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Induction of Cyclooxygenase-2 Signaling by *Stomatococcus mucilaginosus* Highlights the Pathogenic Potential of an Oral Commensal

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***Stomatococcus mucilaginosus* is an oral commensal that has been occasionally reported to cause severe infections in immunocompromised patients. There is no information about the pathogenic role of *S. mucilaginosus* in airway infections. In a cohort of 182 subjects with bronchiectasis, we found that 9% were colonized with *S. mucilaginosus* in their lower airways by culture growth from bronchoalveolar lavage. To address the pathogenic potential of *S.mucilaginosus*, we developed a murine model of *S. mucilaginosus* lung infection. Intratracheal injection of *S. mucilaginosus* in C57BL/6 mice resulted in a neutrophilic influx with production of proinflammatory cytokines, chemokines, and lipid mediators, mainly PGE_2 with induction of cyclooxygenase-2 (COX-2) in the lungs. Presence of TLR2 was necessary for induction of COX-2 and production of PGE_2 by *S. mucilaginosus*. TLR2-deficient mice showed an enhanced clearance of *S. mucilaginosus* compared with wild-type mice. Administration of PGE_2 to $TLR2^{-/-}$ mice resulted in impaired clearance of *S. mucilaginosus*, suggesting a key role for COX-2–induced PGE_2 production in immune response to *S. mucilaginosus*. Mechanistically, induction of COX-2 in macrophages was dependent on the p38-ERK/MAPK signaling pathway. Furthermore, mice treated with *S. mucilaginosus* and *Pseudomonas aeruginosa* showed an increased mortality compared with mice treated with PA103 or *S. mucilaginosus* alone. Inhibition of COX-2 significantly improved survival in mice infected with PA103 and *S. mucilaginosus*. These data provide novel insights into the bacteriology and personalized microbiome in patients with bronchiectasis and suggest a pathogenic role for *S. mucilaginosus* in patients with bronchiectasis. *The Journal of Immunology*, 2013, 191: 3810–3817.**

Bronchiectasis is primarily a disease of the bronchi and bronchioles and has the potential to cause devastating illness by predisposing susceptible individuals to recurrent respiratory infections (1–3). Whereas the lower respiratory tract is normally sterile, conditions such as bronchiectasis and chronic lung illnesses enable colonization, which contributes to lung inflammation and injury. Recent advances in molecular technology have led to unprecedented ability to analyze complex microbial populations, revealing extensive communities of unculturable or previously unidentified organisms. Microbiota and cultures from bronchoalveolar lavage (BAL) from our patients with bronchiectasis showed that 9% were colonized with *Stomatococcus mucilaginosus*. The importance of oral and gut microbiome in the causation of a variety of inflammatory diseases is increasingly recognized. It is also evident that, similar to the human genome, oral and gut microbiome may be personalized and may predispose or protect individuals to certain diseases (4, 5). Because we isolated *S. mucilaginosus* from the lower airways of patients with bronchiectasis, we hypothesized that aspiration of these bacteria in lower airways may contribute to the inflammatory response in this disease and enhance the pathogenicity of other microbes such as *Pseudomonas aeruginosa*.

S. mucilaginosus is an encapsulated Gram-positive coccus found in oral cavity as part of the normal flora that has been occasionally reported to cause severe infections in immunocompromised patients (6–12). It has been implicated in the causation of endocarditis (13, 14), sepsis, and catheter-related bacteremia (8, 15). Similar to *P. aeruginosa*, *S. mucilaginosus* is known to form biofilms; however, there is scant information about the role of *S. mucilaginosus* in lower respiratory infections (16, 17). We pursued studies to identify the pathogenic potential of this bacterium in vitro in primary cultured macrophages and in vivo in a mouse model of *S. mucilaginosus* lung infection.

The host innate response to infections comprises a complex interplay between mediators released by a variety of cell types (18). Engagement of TLRs leads to activation of signaling pathways that results in generation of cytokines, chemokines, and lipid mediators produced by cyclooxygenases that are critical for host immune response (19, 20). We and others have shown that activation of cyclooxygenase-2 (COX-2) and production of lipid mediators play a pivotal immunomodulatory role in fungal, viral, and bacterial infections (21–25). In particular, PGE_2 has immunosuppressive effects and impairs bacterial clearance (22, 23, 26–29), whereas PGD_2 has been shown to have immunostimulatory effects (30, 31).

We identified *S. mucilaginosus* as a cohabitant in the microbiome from the BAL of 9% of patients with bronchiectasis. Because the growth of *S. mucilaginosus* was significant from BAL,

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Abbreviations used in this article: BAL, bronchoalveolar lavage; BMDM, bone marrow–derived macrophage; CF, cystic fibrosis; COX-2, cyclooxygenase-2; i.t., intratracheal; MOI, multiplicity of infection; mPGES, microsomal PGE synthase; MPO, myeloperoxidase.

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we developed a murine model of infection with *S. mucilaginosus* to investigate its pathogenic potential in the lungs.

Materials and Methods

Human data

Patients diagnosed with bronchiectasis using an International Classification of Diseases code (494) were identified between 1999 and 2006. Consent was waived because of the retrospective nature of the study. Patients were included in the study if they were 18 y and older and had confirmed radiological changes suggestive of bronchiectasis (chest x-ray or computerized tomography scan of the chest) reported by radiologist that was independently evaluated by two clinicians. Patients with cystic fibrosis were excluded ($n = 6$). Demographic, radiological, clinical, microbiology data, and usage of antibiotics were collected on patients with confirmed bronchiectasis. Data regarding microbial colonization from the lower airways were collected on all of the patients. Microbiology data from 182 patients with bronchiectasis were reviewed over a 5-y period. Forty percent of patients had a BAL performed during the course of their illness because of persistent symptoms. The growth of *S. mucilaginosus* was considered significant by the microbiologist when the samples showed 4^+ growth. The study was approved by the Institutional Review Board.

