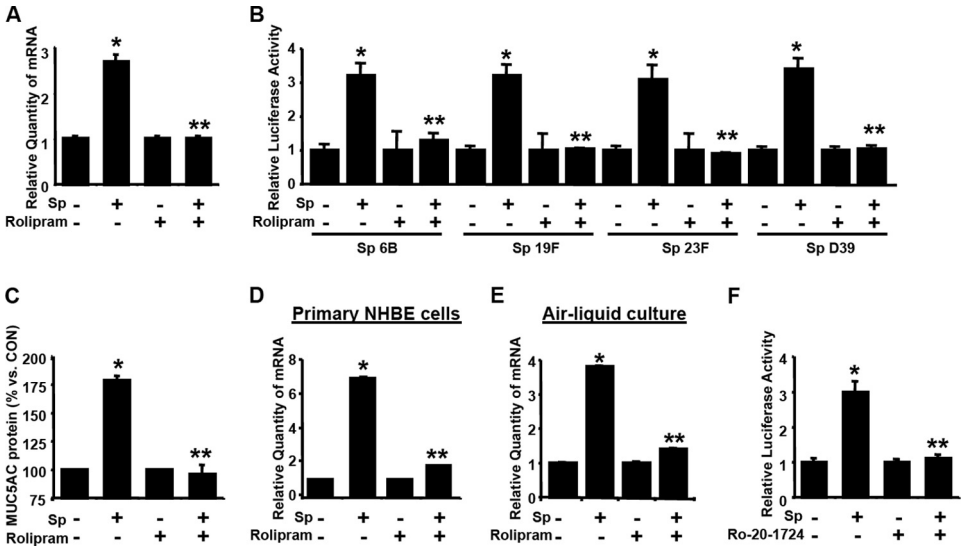


FIGURE 3. **PDE4 plays a critical role in *S. pneumoniae*-induced mucin MUC5AC expression in human middle ear epithelial cells *in vitro*.** *A*, human middle ear epithelial HMEEC cells were pretreated with rolipram, a specific inhibitor for PDE4, for 1 h and treated with *S. pneumoniae* for 5 h. Relative quantity of human MUC5AC mRNA expression was measured by real-time Q-PCR analysis. *B*, cells transfected with MUC5AC-luciferase reporter gene were treated with *S. pneumoniae* strains 6B, 19F, 23F, or D39 for 5 h with or without rolipram pretreatment. MUC5AC transcriptional activity was measured by luciferase assay. *C*, cells were treated with *S. pneumoniae* for 12 h with or without rolipram pretreatment, and expression levels of MUC5AC protein were measured in the cell culture supernatant by direct ELISA assay against MUC5AC. *D* and *E*, primary NHBE cells (*D*) and air-liquid culture of NHBE cells (*E*) were pretreated with rolipram, a specific inhibitor for PDE4, for 1 h and treated with *S. pneumoniae* for 5 h. Relative quantity of human MUC5AC mRNA expression was measured by real-time Q-PCR analysis. *F*, HMEEC cells transfected with MUC5AC-luciferase reporter gene were treated with *S. pneumoniae* for 5 h with or without pretreatment with Ro-20-1724, a specific inhibitor for PDE4. MUC5AC transcriptional activity was measured by luciferase assay. Data represent the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* control; **, $p < 0.05$ *versus S. pneumoniae* (*Sp*) alone. Data are representative of three or more independent experiments.

determined which subfamily of PDE is involved in regulating *S. pneumoniae*-induced MUC5AC expression. Because recent studies showed that among 11 PDE families, PDE4 plays a critical role on immune/inflammatory responses, and PDE4 inhibitor showed anti-inflammatory effects in respiratory inflammatory diseases including chronic obstructive pulmonary disease, we sought to determine whether PDE4 is involved in MUC5AC up-regulation. Interestingly, PDE4-specific inhibitor rolipram but not PDE1-specific inhibitor blocked *S. pneumoniae*-induced up-regulation of MUC5AC expression at the levels of mRNA, transcription, and protein as assessed using Q-PCR, luciferase assay, and ELISA (Fig. 3, *A–C*, and data not shown). In addition to *S. pneumoniae* OM strain 6B shown in Fig. 3, *A* and *C*, the inhibitory effect of rolipram on MUC5AC induction was also observed in *S. pneumoniae* OM strains 19F and 23F as well as in a well characterized strain D39 (Fig. 3*B*). Moreover, the inhibition of MUC5AC by rolipram was also confirmed in human primary airway epithelial NHBE cells (Fig. 3*D*) cultured under routine and air-liquid interface conditions (Fig. 3*E*). Finally, the specific involvement of PDE4 in regulating MUC5AC induction was also confirmed using another PDE4 specific inhibitor Ro-20-1724 (Fig. 3*F*). Collectively, our data suggest that PDE4 indeed plays an important role in regulating MUC5AC induction by *S. pneumoniae in vitro*.

