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*[^5]

Received for publication, December 22, 2011, and in revised form, April 27, 2012. Published, JBC Papers in Press, May 18, 2012, DOI 10.1074/jbc.M111.337378

Jiyun Lee[^1], Kensei Komatsu[^1], Byung Cheol Lee[^2], Jae Hyang Lim[^2], Hirofumi Jono[^3], Haidong Xu[^4], Hirofumi Kai[^4], Z. John Zhang[^5], Chen Yan[^5], and Jian-Dong Li[^5]

From the ^4^Center for Inflammation, Immunity, and Infection and Department of Biology, Georgia State University, Atlanta, Georgia 30303, the ^3^Department of Diagnostic Medicine and ^4^Molecular Medicine, Kumamoto University, Kumamoto 860-8556, Japan, the ^1^School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332, and the ^2^Cardiovascular Research Institute, University of Rochester Medical Center, Rochester, New York 14642

**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 up-regulation of MUC5AC. 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) 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...
PDE4 Mediates MUC5AC Induction via Inhibiting MKP-1

serotypes (15). Moreover, the current treatment of *S. pneumoniae* OM heavily relies 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 mucus 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 mucus 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*-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 inhibited 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 × 10⁶ 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 conducted as described previously (32, 81). All cells were cultured in a humified atmosphere of 5% CO₂ 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 TransIT-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, 44, 81). The primer sequences for human and mouse PDE4A & B are as follows: human PDE4A forward primer, 5’-TCTC-CTCCATCCGTACCTTG-3’; human PDE4A reverse primer, 5’-TGGGCTTGGAGAAAATATGTC-3’; human PDE4B forward primer, 5’-TGATGCTCAGGACATTTCTC-3’; human PDE4B reverse primer, 5’-AGTGGTGAGGGGACCTTTG-3’; mouse PDE4A forward primer, 5’-GCCATGGACAGCT-CAAGATT-3’; mouse PDE4A reverse primer, 5’-ATGGTG- GAGGTCGTCTCCT-3’; mouse PDE4B forward primer, 5’-TGAAGAGCCAGTCCCCATCA-3’; mouse PDE4B reverse primer, 5’-CCACACCCCTAGTGACAGCA-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 μM [³H]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 μl 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 pathe-
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-μm 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 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. 1A, 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 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.
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. 1B, 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. 1C), 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. 2A, 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. 2B). 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. 2C) (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. 2D). The inhibitory effects of IBMX on these pathological changes were also quantitatively analyzed using AxioVision LE image system (Carl Zeiss) (Fig. 2E). 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
PDE4 Mediates MUC5AC Induction via Inhibiting MKP-1

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 < 0.01 versus S. pneumoniae (Sp) alone. Data are representative of three or more independent experiments.

PDE4 Mediates ERK-dependent Up-regulation of MUC5AC

PDE4 Is a Key Positive Regulator for S. pneumoniae-induced MUC5AC Expression in the Middle Ear of Mice in Vivo—Having demonstrated that PDE4 plays a critical role in regulating MUC5AC induction in vitro, 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. 4A) and IF staining using MUC5AC-specific anti-body (Fig. 4B). 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. 4C) (89). Furthermore, rolipram blocked S. pneumoniae-induced pathological changes of OM as assessed by performing otoscopic examination in S. pneumoniae-inoculated mice (Fig. 4D). 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. 4D 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. 4E). The inhibitory effects of rolipram on these pathological changes were also quantitatively analyzed using an AxioVision LE (Fig. 4F). 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 MUC5AC Induction 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-
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. 5A, 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 regulates MKP-1 expression via reducing cAMP. As shown in Fig. 6A, *S. pneumoniae* indeed reduced intracellular cAMP concentrations, 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 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 × 10^9 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 × 10^9 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) (8). 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.
PDE4 Mediates MUC5AC Induction via Inhibiting MKP-1

![Diagram](image)

**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* 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 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. 7A, 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. 7B) 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. 7C). Consistent with these results, PDE4 enzymatic activity was also up-regulated by *S. pneumoniae* (Fig. 7D). 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. 7E, PDE4B expression was markedly reduced by PDE4B-siRNA. Next, we determined the effect of PDE4B-siRNA. As shown in Fig. 7F, 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. 7G). 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...
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. 8A). 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. 8A).