Cell culture and bacteria

RAW 264.7 murine macrophages and bone marrow–derived macrophages (BMDM) from wild-type, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice were cultured, as described (32). Clinical isolates of *S. mucilaginosus* cultured from BAL of patients with bronchiectasis were used for experiments. PA103, which is a well-characterized highly toxic strain of *P. aeruginosa*, was used for survival experiments. Bacteria from frozen stocks were streaked onto trypticase soy agar plates and grown in a deferrated dialysate of trypticase soy broth supplemented with 10 mM nitrilotriacetic acid (Sigma-Aldrich), 1% glycerol, and 100 mM monosodium glutamate at 33˚C for 1–3 h in a shaking incubator. Cultures are centrifuged at $8500 \times g$ for 5 min, and the bacterial pellet was washed twice in Ringer's lactate and diluted into the appropriate number of CFU/ml in Ringer's lactate solution determined by spectrophotometer. The bacterial concentration was confirmed by diluting all samples and plating out the known dilution on sheep blood agar plates.

Animal model

Wild-type C57BL/6J and TLR2 knockout mice (6–8 wk, weighing 20–30 g) were infected with intratracheal (i.t.) *S. mucilaginosus* and intranasal PA103, as described (21, 33, 34). Total and differential cell counts from BAL, lung tissue myeloperoxidase (MPO) activity, and bacterial colony counts were performed, as described before (21, 32–34). BAL protein concentration was determined with bicinchoninic acid method. The studies were approved by the Animal Care Committee and Institutional Biosafety Committee of our Institute.

Survival studies

Mice were treated with i.t. PA103 or *S. mucilaginosus*, or i.t. *S. mucilaginosus* or *Streptococcus gordonii* with intranasal PA103 in 0.9% saline (12 mice/group), monitored every 2 h, and sacrificed when moribund or after 96 h when the observations were terminated.

BAL fluid and total and differential cell counts

After mice were asphyxiated with CO_2, tracheas were cannulated, and lungs were lavaged in situ with sterile pyrogen-free physiological saline that was instilled in four 1-ml aliquots and gently withdrawn with a 1-ml tuberculin syringe. Lung lavage fluid was centrifuged at $400 \times g$ for 10 min. Supernatant was kept at −70˚C, the cell pellet was suspended in serum-free RPMI 1640, and total cell counts were determined on a grid hemocytometer. Differential cell counts were determined by staining cytocentrifuge slides with a modified Wright stain (Diff-Quik; Baxter) and counting 400–600 cells in complete cross-sections.

Lung histology

To collect lung tissue, mice were perfused with saline and lungs were inflated with 1 ml 10% neutral-buffered formalin. After paraffin embedding, 5-μm sections were cut, placed on charged slides, and stained with H&E staining.

Bacterial infection and colony counting

Unless specified, 1 million bacteria (for PA103) and 10^{10} (for *S. mucilaginosus* and *S. gordonii*) in 100 μl PBS were instilled by i.t. route injection with a 27-gauge needle to surgically exposed mouse tracheas. The neck wound was closed with sterile sutures under aseptic conditions. In case of dual infection, mice were first treated with i.t. *S. mucilaginosus*, followed by intranasal PA103. Before harvesting the lungs, the right ventricle was infused with 1 ml sterile PBS to remove blood from the lung tissue, and then the lungs were removed aseptically and homogenized in 3 ml sterile PBS. Lung homogenate was cultured overnight on soy base blood agar plate for bacterial colony counting.

Lung tissue MPO activity

Lung polymorphonuclear neutrophil sequestration was determined by measuring MPO activity, as described previously. At the end of the experiment, lungs were immediately removed, frozen, and stored at −70˚C until assayed. Lungs were homogenized in 5% hexadecyltrimethyl-ammonium bromide buffer, sonicated three times for 15 s on ice, and centrifuged at $16{,}100 \times g$ for 30 min at 4˚C. Protein concentrations were determined by bicinchoninic acid protein assay. A 10-μl sample of the supernatant was loaded into a cuvette plate. *o*-Dianisidine dihydrochloride with 0.0005% hydrogen peroxide in phosphate buffer (182 μl) was then added to samples. Absorbance change was measured at 460 nm for 3 min. MPO activity was expressed as change in absorbance per minute per milligram of protein.

Western blot analysis

Total cell lysate was prepared with radioimmunoprecipitation assay cell lysis buffer (Sigma-Aldrich) supplemented with protease inhibitors (Roche). To obtain proteins from tissue, harvested organs were quickly frozen in liquid nitrogen, and 200 mg tissue was lysed in buffer containing 50 mM HEPES (pH 7.5),150 mM NaCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, and protease inhibitors (Roche). Tissue in the buffer was homogenized and incubated on ice for 15 min with occasional vortexing. Cell debris was removed by centrifugation at $1000 \times g$ for 10 min at 4˚C. Protein content was quantified by the Bradford assay (Bio-Rad), as specified by the manufacturer. After SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad), which was incubated with appropriate Abs. Immune complex was detected by ECL plus (Amersham).

Quantitative real-time RT-PCR

Total RNA was prepared with RNeasy kit (Qiagen), according to manufacturer's manual. Reverse transcription of 2 μg total RNA was performed with SuperScript Π reverse transcriptase and oligo(dT) (Invitrogen) to generate cDNA. Quantitative real-time PCR was carried out by ABI 7900 HT machine, and specific primers of COX-2 and GAPDH and TaqMan Universal PCR Master Mix were purchased from Applied Biosystems. Analyses were done in triplicate, and mean normalized expression was calculated with GAPDH as an internal control.

Cytokine and PGE_2 measurement

Cytokine levels in the lung, BAL, and cell supernatants for TNF-α, IL-1β, IL-6, MIP-1α, MIP-2, KC, IL-12, IL-10, and PGE_2 were determined by ELISA kits, according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

Reagents

Abs against COX-2, microsomal PGE synthase (mPGES)–1, mPGES-2, and NS-398 (COX-2 inhibitor) were purchased from Cayman Chemicals (Ann Arbor, MI); Abs against ERK1/2, p38, and β-actin were purchased from Sigma-Aldrich. ERK1/2 inhibitor (UO126), MEK inhibitor (PD0325901), and p38 inhibitor (SB203580) were purchased from Invivogen.