*PDE4 Is a Key Positive Regulator for S. pneumoniae-induced Mucin MUC5AC Expression in the Middle Ear of Mice in Vivo*—We next determined if PDE4 also plays a critical role in regulating MUC5AC induction *in vivo*. As shown in Fig. 4, *A–C*, PDE4-specific inhibitor rolipram blocked MUC5AC induction at the mRNA and the protein levels in the middle ear of mice inoculated with *S. pneumoniae* as assessed by performing Q-PCR analysis (Fig. 4*A*) and IF staining using MUC5AC-specific antibody (Fig. 4*B*). MUC5AC expression at the protein level in the middle ear tissues of mice was also quantified using a Quantitative Imaging Analysis system Image-Pro Plus (Image-Pro 6.2) (Fig. 4*C*) (89). Furthermore, rolipram blocked *S. pneumoniae*-induced pathological changes of OM as assessed by performing otoscopic examination in *S. pneumoniae*-inoculated mice (Fig. 4*D*). Middle ears of mice inoculated with *S. pneumoniae* but not with saline as a control showed typical symptoms of OM including congestion and swelling of tympanic membrane and mucous effusion accumulation inside bulla as early as 1 day after *S. pneumoniae* inoculation, and OM symptoms were maximal around 3 days and no later than 4 days after *S. pneumoniae* inoculation. By day 7, OM had largely been cleared. Rolipram blocked *S. pneumoniae*-induced overproduction of mucous effusion at all time points (days 1, 3, and 5) after *S. pneumoniae* inoculation (Fig. 4*D* and data not shown). Consistent with these results, rolipram also inhibited *S. pneumoniae*-induced middle ear mucosal thickening and polymorphonuclear neutrophil infiltration in the middle ear mucosa as assessed by histological analysis (Fig. 4*E*). The inhibitory effects of rolipram on these pathological changes were also quantitatively analyzed using an AxioVision LE (Fig. 4*F*). Taken together, our data shown in both Figs. 3 and 4 demonstrated that PDE4 plays a critical role in regulating *S. pneumoniae*-induced MUC5AC up-regulation.

*PDE4 Mediates ERK-dependent Up-regulation of MUC5AC by Inhibiting MKP-1 in Vitro and in Vivo*—Having demonstrated that PDE4 plays an important role in positively regulating MUC5AC induction by *S. pneumoniae*, still unknown is how PDE4 regulates MUC5AC induction. *S. pneumoniae* induces MUC5AC expression via a TLR4-MyD88-TRAF6-dependent signaling pathway as knockdown of TLR4, MyD88, and TRAF6 using specific siRNAs markedly inhibited *S. pneu-*