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...
**PDE4 Mediates MUC5AC Induction via Inhibiting MKP-1**

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 via a cAMP-PKA-dependent mechanism. Data are the mean ± S.D. (n = 3). *, p < 0.05 versus control inoculation; **, p < 0.05 versus *S. pneumoniae* (Sp) alone. Data are representative of three or more independent experiments.

![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.](image)

**FIGURE 8. Topical and post-infection treatment with PDE4 inhibitor inhibits mucin MUC5AC expression in a mouse model of *S. pneumoniae*-induced OM.** A, middle ear mucosa was inoculated with *S. pneumoniae*, and rolipram was administrated 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 MUC5AC by 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.

![FIGURE 8.](image)
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 isoform 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.

**REFERENCES**

1. Bluestone, C. D. (1982) Otitis media in children. To treat or not to treat? *N. Engl. J. Med.* **306**, 1399–1404

2. Giebink, G. S. (1991) Immunophagocytosis of otitis media. *Adv. Exp. Med. Biol.* **303**, 149–158

3. Murphy, T. F., and Apicella, M. A. (1987) Nontyphoidal *Haemophilus influenzae*. A review of clinical aspects, surface antigens, and the human immune response to infection. *Rev. Infect. Dis.* **9**, 1–15

4. Gates, G. A. (1996) Cost-effectiveness considerations in otitis media treatment. *Otolaryngol. Head Neck Surg.* **114**, 525–530

5. Klein, J. O. (2000) The burden of otitis media. *Vaccine* **19**, S2–S8

6. Teole, D. W., Klein, J. O., and Rosner, B. (1989) Epidemiology of otitis media during the first seven years of life in children in greater Boston. A prospective, cohort study. *J. Infect. Dis.* **160**, 83–94

7. Reichman, J., and Healey, W. C. (1983) Learning disabilities and conductive hearing loss involving otitis media. *J. Learn. Disabil.* **16**, 272–278

8. Bluestone, C. D., Beery, Q. C., and Paradise, J. L. (1973) Audiometry and tympanometry in relation to middle ear effusions in children. *Laryngoscope* **83**, 594–604

9. Foxwell, A. R., Kyd, J. M., and Cripps, A. W. (1998) Nontyphoidal *Haemophilus influenzae*. Pathogenesis and prevention. *Microbiol. Mol. Biol. Rev.* **62**, 294–308

10. Straetemans, M., Sanders, E. A., Veenhoven, R. H., Schilder, A. G., Damoiseaux, R. A., and Zhielhus, G. A. (2003) Review of randomized controlled trials on pneumococcal vaccination for prevention of otitis media. *Pediatr. Infect. Dis. J.* **22**, 515–524

11. Veenhoven, R., Bogaert, D., Uiterwaal, C., Brouwer, C., Kiezbrink, H., Bruin, J., IJzerman, E., Hermans, P., de Groot, R., Zegers, B., Hansen, J. R., Elvin, L., Ensor, K. M., Hackell, J., Siber, G., Malinoski, F., Madore, D., Chang, I., Kohberger, R., Watson, W., Austrian, R., and Edwards, K. (2000) Efficacy, safety, and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr. Infect. Dis. J.* **19**, 187–195

12. Black, S., Shinefield, H. R., Hansen, J., Elvin, L., Lauder, D., and Malinoski, F. (2001) Postlicensure evaluation of the effectiveness of seven valent pneumococcal conjugate vaccine. *Pediatr. Infect. Dis. J.* **20**, 1105–1107