Statistical analysis

Data are expressed as mean ± SEM, unless specified. Statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software). All experiments were repeated at least three to five times. Student *t* tests were used for two-group comparisons, ANOVA with Bonferroni posttests for multiple group comparisons, and a p value < 0.05 was considered significant. Survival data were analyzed by the construction of Kaplan–Meier plots and use of log-rank test.

Results

S. mucilaginosus *is a cohabitant in lower airways of patients with bronchiectasis*

Microbiology data from 182 patients with bronchiectasis were reviewed over a 5-y period. Seventy-three percent were women,

and 27% were men (2.8:1). Forty-two percent smoked with average 22.2 pack years. Thirty-five percent recalled a childhood infection with pneumonia, 12 had tuberculosis, and 6 had pertussis. Only 1% had a family history of bronchiectasis. Fourteen percent had associated rheumatological condition such as rheumatoid arthritis, and only 4 were diagnosed with HIV. Forty percent of patients had a BAL performed during the course of their illness because of persistent symptoms. Eleven patients (5.7%) had a new opacification seen on radiology with exacerbation. Pathogens that were isolated are shown in Fig. 1. We found that 9% of patients showed a significant growth of *S. mucilaginosus* from their BAL (Fig. 1). The growth of *S. mucilaginosus* was reported by the microbiologist when cultures from BAL showed 4^+ growth. To our surprise, this bacteria has been reported as a lower respiratory pathogen in only two previous studies (16, 17). We hypothesized that the presence of this bacterium in the lower airways may create an inflammatory milieu seen in patients with bronchiectasis. Therefore, we investigated whether *S. mucilaginosus* induces an inflammatory response and whether it contributes to the pathogenicity of other microbes in the lower airways by employing a mouse model.

Intratracheal administration of S. mucilaginosus *induces an inflammatory response in vivo in lungs of mice*

S. mucilaginosus is a Gram-positive coccus that has been reported to cause infections in immunocompromised patients and occasionally reported to cause severe infections in immunocompetent hosts (6–8, 16, 17). This bacterium has not been previously studied in an experimental model. To investigate the pathogenic potential of *S. mucilaginosus*, we infected wild-type mice with bacteria. We first infected mice i.t. with multiplicity of infection (MOI) of 10^5, 10^6, 10^7, 10^8, 10^9, and 10^{10} CFU. We were able to detect an inflammatory response with neutrophilic influx at a dose of 10^{10} CFU at 24 h (Fig. 2A).

After establishing a dose response, we performed additional experiments with a dose of 10^{10} CFU *S. mucilaginosus.* Control mice were treated with Gram-positive commensal *S. gordonii* (10^{10} CFU). Mice infected with *S. mucilaginosus* showed a significant increase in total cell count, neutrophils, MPO activity (Fig. 2B) with retrievable colony counts from lungs, and BAL (Fig. 2C), whereas control mice showed minimal inflammatory response at a comparable dose. There was also a significant increase in BAL protein (Fig. 2) with increase in proinflammatory cytokines and chemokines from BAL and lungs of mice infected with *S. mucilaginosus* (data not shown). Control mice did not show a significant increase in cells or proteins in BAL or lungs.

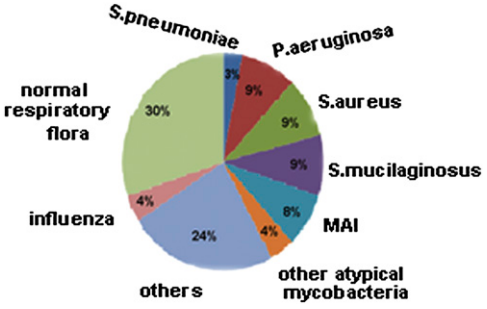

FIGURE 1. *S. mucilaginosus* is a cohabitant from lower airways of patients with bronchiectasis. Microbiota study from sputum and BAL from 192 patients with bronchiectasis showed that 9% of patients were colonized with *S. mucilaginosus*, 9% *P. aeruginosa*, 9% *Staphylococcus aureus*, 8% mycobacterium avium intracellulare, 4% other mycobacteria, 4% *Haemophilus influenzae*, 3% *Streptococcus pneumoniae*, and 30% normal respiratory flora.

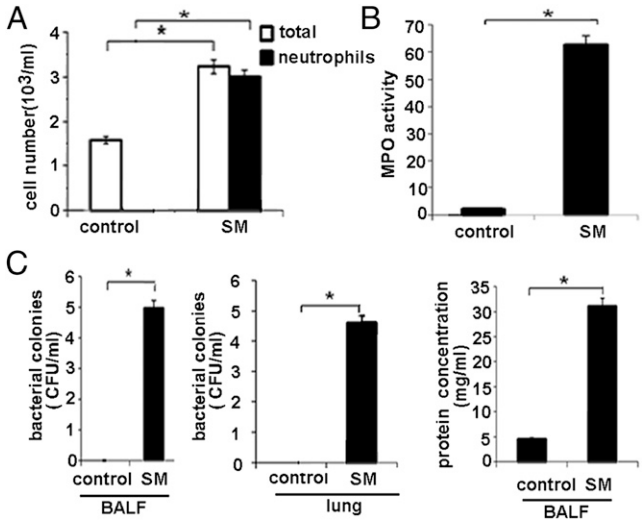

FIGURE 2. *S. mucilaginosus* induces a neutrophilic inflammatory response in lungs of mice. Wild-type mice were treated with i.t. *S. mucilaginosus* or *S. gordonii* (control mice) 10^{10} CFU. Mice were euthanized 24 h postinfection. (**A**) Total and neutrophil counts from BAL; (**B**) MPO assay from lungs; (**C**) bacterial colony counts from BAL fluid and lungs ($\times 10^3$ CFU/ml BAL fluid or right middle lobe of the lung); protein content from BAL. $n = 4–5$, $^*p < 0.01$.