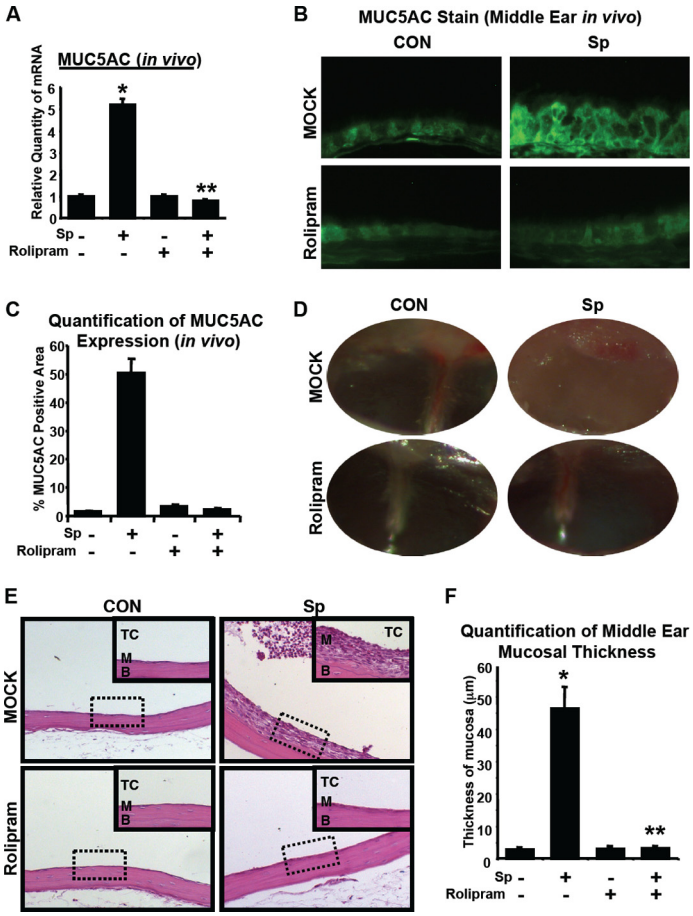


FIGURE 4. **PDE4 is a key positive regulator for *S. pneumoniae*-induced mucin MUC5AC expression in the middle ear of mice *in vivo*.** *A*, C57BL/6 mice were intraperitoneally inoculated with rolipram (10 mg/kg body weight). Two hours after rolipram inoculation, *S. pneumoniae* ($1 \times 10^6$ cfu per ear) was inoculated into the middle ear of mice via tympanic membrane. Total RNA was extracted from the bullae 12 h after *S. pneumoniae* inoculation, and relative quantity of mouse MUC5AC mRNA expression was measured by real-time Q-PCR analysis. *B* and *C*, middle ears of the mice inoculated with *S. pneumoniae* ($1 \times 10^6$ cfu per ear) with or without rolipram inoculation were isolated, fixed with formaldehyde, decalcified, and embedded in paraffin. Middle ear tissue sections of mice inoculated with *S. pneumoniae* with rolipram or vehicle were stained with antibody against MUC5AC, probed with FITC-conjugated goat anti-mouse IgG, and imaged with AxioVert (magnification ×400) (*B*). Expression of MUC5AC in IF-stained middle ear tissues was quantitated using Image-Pro Plus system (Image-Pro 6.2) (*C*). *D*, mice were trans-tympanically inoculated with *S. pneumoniae* with or without rolipram pretreatment, and tympanic cavity was observed and recorded under the otoscope. *E* and *F*, middle ear tissue sections of mice inoculated with *S. pneumoniae* with rolipram or vehicle were stained with hematoxylin and eosin stain (magnification ×200 in large frame; ×400 in inserted frame) and visualized with AxioVert (*E*). Thickness of middle ear mucosa was measured from 10 middle ear tissue sections per experimental group (*F*). Data represent in *A*, *C*, and *F* are the mean ± S.D. ($n = 3$ in *A*, $n = 10$ in *C* and *F*). *, $p < 0.05$ *versus* control inoculation; **, $p < 0.05$ *versus S. pneumoniae* (Sp) alone. *CON*, control; *TC*, tympanic cavity; *B*, bone; *M*, mucosa. Data are representative of three or more independent experiments.

*moniae*-induced MUC5AC expression in HMEEC cells (supplemental Fig. S1). We previously demonstrated that *S. pneumoniae* up-regulates MUC5AC via activation of ERK (38). Interestingly, ERK activation by *S. pneumoniae* also up-regulates MKP-1 as blocking ERK activation using specific chemical inhibitor inhibited *S. pneumoniae*-induced MKP-1 expression, which in turn led to inhibition of ERK (supplemental Fig. S2) (39). Because of the critical role of MKP-1-ERK in MUC5AC induction, we first determined if PDE4 regulates MUC5AC induction via activation of ERK. Indeed, as shown in Fig. 5*A*, PDE4 inhibitor rolipram blocked *S. pneumoniae*-induced activation of ERK. This interesting result led us to further determine whether PDE4 mediates ERK-dependent induction of MUC5AC via up-regulating MKP-1 expression. As expected, rolipram greatly enhanced *S. pneumoniae*-induced MKP-1 expression at mRNA and protein levels in HMEEC *in vitro* and mouse middle ear *in vivo* as assessed using Q-PCR and Western blot analysis (Fig. 5, *B–D*). Because overexpressing wild-type MKP-1 also reduced MAPK JNK phosphorylation, our data do not preclude the possibility that inhibition of JNK may also affect the expression level of MUC5AC (data not shown). Nonetheless, these data demonstrate that PDE4 does positively regulate ERK-dependent MUC5AC up-regulation by *S. pneumoniae* likely via inhibiting MKP-1, the negative regulator for ERK.