13. Fletcher, M. A., and Fritzell, B. (2007) Brief review of the clinical effectiveness of PREVENAR against otitis media. *Vaccine* **25**, 2507–2512

14. Moore, M. R., and Whitney, C. G. (2008) Emergence of nonvaccine serotypes after introduction of pneumococcal conjugate vaccine. *Cause and effect? Clin. Infect. Dis.* **46**, 183–185

15. Van Bambake, F., Reinert, R. R., Appelbaum, P. C., Tulkens, P. M., and Peertman, W. E. (2007) Multidrug-resistant *Streptococcus pneumoniae* infections. Current and future therapeutic options. *Drugs* **67**, 2355–2382

16. Rose, A. S., Prazma, J., Randell, S. H., Baggett, H. C., Lane, A. P., and Pillsbury, H. C. (1997) Nitric oxide mediates mucin secretion in endotoxin-induced otitis media with effusion. *Otolaryngol. Head Neck Surg.* **116**, 308–316

17. Majima, Y., Hamaguchi, Y., Hirata, K., Takeuchi, K., Morishita, A., and Sakakura, Y. (1988) Hearing impairment in relation to viscoelasticity of middle ear effusions in children. *Ann. Otol. Rhinol. Laryngol.* **97**, 272–274

18. Knowles, M. R., and Boucher, R. C. (2002) Mucus clearance as a primary innate defense mechanism for mammalian airways. *J. Clin. Invest.* **109**, 571–577

19. Carrie, S., Hutton, D. A., Birchall, J. P., Green, G. G., and Pearson, J. P. (1992) Otitis media with effusion. Components that contribute to the viscous properties. *Acta Otolaryngol.* **112**, 504–511

20. Kerschner, J. E. (2007) Mucin gene expression in human middle ear epithelium. *Laryngoscope* **117**, 1666–1676

21. Rose, M. C., Nickola, T. J., and Vosnou, J. A. (2001) Airway mucus obstruction. Mucin glycoproteins, MUC gene regulation, and goblet cell hyperplasia. *Ann. J. Respir. Cell Mol. Biol.* **25**, 533–537

22. Basbaum, C., Lemjabbar, H., Longpre, M., Li, D., Gensch, E., and McNamara, N. (1999) Control of mucin transcription by diverse injury-induced signaling pathways. *Am. J. Respir. Crit. Care Med.* **160**, 544–548

23. Wang, B., Lim, D. J., Cha, Y., Son, C. Q., Li, S., and Amin, M. B. (2002) Isolation and characterization of the major form of human MUC18 cDNA gene and correlation of MUC18 overexpression in prostate cancer cell lines and tissues with malignant progression. *Gene* **279**, 17–31

24. Gendler, S. J., and Reiner, A. (1995) Epithelial mucin genes. *Annu. Rev. Physiol.* **57**, 607–634

25. Basbaum, C. B., and Li, J. D. (1998) In *Cilia, Mucus, and Mucociliary Interactions* (Baum, G. L., et al., eds) pp. 349–352, Marcel Dekker, Inc., New York

26. Li, J. D. (2003) Exploitation of host epithelial signaling networks by respiratory bacterial pathogens. *J. Pharmacol. Sci.* **91**, 1–7

27. Wang, B., Lim, D. J., Han, J., Kim, Y. S., Basbaum, C. B., and Li, J. D. (2002) Novel cytoplasmic proteins of nontypeable *Haemophilus influenzae* up-regulate human MUC18 mucin transcription via a positive p38 mitogen-activated protein kinase pathway and a negative phosphoinositide 3-kinase-Akt pathway. *J. Biol. Chem.* **277**, 949–957

28. Jono, H., Shuto, T., Xu, H., Kai, H., Lim, D. J., Gum, J. R., Jr., Kim, Y. S., Yamaoka, S., Feng, X. H., and Li, J. D. (2002) Transforming growth factor-β-Smad signaling pathway cooperates with NF-kB to mediate nontypeable *Haemophilus influenzae*-induced MUC2 mucin transcription. *J. Biol. Chem.* **277**, 45547–45557