These data suggest that inhalation of large doses of *S. mucilaginosus* in the lungs can induce an inflammatory response, which is similar to that seen with pathogens such as *P. aeruginosa* (21, 33).

S. mucilaginosus *induces COX-2 in vitro in macrophages and in vivo in lungs of mice with increased production of* PGE_2

Cumulative evidence from in vitro and in vivo models of infection implicates COX-2 and lipid mediators as important regulators of host defense/inflammatory networks and determinants of pathogenetic mechanisms. PGs produced by induction of COX-2 exhibit strong immunomodulatory activity with autocrine and paracrine effects that alter the host ability to clear pathogens (21, 22). Because *S. mucilaginosus* induced a neutrophilic influx with an inflammatory response in the lungs, we hypothesized that activation of COX-2 may be a contributor to the pathogenic potential of *S. mucilaginosus*. Therefore, we sought to investigate whether *S. mucilaginosus* induces COX-2 in vitro and in vivo.

We first examined whether COX-2 is induced in vitro by *S. mucilaginosus* and defined the time course and dose response of bacteria needed. BMDM from wild-type mice were treated with *S. mucilaginosus* (MOI 10 and 100). Cell lysates were extracted at 4, 8, 12, 24, 36, and 48 h postinfection. Western blotting for COX-2 confirmed that COX-2 protein was induced as early as 4 h and lasted up to 24 h postinfection (data not shown). The minimum MOI needed for induction of COX-2 by *S. mucilaginosus* was 100 (data not shown). PGE_2 and PGD_2 production from cell culture supernatants were measured. There was an increased production of PGE_2 at all the given time points, whereas the levels of PGD_2 were not detectable (data not shown).

Next, we investigated the ability of *S. mucilaginosus* to induce COX-2 in vivo. Wild-type mice were treated with i.t. *S. mucilaginosus* (10^{10} CFU) and euthanized 24 h postinfection. Mice treated with *S. mucilaginosus* showed an increase in COX-2 protein (Fig. 3A) and mRNA (Fig. 3B) in a time-dependent manner with increased production of PGE_2 (Fig. 3C). Control mice did not show a significant induction of COX-2 or production of PGE_2. Because PGE_2 can be produced by induction of microsomal PGE synthases, we performed Western blot analysis for mPGES-1 and mPGES-2

from lungs to determine the source of PGE_2 production. At the given time points, we were unable to detect an induction for mPGES-1 or mPGES-2 in the lung specimens infected with *S. mucilaginosus* (data not shown). Together these data show that *S. mucilaginosus* induces COX-2 in lungs with production of PGE_2.

Inhibition of COX-2 enhances the bacterial clearance of S. mucilaginosus *in vivo in mice*

Inhibition of COX-2 has shown to improve immune response to viral infections and vaccinations (35–38). We and others have shown that induction of COX-2 in the lungs in response to *P. aeruginosa* is immunosuppressive in a PGE_2-dependent manner. Furthermore, inhibition of COX-2 enhances clearance of *P. aeruginosa* (21, 22, 39). Because *S. mucilaginosus* induces COX-2 with increased production of PGE_2, we investigated whether inhibition of COX-2 has an impact on host immune response and bacterial clearance. For these experiments, mice were treated with NS-398 (specific COX-2 inhibitor) (15 mg/kg given 2 i.p. doses) prior to infection with *S. mucilaginosus*. Control mice were treated with i.p. vehicle (2 doses) prior to infection. As shown in Fig. 4A, administration of NS-398 to mice inhibited COX-2 expression and PGE_2 production (Fig. 4B). The clearance of *S. mucilaginosus* was enhanced by administration of NS-398, as demonstrated by bacterial colony counts in the lungs (Fig. 4C). However, to our surprise, there was no significant difference in the BAL cell counts in mice treated with NS-398 (data not shown). We also measured the proinflammatory cytokines in BAL and lungs of mice that were treated with *S. mucilaginosus* and NS-398. The production of IL-6 (Fig. 4D) and IL-1β (Fig. 4E) was significantly reduced by NS-398, whereas there was no difference in the production of MIP-1α (Fig. 4F) in mice that were treated with NS-398. There was no difference in the production of TNF-α, MIP-2, KC, IL-10, or IL-12 (data not shown). These data suggest that inhibition of COX-2 enhances the clearance of *S. mucilaginosus*. Furthermore, the enhanced bacterial clearance does not seem to be related to a difference in inflammatory cell count; however, it may be related to decreased production of PGE_2 and inhibition of select proinflammatory cytokines in the lungs by COX-2 inhibition.

TLR2 signaling is necessary for induction of COX-2 by S. mucilaginosus

Next, we investigated the signaling mechanisms that lead to the induction of COX-2 by *S. mucilaginosus*. TLRs are central to defining the immune response to infectious pathogens. COX-2

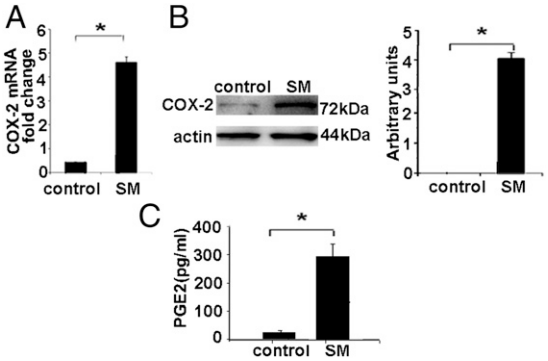

FIGURE 3. *S. mucilaginosus* induces COX-2 in lungs with production of PGE_2 in vivo. Wild-type mice were treated with i.t. *S. mucilaginosus* or *S. gordonii* (control mice) 10^{10} CFU. Mice were euthanized 24 h postinfection. (**B**) Western blot analysis of COX-2 expression in the lungs with densitometry; (**A**) real-time RT-PCR for COX-mRNA; (**C**) PGE_2 in the lungs measured by ELISA. $n = 4–5$, $^*p < 0.01$.