*PDE4 Mediates S. pneumoniae-induced Mucin MUC5AC Expression by Inhibiting Expression of MKP-1 via a cAMP-PKA-dependent Mechanism*—PDE4 exerts its cellular functions by catalyzing and degrading cAMP and thus controlling its intracellular concentrations (59–63, 75, 76). Thus we next sought to determine if PDE4 induced by *S. pneumoniae* down-regulates MKP-1 expression via reducing cAMP. As shown in Fig. 6*A*, *S. pneumoniae* indeed reduced intracellular cAMP concentration, and inhibition of PDE4 using rolipram restored cAMP concentration to the control level as assessed by performing cAMP ELISA assay. These data suggest that cAMP may play an

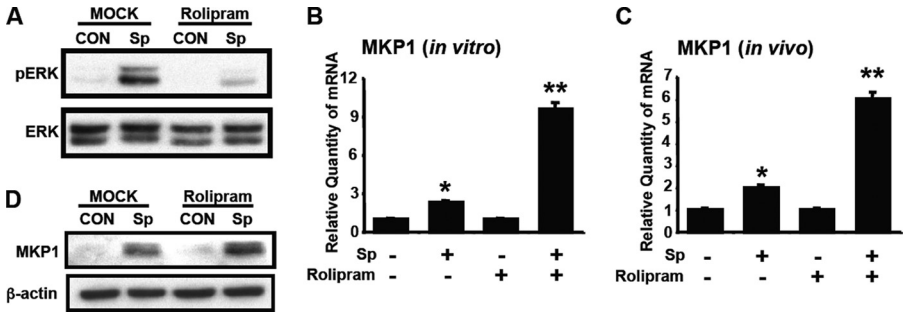


FIGURE 5. **PDE4 mediates ERK-dependent up-regulation of MUC5AC by inhibiting MKP-1 *in vitro* and *in vivo*.** *A*, cells were treated with *S. pneumoniae* with or without rolipram pretreatment. Cell lysate was immunoblotted against phospho-ERK and total-ERK by Western blotting analysis. *B*, cells were pretreated with rolipram, a specific inhibitor for PDE4, for 1 h and treated with *S. pneumoniae* for 90 min. Relative quantity of human MKP-1 mRNA expression was measured by real-time Q-PCR analysis. *C*, mice were intraperitoneally inoculated with rolipram (10 mg/kg of body weight). Two hours after rolipram inoculation, *S. pneumoniae* ($1 \times 10^6$ cfu per ear) was inoculated into the middle ear of mice via tympanic membrane. Total RNA was extracted from the bullae 9 h after *S. pneumoniae* inoculation, and relative quantity of mouse MKP-1 mRNA expression was measured by real-time Q-PCR analysis. *D*, cells were treated with *S. pneumoniae* for 90 min with or without rolipram pretreatment. Cell lysate was immunoblotted against MKP-1 and β-actin by Western blotting analysis. Data in *B* and *C* are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* control inoculation; **, $p < 0.05$ *versus S. pneumoniae* (*Sp*) alone. *CON*, control. Data are representative of three or more independent experiments.

important role in positively regulating MKP-1 expression and PDE4 negatively regulates MKP-1 expression by reducing intracellular cAMP concentration. To further determine if cAMP does play an important role in inhibiting ERK-dependent MUC5AC induction via enhancing MKP-1 expression, we next evaluated the effect of 8-bromo-cAMP, a cell-permeable cAMP analog that is more resistant to PDE4 than cAMP, on *S. pneumoniae*-induced MUC5AC and MKP-1 expression. As shown in Fig. 6, *B* and *C*, 8-bromo-cAMP inhibited MUC5AC induction by *S. pneumoniae* and also markedly enhanced MKP-1 induction by *S. pneumoniae*. We further confirmed the involvement of cAMP in *S. pneumoniae*-induced MUC5AC and MKP-1 expression by using forskolin, a cell-permeable diterpenoid, which has been commonly used to raise levels of intracellular cAMP. As shown in Fig. 6, *D* and *E*, forskolin blocked *S. pneumoniae*-induced up-regulation of MUC5AC and markedly enhanced MKP-1 induction.

Because PKA represents the major downstream signaling effector of cAMP, and PKA was known to be involved in mucin regulation, we next determined if PDE-mediated inhibition of MKP-1 and subsequent up-regulation of MUC5AC expression is dependent on cAMP-dependent PKA activation by assessing the effect of PKA inhibitor PKI on *S. pneumoniae*-induced MUC5AC and MKP-1 expression. As shown in Fig. 6, *F–H*, PKI enhanced *S. pneumoniae*-induced MUC5AC expression at both mRNA and protein levels and inhibited MKP-1 mRNA expression, suggesting that PDE-mediated up-regulation of MUC5AC expression by *S. pneumoniae* via inhibition of MKP-1 is dependent on cAMP-PKA pathway.