29. Jono, H., Xu, H., Kai, H., Lim, D. J., Kim, Y. S., Feng, X. H., and Li, J. D. (2003) Transforming growth factor-β-Smad signaling pathway negatively regulates nontypeable *Haemophilus influenzae*-induced MUC18 mucin transcription via mitogen-activated protein kinase (MAPK) phosphatase-1-dependent inhibition of p38 MAPK. *J. Biol. Chem.* **278**, 27811–27819
PDE4 Mediates MUC5AC Induction via Inhibiting MKP-1

35. Takeuchi, K., Yagawa, M., Ishinaga, H., Kishioka, C., Harada, T., and Ma- jima, Y. (2003) Mucin gene expression in the effusions of otitis media with effusion. Int. J. Pediatr. Otorhinolaryngol. 67, 53–58

36. Smirnova, M. G., Birchall, J. P., and Pearson, J. P. (2000) TNF-α in the regulation of MUC5AC secretion. Some aspects of cytokine-induced mucin hypersecretion on the in vitro model. Cytokine 12, 1732–1736

37. Jung, H. H., Lee, J. H., Lim, H. H., and Chung, C. (1999) Recent Advances in Otitis Media with Effusion: Proceedings of the Seventh International Symposium, Ft. Lauderdale, FL, June 1–5, 1999 (Lim, D. J., Bluestone, C. D., and Cassetell, M. L., eds) p. 251, B.C. Decker, Inc., Hamilton, ON, Canada

38. Ha, U., Lim, J. H., Jono, H., Koga, T., Srivastava, A., Malley, R., Pagés, G., Pouysségur, J., and Li, J. D. (2007) A novel role for IkBa kinase (IKK) ε and IKKβ in ERK-dependent up-regulation of MUC5AC mucin transcription by Streptococcus pneumoniae. J. Immunol. 178, 1736–1747

39. Ha, U. H., Lim, J. H., Kim, H. J., Wu, W., Jin, S., Xu, H., and Li, J. D. (2008) MKP1 regulates the induction of MUC5AC mucin by Streptococcus pneumoniae pneumolysin by inhibiting the PAK4-IκB signaling pathway. J. Biol. Chem. 283, 30624–30631

40. Komatsu, K., Jono, H., Lim, J. H., Imasato, A., Xu, H., Kai, H., Yan, C., and Li, J. D. (2007) Opposing roles of PAK2 and PAK4 in synergistic induction of MUC5AC mucin by bacterium NTHI and EGF. Biochem. Biophys. Res. Commun. 359, 691–696

41. Chen, R., Lim, J. H., Jono, H., Gu, X. X., Kim, Y. S., Baasum, C. B., Murphy, T. F., and Li, J. D. (2004) Nontypeable Haemophilus influenzae lipoprotein P6 induces MUC5AC mucin transcription via TLR2-TAK1-dependent p38 MAPK-AP1 and IKKβ-IκBα-NF-κB signaling pathways. Biochem. Biophys. Res. Commun. 324, 1087–1094

42. Lim, J. H., Kim, H. J., Komatsu, K., Ha, U., Huang, Y., Jono, H., Kweon, S. M., Lee, J., Xu, X., Zhang, G. S., Shen, H., Kai, H., Zhang, W., Xu, H., and Li, J. D. (2009) Differential regulation of Streptococcus pneumoniae-induced human MUC5AC mucin expression through distinct MAPK pathways. Am. J. Respir. Crit. Care Med. 180, 300–311

43. Zhang, J., Shen, B., and Lin, A. (2007) Novel strategies for inhibition of the p38 MAPK pathway. Trends Pharmacol. Sci. 28, 286–295