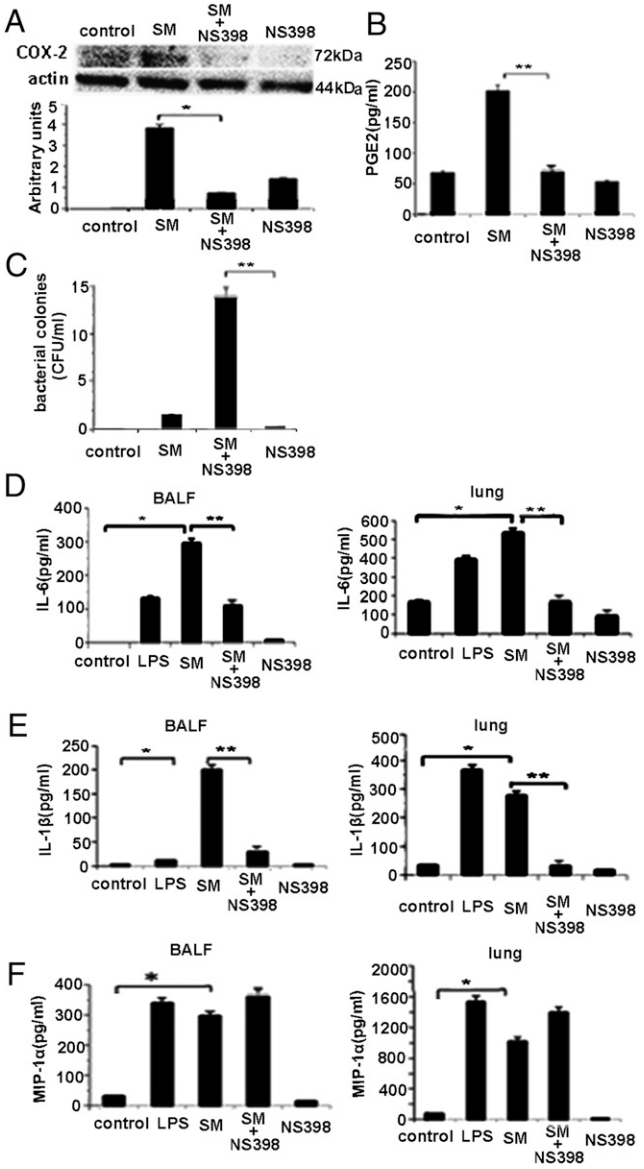

FIGURE 4. Inhibition of COX-2 enhances bacterial clearance of *S. mucilaginosus* in vivo. Wild-type mice were treated with NS-398 (specific COX-2 inhibitor) 15 mg/kg i.p. (2 doses) prior to i.t. infection of mice with *S. mucilaginosus* 10^{10} CFU. (**A**) Western blot analysis for COX-2 protein with densitometry; (**B**) PGE_2 in BAL fluid; (**C**) bacterial colony counts from right middle lobe of the lungs ($\times 10^3$ CFU/ml); (**D**) IL-6 in BAL and lungs; (**E**) IL-1β in BAL and lungs; (**F**) MIP-1α in BAL and lungs. $n = 4–5$, $^*p < 0.05$, $^{**}p < 0.001$.

induction by viruses, bacteria, and microbial products is mediated by activation of TLRs and is transcriptionally regulated in macrophages (40–43). There are no previous reports to suggest mechanisms by which *S. mucilaginosus* can induce inflammatory response. Because *S. mucilaginosus* is a Gram-positive coccus, we questioned whether TLR2 signaling is necessary for the induction of COX-2. Macrophages play a pivotal role in the host immune response (18); we therefore performed in vitro studies in primary cultured macrophages from bone marrow (BMDM) of mice. BMDM from wild-type, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice were treated with *S. mucilaginosus* (MOI 100, dose derived from our initial studies). Macrophages from $TLR4^{-/-}$ mice induced COX-2 similar to wild-type macrophages in response to *S. mucilaginosus*, whereas macrophages from $TLR2^{-/-}$ mice showed a significantly reduced expression of COX-2 mRNA (Fig. 5A) with attenuated

production of PGE_2 (Fig. 5B). These data suggest that, in macrophages, presence of TLR2 is necessary for the induction of COX-2 by *S. mucilaginosus.*

We then performed in vivo experiments in mice to define the role of TLR2 in the induction of COX-2 in the lungs by *S. mucilaginosus*. Wild-type and TLR2 knockout mice were treated with i.t. *S. mucilaginosus* 10^{10} CFU. Control mice were treated with heat-killed *S. mucilaginosus* (10^{10} CFU). Mice were euthanized 24 h postinfection. TLR2 knockout mice showed a decreased induction of COX-2 protein (Fig. 5C), message (Fig. 5D), with an attenuated production of PGE_2 (Fig. 5E). To define the functional significance of COX-2 induction in TLR2 knockout mice, we measured bacterial clearance of *S. mucilaginosus*. We found that TLR2 knockout mice showed a significantly enhanced clearance of *S. mucilaginosus* (Fig. 5E). To determine whether the enhanced bacterial clearance was related to the production of PGE_2, we administered i.t. PGE_2 (200 μg/kg) to TLR2 knockout and wild-type mice and measured bacterial colony counts in the lungs. Wild-type mice that were treated with PGE_2 along with *S. mucilaginosus* showed a trend toward further increase in colony counts. However, TLR2 knockout mice showed an increase in bacterial colony counts, thus suggesting that the protective effect seen in $TLR2^{-/-}$ is mediated by a decreased production of PGE_2 (Fig. 5E). Thus, these data for the first time, to our knowledge, show that *S. mucilaginosus* induces COX-2 in a TLR2-dependent manner that modulates the host immune response because of altered production of PGE_2.