*PDE4B Mediates S. pneumoniae-induced Mucin MUC5AC Expression by Inhibiting Expression of MKP-1, Which in Turn Leads to the Enhanced Activation of MAPK ERK*—Having demonstrated a critical role of PDE4 in regulating MUC5AC induction, we next sought to determine which PDE4 subfamily member is specifically involved. PDE4 consists of four subfamily genes, PDE4A-D, encoding rolipram-sensitive PDEs. We first determined if PDE4 is up-regulated by *S. pneumoniae.* As shown in Fig. 7*A*, PDE4B, but not -A, is markedly up-regulated by *S. pneumoniae* as assessed using Q-PCR in human middle ear HMEEC cells. A similar result was also observed in the middle ear mucosa of mouse (Fig. 7*B*) as well as in human primary NHBE cells and airway epithelial cell line A549 (data not shown). In addition, up-regulation of PDE4B by *S. pneumoniae* at the protein level was observed in HMEEC cells as assessed by performing Western blot analysis (Fig. 7*C*). Consistent with these results, PDE4 enzymatic activity was also up-regulated by *S. pneumoniae* (Fig. 7*D*). Together, these data suggest that PDE4B may play an important role in regulating *S. pneumoniae*-induced mucin MUC5AC up-regulation. To further explore if PDE4B is required, we next performed siRNA knockdown of PDE4B. We first determined the efficiency of PDE4B-siRNA in reducing PDE4B expression. As shown in Fig. 7*E*, PDE4B expression was markedly reduced by PDE4B-siRNA. Next, we determined the effect of PDE4B-siRNA. As shown in Fig. 7*F*, PDE4B-siRNA markedly inhibited *S. pneumoniae*-induced up-regulation of MUC5AC at mRNA level in human middle ear epithelial HMEEC cells. Furthermore, knockdown of PDE4B using PDE4B-siRNA significantly enhanced *S. pneumoniae*-induced MKP-1 mRNA expression (Fig. 7*G*). Taken together, these data provide direct evidence that PDE4B plays an important role in regulating *S. pneumoniae*-induced up-regulation of mucin MUC5AC via inhibiting MKP-1.

*Topical and Post-infection Treatment with PDE4 Inhibitor Inhibits Mucin MUC5AC Expression in a Mouse Model of S. pneumoniae-induced OM*—We have showed that PDE general inhibitor IBMX- and PDE4-specific inhibitor rolipram, when preadministered systemically, inhibited *S. pneumoniae*-induced MUC5AC up-regulation in the middle ear of an OM mouse model (Figs. 2 and 4). However, it is still unclear if post-infection treatment of rolipram, which is more physiologically relevant to the clinical situation, also inhibits MUC5AC up-regulation by *S. pneumoniae*. We thus tested if post-infection administration of PDE4 inhibitor rolipram has any therapeutic effect on treating mucin overproduction under clinically relevant condition. Topical administration works efficiently when the eardrum is perforated either pathologically or surgically by tympanostomy tube insertion (90–94). Thus we determined if topical administration of rolipram via tympanic membrane inhibits MUC5AC up-regulation in the middle ear of mice post