44. Kyriakos, J. M., and Avruch, J. (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev. 81, 807–869

45. Herlaar, E., and Brown, Z. (1999) p38 MAPK signaling cascades in inflammatory disease. Mol. Med. Today 5, 439–447

46. Palacios, S. D., Pak, K., Kayali, A. G., Rivkin, A. Z., Aletsee, C., Melhus, A., Webster, N. J., and Ryan, A. F. (2002) Participation of Ras and extracellular-regulated kinase in the hyperplastic response of middle-ear mucosa during bacterial otitis media. J. Infect. Dis. 186, 1761–1769

47. Palacios, S. D., Pak, K., Rivkin, A. Z., Bennett, T., and Ryan, A. F. (2002) Growth factors and their receptors in the middle ear mucosa during otitis media. Laryngoscope 112, 420–423

48. Bender, A. T., and Beavo, J. A. (2006) Cyclic nucleotide phosphodiesterases. Molecular regulation to clinical use. Pharmacol. Rev. 58, 488–520

49. Chi, H., Barry, S. P., Roth, R. J., Wu, J. J., Jones, E. A., Bennett, A. M., and Flavell, R. A. (2006) Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses. Proc. Natl. Acad. Sci. U.S.A. 103, 2274–2279

50. Konod, K., and Nishida, E. (2007) Regulation of MAP kinases by MAP kinase phosphatases. Biochim. Biophys. Acta 1773, 1227–1237

51. Liu, Y., Shepherd, E. G., and Nelin, L. D. (2007) MAPK phosphatase-regulating the immune response. Nat. Rev. Immunol. 7, 202–212

52. Beavo, J. A. (1995) Cyclic nucleotide phosphodiesterases. Functional implications of multiple isoforms. Physiol. Rev. 75, 725–748

53. Beavo, J. A., and Brunton, L. L. (2002) Cyclic nucleotide research. Still expanding after half a century. Nat. Rev. Mol. Cell Biol. 3, 710–718

54. Rehmann, H., Wittinghofer, A., and Bos, J. L. (2007) Capturing cyclic nucleotides in action. Snapshots from crystallographic studies. Nat. Rev. Mol. Cell Biol. 8, 63–73

55. Dychok, O., Isakov, Y., Sægotorp, J., and Tengholm, A. (2006) Oscillations of cyclic AMP in hormone-stimulated insulin-secreting β-cells. Nature 439, 349–352

56. Fischeimer, R., Castro, L. R., Abi-Gerges, A., Rochais, F., Jurevicus, J., Leroy, J., and Vandecasteele, G. (2006) Compartmentation of cyclic nucleotide signaling in the heart. The role of cyclic nucleotide phosphodiesterases. Circ. Res. 99, 816–828

57. Beavo, J. A., and Brunton, L. L. (2002) Cyclic nucleotide research. Still expanding after half a century. Nat. Rev. Mol. Cell Biol. 3, 710–718

58. Rehmann, H., Wittinghofer, A., and Bos, J. L. (2007) Capturing cyclic nucleotides in action. Snapshots from crystallographic studies. Nat. Rev. Mol. Cell Biol. 8, 63–73

59. Dychok, O., Isakov, Y., Sægotorp, J., and Tengholm, A. (2006) Oscillations of cyclic AMP in hormone-stimulated insulin-secreting β-cells. Nature 439, 349–352

60. Fischeimer, R., Castro, L. R., Abi-Gerges, A., Rochais, F., Jurevicus, J., Leroy, J., and Vandecasteele, G. (2006) Compartmentation of cyclic nucleotide signaling in the heart. The role of cyclic nucleotide phosphodiesterases. Circ. Res. 99, 816–828

61. Beavo, J. A., and Brunton, L. L. (2002) Cyclic nucleotide research. Still expanding after half a century. Nat. Rev. Mol. Cell Biol. 3, 710–718

62. Rehmann, H., Wittinghofer, A., and Bos, J. L. (2007) Capturing cyclic nucleotides in action. Snapshots from crystallographic studies. Nat. Rev. Mol. Cell Biol. 8, 63–73
Janssens, S. P. (2009) Ventricular phosphodiesterase-5 expression is increased in patients with advanced heart failure and contributes to adverse ventricular remodeling after myocardial infarction in mice. *Circulation* **119**, 408–416.