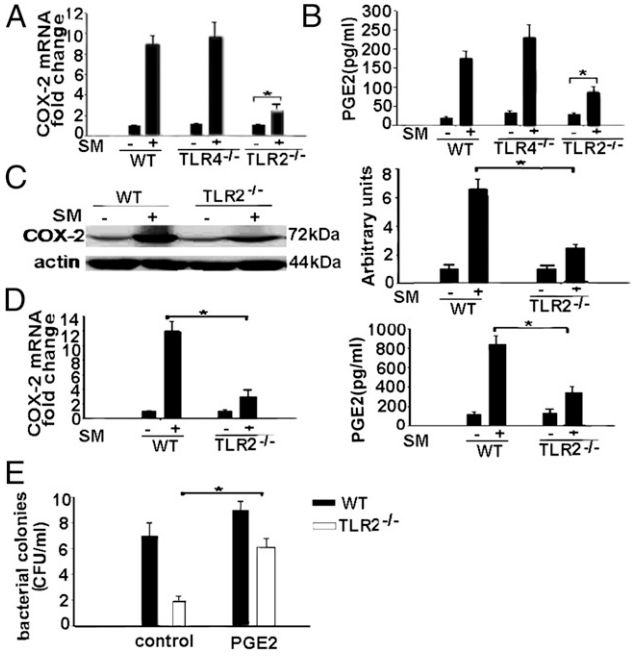

FIGURE 5. Presence of TLR2 is necessary for the induction of COX-2 and production of PGE_2 by *S. mucilaginosus*. BMDM from wild-type, TLR4, and TLR2 knockout mice were infected with *S. mucilaginosus* or heat-killed bacteria (MOI of 100). (**A**) Fold induction of COX-2 mRNA. (**B**) PGE_2 production from cell supernatant. Wild-type and TLR2 knockout mice were treated with i.t. *S. mucilaginosus* 10^{10} CFU. Mice were euthanized 24 h postinfection. (**C**) Western blot analysis for COX-2 protein with densitometry. Fold induction of COX-2 mRNA. (**D**) PGE_2 production in the lungs was significantly attenuated in TLR2 knockout macrophages. TLR2 knockout and wild-type mice were treated with i.t. PGE_2 (200 μg/kg) prior to infection with *S. mucilaginosus* (10^{10} CFU). (**E**) Bacterial colony counts measured from the right middle lobe of the lungs ($\times 10^3$ CFU/ml) (n = 4–5, $^*p < 0.01$).

Induction of COX-2 is dependent on p38-ERK/MAPK signaling in vitro

Next, we investigated the downstream signaling that regulates the expression of COX-2 in macrophages in response to *S. mucilaginosus*. Ligation of TLRs by microbial products congregates on downstream signaling through activation of NF-κB signaling pathway. Induction of COX-2 in response to LPS and bacterial products is regulated by p38-MAPK signaling (44). We therefore investigated the role of MAPK signaling in the induction of COX-2 by *S. mucilaginosus*.

BMDM and RAW cells were treated with *S. mucilaginosus* for specified time points. Western blot analysis for phosphorylated p38 and p44/42 was performed to detect activation of MAPKs. Within 10 min postinfection of BMDM with *S. mucilaginosus* (MOI 100), expression of the phosphorylated p38 and ERK1/2 (p44/42) MAPKs was detected, which was sustained for 45 min (data not shown). To investigate whether activation of p38-ERK/MAPK pathway is necessary to induce COX-2, we treated BMDM and RAW cells with p38-ERK–specific inhibitors prior to infection with *S. mucilaginosus*. Inhibition of p38 (SB203580, 10 μmol), ERK (PD098059, 10 μmol), and MEK (UO126, 10 μmol) attenuated induction of COX-2 in BMDM (Fig. 6C) and RAW cells (Fig. 6). These data suggest that the induction of COX-2 by *S. mucilaginosus* is dependent on the p38-ERK/MAPK signaling pathway.

S. mucilaginosus *increases mortality of mice treated with* P. aeruginosa *that is rescued by inhibition of COX-2*

Our data to date show that *S. mucilaginosus* induces a neutrophilic influx and generates an inflammatory response in vivo, including induction of COX-2 with increased production of PGE_2. We questioned whether the presence of *S. mucilaginosus* alters the pathogenicity of other commonly isolated microbes in patients with bronchiectasis. Because *P. aeruginosa* is a commonly found resistant infection in patients with bronchiectasis, we simulated a murine model by infecting mice first with i.t. *S. mucilaginosus* or

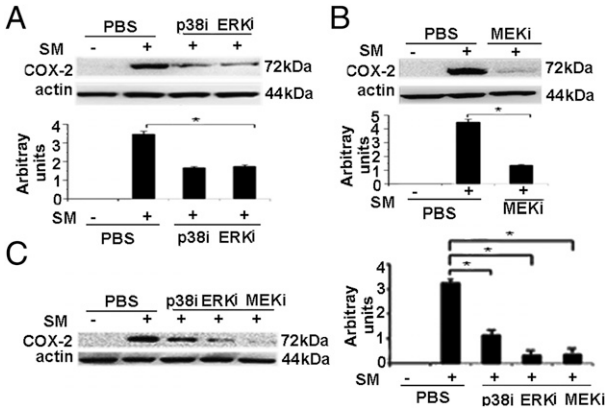

FIGURE 6. Induction of COX-2 by *S. mucilaginosus* is dependent on activation of MAPK signaling in macrophages. BMDM or RAW cells were treated with *S. mucilaginosus* (MOI of 100). Western blot analysis was performed from cell lysates obtained from BMDM at specified time points. (**A**) Phosphorylation of p38 and (**B**) phosphorylation of p44/42. Western blot analysis from RAW cells treated with *S. mucilaginosus* at specified time points. (**C**) Phosphorylation of p38. Phosphorylation of p44/42. BMDM from wild-type mice were treated with p38, ERK, or MEK inhibitors prior to infection. Western blot analysis for COX-2 protein after treatment with p38 and ERK inhibitor (10 μmol). Western blot analysis for COX-2 after treatment with MEK inhibitor. RAW cells were treated with p38, ERK, and MEK inhibitors (10 μmol). Western blot analysis for COX-2 protein (n = 4–5, $^*p < 0.01$).