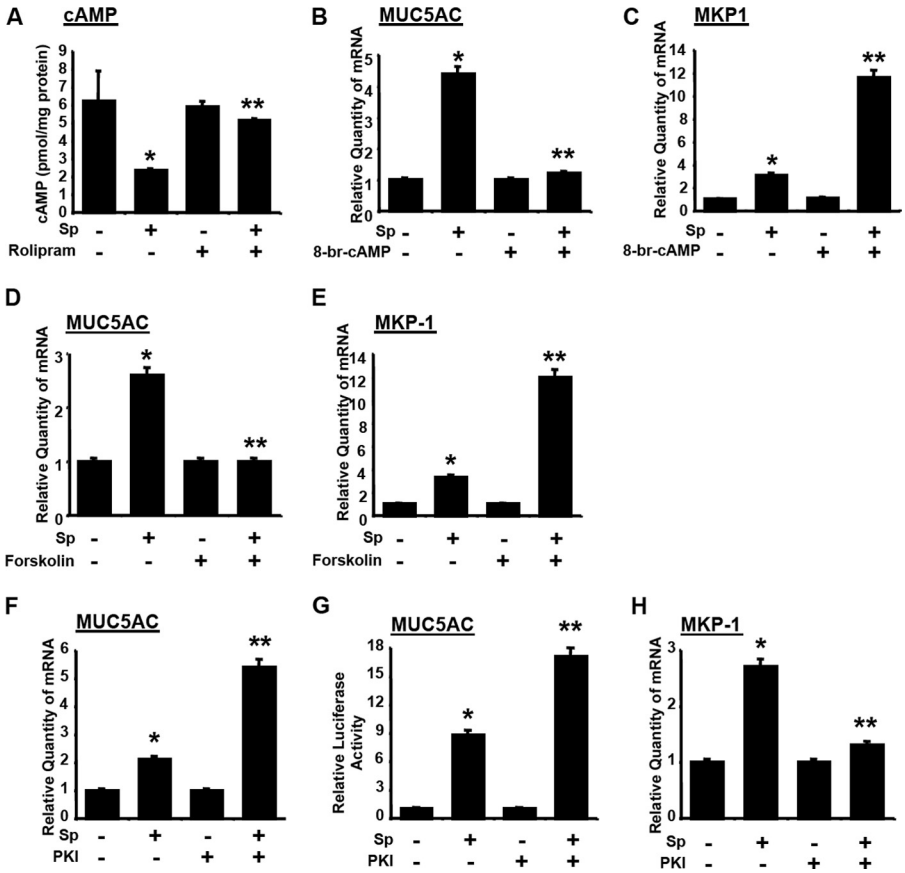


FIGURE 6. **PDE4 mediates *S. pneumoniae*-induced mucin MUC5AC expression by inhibiting expression of MKP-1 via a cAMP-PKA-dependent mechanism.** *A*, cells were treated with *S. pneumoniae* with rolipram or vehicle, and intracellular level of cAMP was measured in the cell lysate by using cAMP "PLUS" EIA kit by following the manufacturer's instruction. *B* and *C*, cells were pretreated with 8-bromo-cAMP, a cell-permeable cAMP analog, for 1 h and treated with *S. pneumoniae* for 5 h (*B*) or 90 min (*C*). Relative quantity of human MUC5AC (*B*) and MKP-1 (*C*) mRNA expression was measured by real-time Q-PCR analysis. *D* and *E*, cells were pretreated with forskolin, a cell-permeable diterpenoid, for 1 h and treated with *S. pneumoniae* for 5 h (*D*) or 90 min (*E*). Relative quantity of human MUC5AC (*D*) and MKP-1 (*E*) mRNA expression was measured by Q-PCR analysis. *F*, cells were pretreated with PKI, PKA inhibitor for 1 h and treated with *S. pneumoniae* for 5 h. Relative quantity of human MUC5AC mRNA expression was measured by Q-PCR analysis. *G*, cells transfected with MUC5AC-luciferase reporter gene were treated with *S. pneumoniae* for 5 h with or without PKI pretreatment. MUC5AC transcriptional activity was measured by luciferase assay. *H*, cells were pretreated with PKI for 1 h and treated with *S. pneumoniae* for 90 min. Relative quantity of human MKP-1 mRNA expression was measured by Q-PCR analysis. Data are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* control inoculation; **, $p < 0.05$ *versus S. pneumoniae* (*Sp*) alone; *8-Br-cAMP*, 8-bromo-cAMP. Data are representative of three or more independent experiments.

*S. pneumoniae* inoculation. Interestingly, topical and post-infection administration of rolipram to the middle ear cavity markedly inhibited *S. pneumoniae*-induced MUC5AC up-regulation in a mouse model of OM *in vivo* (Fig. 8*A*). This finding is of particular translational interest and significance because some of the PDE4 inhibitors have been already developed in the clinic for treating asthma, chronic obstructive pulmonary disease, and multiple sclerosis.

## DISCUSSION

In this study we showed that PDE4B positively regulates *S. pneumoniae*-induced MUC5AC up-regulation in the middle ear *in vitro* and *in vivo*. Interestingly, PDE4B mediates *S. pneumoniae*-induced ERK-dependent up-regulation of MUC5AC by inhibiting MKP-1, a critical negative regulator for MAPK ERK. Moreover, *S. pneumoniae* induces PDE4B up-regulation, which in turn reduces intracellular level of cAMP and inhibits activity of PKA, a downstream effector molecule of cAMP, leading to the enhancement of *S. pneumoniae*-induced MKP-1 expression. Inhibition of PDE4 by rolipram, a specific inhibitor for PDE4, thus leads to the inhibition of *S. pneumoniae*-induced MUC5AC expression by up-regulating MKP-1 expression. Importantly, not only pretreatment with rolipram, but also topical and post-infection treatment in mice inhibited *S. pneumoniae*-induced MUC5AC up-regulation (Fig. 8*B*).