76. Takimoto, E., Champion, H. C., Li, M., Belardi, D., Ren, S., Rodriguez, E. R., Bedja, D., Gabrielson, K. L., Wang, Y., and Kass, D. A. (2005) Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat. Med.* **11**, 214–222.

77. Koga, T., Lim, J. H., Jono, H., Ha, U. H., Xu, H., Ishinaga, H., Morino, S., Xu, X., Yan, C., Kai, H., and Li, J. D. (2008) Tumor suppressor cylindromatosis acts as a negative regulator for Streptococcus pneumoniae-induced NFAT signaling. *J. Biol. Chem.* **283**, 12546–12554.

78. Lim, J. H., Ha, U., Sakai, A., Woo, C. H., Kweon, S. M., Xu, H., and Li, J. D. (2008) *Streptococcus pneumoniae* synergizes with nontypeable Haemophilus influenzae to induce inflammation via up-regulating TLR2. *BMC Immunol.* **9**, 40.

79. Lim, J. H., Stirling, B., Derry, J., Koga, T., Jono, H., Woo, C. H., Xu, H., Bourne, P., Ha, U. H., Ishinaga, H., Xu, H., Andalibi, A., Feng, X. H., Zhu, H., Huang, Y., Zhang, W., Weng, X., Yan, C., Yin, Z., Briles, D. E., Davis, R. J., Flavell, R. A., and Li, J. D. (2008) Tumor suppressor CYLD regulates acute lung injury in lethal *Streptococcus pneumoniae* infections. *Immunity* **27**, 349–360.

80. Chun, Y. M., Moon, S. K., Lee, H. Y., Webster, P., Brackmann, D. E., Rhim, J. S., and Lim, D. J. (2002) Immortalization of normal adult human middle ear epithelial cells using a retrovirus containing the E6/E7 genes of human papillomavirus type 16. *Ann. Otol. Rhinol. Laryngol.* **111**, 507–517.

81. Imasato, A., Desbois-Mouthon, C., Han, J., Kai, H., Cato, A. C., Akira, S., and Li, J. D. (2002) Inhibition of p38 MAPK by glucocorticoids via induction of MAPK phosphatase-1 enhances nontypeable Haemophilus influenzae-induced expression of toll-like receptor 2. *J. Biol. Chem.* **277**, 47444–47450.

82. Ishinaga, H., Jono, H., Lim, J. H., Komatsu, K., Xu, X., Lee, J., Woo, C. H., Xu, H., Feng, X. H., Chen, L. F., Yan, C., and Li, J. D. (2009) Synergistic induction of nuclear factor-κB by transforming growth factor-β and tumor necrosis factor-α is mediated by protein kinase A-dependent RelA acetylation. *Biochem. J.* **417**, 583–591.

83. Lim, J. H., Ha, U. H., Woo, C. H., Xu, H., and Li, J. D. (2008) CYLD is a crucial negative regulator of innate immune response in *Escherichia coli* pneumonia. *Cell Microbiol.* **10**, 2247–2256.

84. Lim, J. H., Jono, H., Koga, T., Woo, C. H., Ishinaga, H., Bourne, P., Xu, H., Ha, U. H., Xu, H., and Li, J. D. (2007) Tumor suppressor CYLD acts as a negative regulator for non-typeable Haemophilus influenzae-induced inflammation in the middle ear and lung of mice. *PLoS One* **2**, e1032.