S. gordonii and then administering *P. aeruginosa* via intranasal route. We have previously shown that, in C57BL/6 mice, the LD_{50} for *P. aeruginosa* is 10^7 CFU, whereas a dose of 10^6 CFU is sufficient to cause lung infection with neutrophilic influx (21, 33, 34). Therefore, we used a dose of 10^6 CFU for *P. aeruginosa* infection. To our surprise, control mice that were infected with *S. gordonii* (10^{10} CFU) and sublethal *P. aeruginosa* (10^6 CFU) all survived, whereas mice that were treated with *S. mucilaginosus* (10^{10} CFU) and *P. aeruginosa* (10^6 CFU) died within 48 h of infection (Fig. 7). Because inhibition of COX-2 increases the clearance of *S. mucilaginosus*, we administered NS-398 (15 mg/kg in 2 doses given i.p.) to mice prior to infection with *S. mucilaginosus* and *P. aeruginosa*. Mice treated with NS-398 showed an improved survival (Fig. 7). Additional experiments were performed in which mice were euthanized at 8 h postinfection. There was no significant difference in the production of TNF-α, MIP-2, KC, IL-10, IL-12, or PGE_2 at this time point (data not shown). Together these data suggest that *S. mucilaginosus* increases the pathogenic potential of PA103 by inducing COX-2 that is rescued by inhibition of COX-2.

Discussion

In this study, we found that *S. mucilaginosus* was colonized in the lower airways of 9% of patients with bronchiectasis. To our knowledge, this is the first report that shows the growth of *S. mucilaginosus* in lower airways of patients with bronchiectasis. In a murine model, we show that mice treated with i.t. *S. mucilaginosus* generate a neutrophilic influx/inflammation with induction of COX-2, production of proinflammatory cytokines, and lipid mediators, mainly PGE_2. Presence of TLR2 was necessary for the induction of COX-2 in vitro in macrophages and in vivo in lungs of mice infected with *S. mucilaginosus.* TLR2 knockout mice showed an enhanced clearance of *S. mucilaginosus*, which was PGE_2 dependent. In vitro studies in primary cultured macrophages showed that induction of COX-2 is dependent on p38-ERK/MAPK signaling pathway. Most importantly, we demonstrate that mice infected with *S. mucilaginosus* and sublethal dose of *P. aeruginosa* showed an increased mortality that is rescued by inhibition of COX-2. To our knowledge, these are the first studies that have investigated the contribution of *S. mucilaginosus* in lung infection in an experimental model and demonstrate the pathogenic potential of an oral commensal.

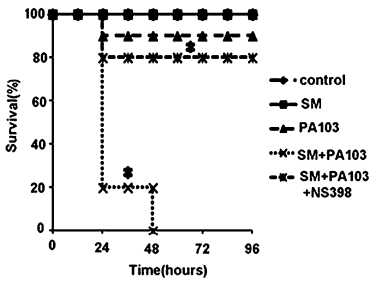

FIGURE 7. *S. mucilaginosus* increases mortality in mice treated with *P. aeruginosa*, which is rescued by inhibition of COX-2. Wild-type mice were treated with *S. mucilaginosus* (10^{10} CFU), PA103 (10^6 CFU), or PA103 (10^6 CFU) with *S. mucilaginosus* (10^{10} CFU) or *S. gordonii* (10^{10} CFU) with or without NS-398 (COX-2 inhibitor) (15 mg/kg). Mice treated with PA103 or *S. mucilaginosus* alone all survived, whereas mice treated with *S. mucilaginosus* and PA103 succumbed within 48 h. Mice treated with PA103 and *S. mucilaginosus* with NS-398 showed an improved survival. The results are represented by Kaplan-Meier curve ($p < 0.01$ log-rank test).

S. mucilaginosus belongs to the family Micrococcaceae and is a Gram-positive, encapsulated, coagulase-negative coccus that is part of the normal oropharyngeal flora. Infections with *S. mucilaginosus* are being increasingly reported in immunocompromised patients. Reports of bacteremia, central venous catheter sepsis, pneumonia, and meningitis with *S. mucilaginosus* have been seen in neutropenic patients (6–8, 16, 17). *S. mucilaginosus* pneumonia has also been reported in patients with HIV infection and in a patient following liver transplant (7, 11, 16). Few cases of infections by *S. mucilaginosus* have been reported in immunocompetent host (10). In a previous study, Korsholm et al. (17) isolated *S. mucilaginosus* from eight patients suffering from lower respiratory tract infections over a 4-y period. In their series, infections ranged from mild cases of pneumonia to life-threatening recurrent lung abscesses in a neutropenic patient. The various strains of *S. mucilaginosus* in their study were cultured from specimens obtained by bronchoscopy, blood, and sputum specimens.

Our study raises the question of significance of isolating *S. mucilaginosus* in lower airways. It is difficult to culture *S. mucilaginosus* from sputum samples as the bacteria may be scant because they are often overgrown by the faster growing pathogens. Alternatively, when *S. mucilaginosus* is grown from sputum, it is invariably ignored because it is considered to be a contaminant. In this study, *S. mucilaginosus* was isolated from BAL of patients. Furthermore, our in vivo data in mice confirmed that *S. mucilaginosus* is able to generate a proinflammatory response and may exhibit pathogenic potential in an appropriate clinical setting, such as in patients with bronchiectasis in which chronic airway damage may allow these bacteria to colonize and form biofilms. Our study suggests that isolating *S. mucilaginosus* especially in large numbers from BAL should not be ignored.