OM is characterized by mucus overproduction in the middle ear, an important factor contributing to the development of conductive hearing loss (3, 7, 9, 19–22). Mucin represents the major component in mucus (25, 38, 39, 43, 95, 96). Currently there are no effective therapeutic agents available that directly inhibit the production of mucin. Thus, effective and specific inhibition of up-regulation of mucin is urgently needed. Among all known mucin genes, MUC5AC represents one of the most important mucin genes in the pathogenesis of OM (25, 38, 39, 43, 95, 96). *S. pneumoniae* is the most common Gram-positive bacterial pathogen causing OM and has been shown to up-regulate MUC5AC expression in the middle ear during OM (38, 39, 42). However, the molecular mechanisms by which *S. pneumoniae* induces MUC5AC up-regulation in the middle ear in OM has yet to be understood. Previously we found that *S. pneumoniae* up-regulates MUC5AC transcription via activation of

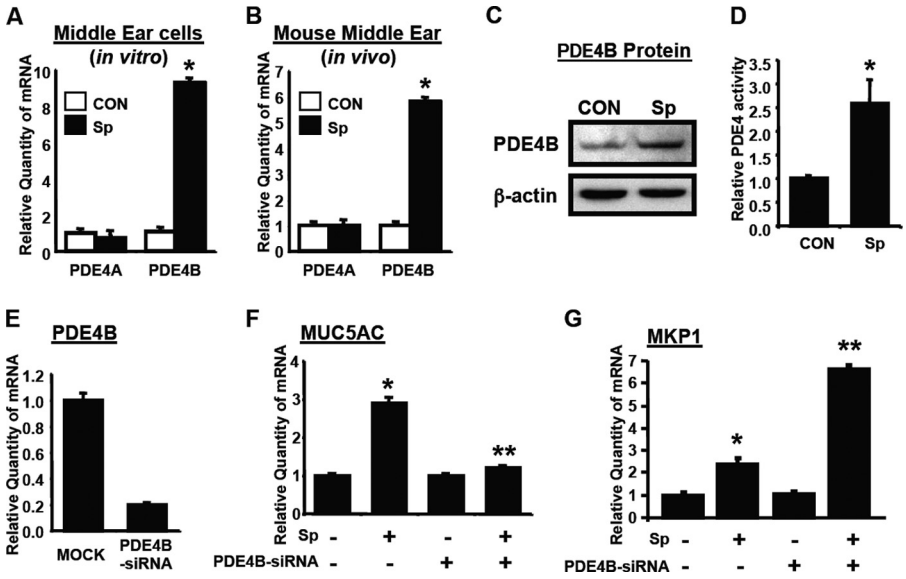


FIGURE 7. **PDE4B mediates *S. pneumoniae*-induced mucin MUC5AC expression by inhibiting expression of MKP-1, which in turn leads to the enhanced activation of MAPK ERK.** *A*, human middle ear epithelial HMEEC cells were treated with *S. pneumoniae* for 5 h. Relative quantity of human PDE4A and PDE4B mRNA expression was measured by real-time Q-PCR analysis. *B*, *S. pneumoniae* ($1 \times 10^6$ cfu per ear) was inoculated into the middle ear of mice via tympanic membrane. Total RNA was extracted from the bullae 6 h after *S. pneumoniae* inoculation, and relative quantity of mouse PDE4A and PDE4B mRNA expression was measured by Q-PCR analysis. *C*, cells were treated with *S. pneumoniae*, and cell lysate was immunoblotted against PDE4B and β-actin by Western blotting analysis. *D*, cAMP-specific PDE activity was measured in cell lysate in the presence of vehicle or rolipram with 0.3 μM [$^3$H]cAMP as substrate. PDE4 specific activity was determined by subtracting PDE-specific inhibited activity from total PDE activity. *E*, cells were transfected with PDE4B-siRNA or control-siRNA, and relative quantity of PDE4B mRNA expression was measured by Q-PCR analysis. *F* and *G*, cells transfected with PDE4B-siRNA or control-siRNA were treated with *S. pneumoniae* for 5 h (*F*) or 90 min (*G*). Relative quantity of MUC5AC (*F*) and MKP-1 (*G*) mRNA expression was measured by Q-PCR analysis. Data are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* control inoculation; **, $p < 0.05$ *versus S. pneumoniae* (*Sp*) in control-siRNA. *CON*, control; *MOCK*, control-siRNA. Data are representative of three or more independent experiments.