85. Miller, C. L., Oikawa, M., Cai, Y., Wojtovich, A. P., Nagel, D. J., Xu, X., Xu, H., Florio, V., Rybalkin, S. D., Beavo, J. A., Chen, Y. F., Li, J. D., Blaxall, B. C., Abe, J., and Yan, C. (2009) Role of Ca²⁺/calmodulin-stimulated cyclic nucleotide phosphodiesterase 1 in mediating cardiomyocyte hypertrophy. *Circ. Res.* **105**, 956–964.

86. Surapisitchat, J., Jeon, K. I., Yan, C., and Beavo, J. A. (2007) Differential regulation of endothelial cell permeability by cGMP via phosphodiesterases 2 and 3. *Circ. Res.* **101**, 811–818.

87. Nagel, D. J., Aizawa, T., Jeon, K. I., Liu, W., Mohan, A., Wei, H., Miano, J. M., Florio, V. A., Gao, P., Korshunov, V. A., Berk, B. C., and Yan, C. (2006) Role of nuclear Ca²⁺/calmodulin-stimulated phosphodiesterase 1A in vascular smooth muscle cell growth and survival. *Circ. Res.* **98**, 777–784.

88. Ishinaga, H., Jono, H., Lim, J. H., Kweon, S. M., Xu, H., Ha, U. H., Xu, H., Koga, T., Yan, C., Feng, X. H., Chen, L. F., and Li, J. D. (2007) TGF-β induces p65 acetylation to enhance bacteria-induced NF-κB activation. *EMBO J.* **26**, 1150–1162.

89. Chua, T. C., Yao, P., Akther, J., Young, L., Bao, S., Samaraweera, U., Yan, T. D., and Morris, D. L. (2009) Differential expression of Ki-67 and sex steroid hormone receptors between genders in peritoneal mesothelioma. *Pathol. Oncol. Res.* **15**, 671–678.

90. Herr, B. D., and Marzo, S. J. (2005) Intratracheal steroid perfusion for refractory sudden sensorineural hearing loss. *Otolaryngol. Head Neck Surg.* **132**, 527–531.

91. Miró, N. (2000) Controlled multicenter study on chronic suppressive otitis media treated with topical applications of ciprofloxacin 0.2% solution in single-dose containers or combination of polymyxin B, neomycin, and hydrocortisone suspension. *Otolaryngol. Head Neck Surg.* **123**, 617–623.

92. Rahman, A., Rizwan, S., Waycaster, C., and Wall, G. M. (2007) Pooled analysis of two clinical trials comparing the clinical outcomes of topical ciprofloxacin/dexamethasone otic suspension and polymyxin B/neomycin/hydrocortisone otic suspension for the treatment of acute otitis externa in adults and children. *Clin. Ther.* **29**, 1950–1956.

93. Stenner, M., Jecker, P., Gouveris, H., and Mann, W. (2006) Treatment of sensorineural hearing loss in acute viral otitis media with intratympanic dexamethasone and hyaluronic acid in comparison with intravenous therapy. *Laryngorhinootologie* **85**, 32–37.

94. Wall, G. M., Stroman, D. W., Roland, P. S., and Dohar, J. (2009) Ciprofloxacin 0.3%/dexamethasone 0.1% sterile otic suspension for the topical treatment of ear infections. A review of the literature. *Pediatr. Infect. Dis. J.* **28**, 141–144.

95. Kerschmer, J. E., Horsey, E., Ahmed, A., Erbe, C., Kham pang, P., Ciof f, I., Hu, F. Z., Post, J. C., and Ehrlich, G. D. (2009) Gene expression differences in infected and noninfected middle ear complementary DNA libraries. *Arch Otolaryngol. Head Neck Surg.* **135**, 33–39.

96. Li, J. D., Feng, W., Gallup, M., Kim, J. H., Gum, J., Kim, Y., and Basbaum, C. (1998) Activation of NF-κB via a Src-dependent Ras-MAPK-pp90rsk pathway is required for *Pseudomonas aeruginosa*-induced mucin overproduction in epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5718–5723.