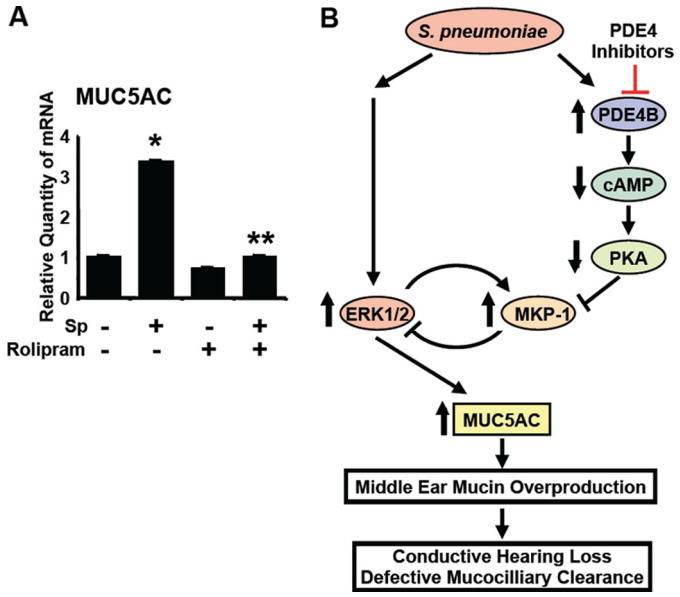


FIGURE 8. **Topical and post-infection treatment with PDE4 inhibitor inhibits mucin MUC5AC expression in a mouse model of *S. pneumoniae*-induced OM.** *A*, the middle ear of mice was inoculated with *S. pneumoniae*, and rolipram was administered to the middle ear cavity through tympanic membrane 9 h after *S. pneumoniae* infection. Total RNA was extracted from the middle ear of mice, and mRNA expression of MUC5AC was measured by Q-PCR. *B*, shown is a schematic representation of regulation of *S. pneumoniae*-induced up-regulation of MUC5AC by PDE4B. *S. pneumoniae* selectively up-regulates the expression of PDE4B. PDE4B acts as a positive regulator for *S. pneumoniae*-induced mucin MUC5AC expression by inhibiting MKP-1 via a cAMP-PKA-dependent mechanism. Data are the mean ± S.D. ($n = 3$). *, $p < 0.05$ *versus* control; **, $p < 0.05$ *versus S. pneumoniae* (Sp) alone. Data are representative of three or more independent experiments.

MAPK ERK. On the other hand, *S. pneumoniae* also induces MKP-1 up-regulation, which in turn inhibits *S. pneumoniae*-dependent activation of ERK and the subsequent induction of MUC5AC (38, 39). This finding is of particular translational interest and significance because increasing the expression of the negative regulators has long been thought as an attractive and effective therapeutic strategy for treating overactive host response such as mucus overproduction without causing serious adverse effects (52, 54, 55). Therefore, understanding the molecular mechanisms by which MKP-1 is up-regulated may not only bring novel insights into the tight regulation of mucin overproduction but also lead to the identification of novel therapeutic targets for controlling mucus overproduction in OM.

Of particular interest in the current study is that we found that PDE4B positively regulates *S. pneumoniae*-induced ERK-dependent MUC5AC expression by inhibiting MKP-1 as PDE4 inhibitor rolipram, and PDE4B knockdown using PDE4B-siRNA enhances *S. pneumoniae*-induced MKP-1 expression and inhibits MUC5AC expression. To date, a number of PDE inhibitors have been already successfully developed as effective therapeutic agents used in clinic, including the drug that has been recently approved for treating chronic obstructive pulmonary disease and asthma (51, 65, 71). Thus, identifying PDE4B as a therapeutic target for inhibiting MUC5AC up-regulation via increasing the negative regulator MKP-1 may have significant therapeutic potential for treating OM. It is noted that some patients exhibited severe gastrointestinal side effects including nausea, emesis, and diarrhea when taking PDE4 inhibitors. Recent studies suggest that PDE4D is responsible for these gastrointestinal side effects (67). To further

improve the clinical efficacy and reduce the side effects, a number of strategies are currently being pursued. Because among four PDE4 isotypes, PDE4B was found as the major isotype mediating up-regulation of mucin MUC5AC via regulation of MKP-1 expression, further study to develop the PDE4B-specific inhibitors is needed toward developing promising therapeutics for mucus overproduction in the OM without serious gastrointestinal side effects. In addition, taking anatomical advantage of the middle ear for being topically accessible, we found that ototopical application of PDE4B inhibitor rolipram efficiently blocked MUC5AC up-regulation by *S. pneumoniae* when administered in both pre-administration and post-infection experiments (Fig. 8). This finding is of particular therapeutic importance because ototopical administration of rolipram could significantly reduce the dose of rolipram, which will help minimize the side effects often seen with systemic administration. Finally, it would be also interesting to explore the possibility of reducing the dose of the PDE4 inhibitor by using targeted drug delivery.

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