Host immune factors are critical to define outcomes in infections (18). Activation of TLRs and transcription factors are key elements of innate immunity that promote the expression of genes involved in host defense, such as proinflammatory cytokines, and enzymes such as COX-2 (19–20). PGs and lipid mediators produced by induction of COX-2 are being recognized as key immunomodulators in infections and cancer (45). COX-2 has been shown to play a pivotal regulatory role in a variety of infections, including viruses, bacteria, fungi, and parasites (44, 46–49). COX-2–deficient mice display resistance against detrimental effects of endotoxemia (50, 51). Modulation of immune response by COX-2 is largely related to an increased production of PGE_2, which has been shown to be immunosuppressive in animal models of bacterial pneumonias and sepsis (21, 23, 52–54). PGE_2 can also be produced by induction of mPGE synthases, depending on the stimulus (55, 56). In this study, we were unable to detect expression of mPGES-1 or 2 in response to *S. mucilaginosus* despite increased production of PGE_2. Thus, our data indicate that increased production of PGE_2 is predominantly related to induction of COX-2 in response to *S. mucilaginosus*.

There are several potential mechanisms by which PGE_2 mediates immunosuppression, which include inhibition of production of NO, reactive oxygen species, and IL-12, which have microbicidal properties against bacteria and viruses (21). PGE_2 also inhibits B cell proliferation and Ig production; enhances production of immunosuppressive cytokines IL-10 and IL-6; inhibits leukocyte chemotaxis and leukotriene synthesis; and inhibits phagocytosis of bacteria in macrophages (22, 23, 53). We speculate that these mechanisms play a key role in PGE_2-induced immunosuppression in response to *S. mucilaginosus*.

The harmful effects of COX-2 have been supported by studies that have shown that administration of COX-2 inhibitors suppresses viral replication and enhances immunity in H5N1 and vaccinia

murine models (35, 49). We and others have previously shown that inhibition of COX-2 improves mortality in a lethal mouse and bone marrow transplant model of *P. aeruginosa* lung infection (21, 53). In this study, inhibition of COX-2 improved survival in mice treated with PA103 and *S. mucilaginosus*, which suggests that pathogenic effects of *S. mucilaginosus* are related to induction of COX-2. Furthermore, data from TLR2 knockout mice are in agreement with these findings because we found a decreased induction of COX-2, attenuated production of PGE_2 in TLR2 knockout mice with enhanced clearance of *S. mucilaginosus* from the lungs. Together these data strongly favor a role for PGE_2-induced immunosuppression in this model.

Although understanding of the roles of COX-2 and its mediators in microbial host defense mechanisms is expanding, there are yet relatively few reports pertaining to its significance in human diseases. Our study has important clinical implications because increased prostanoid release has been reported in patients with bronchiectasis associated with cystic fibrosis (57, 58). Furthermore, some studies suggest that inhibition of COX-2 delays the progression of lung disease in patients with cystic fibrosis (59), although the mechanisms are not fully understood. To date there are no studies that have investigated the role of COX-2 inhibition in noncystic fibrosis (CF) bronchiectasis. Although speculative data from this study suggest that low-grade pathogens such as *S. mucilaginosus* may colonize lower airways, leading to increased production of prostanoids that may suppress host immunity, thus making it congenial for other microbes to establish infection, further studies to define the microbiome and the role of low-grade pathogens such as *S. mucilaginosus* in patients with chronic lower airway diseases such as cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary disease are needed.

Because there is lack of data about the pathogenic role of *S. mucilaginosus*, there is a gap in knowledge on how this pathogen can initiate host immune response. To our knowledge, our study for the first time shows that *S. mucilaginosus* induces host immune response by activating TLR2. TLRs play a central role in mounting a host immune response to infections (19, 20). To our knowledge, this is the first study to define an association of TLR signaling in *S. mucilaginosus* immune response.

We also sought to determine the downstream signaling pathways that lead to induction of COX-2. Previous studies with LPS, *Salmonella*, *P. aeruginosa*, and mycobacteria have shown that regulation of COX-2 expression depends on the activation of NF-κB and MAPKs (60). We assessed the activation of MAPKs by detection of the phosphorylated forms of ERK1/2 and p38 in the extracts of macrophages infected with *S. mucilaginosus*. Our study shows that inhibition of p38-ERK MAPKs abrogated induction of COX-2 in vitro in macrophages, thus confirming that induction of COX-2 is dependent on p38-ERK/MAPK signaling pathway. To our knowledge, these are the first studies to investigate the signaling mechanisms by which *S. mucilaginosus* initiates and induces an inflammatory response.

Bronchiectasis is a chronic, debilitating, airways disease characterized by a vicious cycle of inflammation and bacterial colonization (1–3). Infections contribute to development of recurrent exacerbations and premature death. The definitive etiology of bronchiectasis is established in very few patients, and, in general, there are no effective treatments apart from antibiotics and chest clearance techniques (39). It is unclear why some patients develop chronic bacterial colonization while others experience a more benign course. We reasoned that some patients may aspirate their oral contents, allowing commensals such as *S. mucilaginosus* to have access to the lower airways. These bacteria may then act as low-grade pathogens and over time create an environment congenial for the growth of other microbes such as *P. aeruginosa*. Future studies will define the specific bacterial factors present in *S. mucilaginosus* that are responsible for the inflammatory and immunogenic potential.

The CF pulmonary microbiome has identified complex bacterial communities, including traditional pathogens, anaerobic bacteria, and other less known pathogens in lower airways of patients with CF (61, 62). There is a lack of similar studies in patients with non-CF bronchiectasis. Based on culture growth, we have identified an oral pathogen from BAL of significant number of patients with bronchiectasis. To profile the mixed-species biomarker gene, amplicons generated by culture-independent approaches are being developed. Molecular approaches to phylogenetically profile mixed species in a given microbial community are typically DNA based and rely on PCR amplification techniques. These techniques are more specific and sensitive and in the future will help identify mixed communities in lower airways of patients with bronchiectasis. Our study is limited because of its retrospective nature. Although we have identified one oral pathogen, it is plausible that there are multiple pathogens forming communities in airways of these patients that are not reported or are ignored. Prospective studies using more sensitive techniques are sorely needed in patients with non-CF bronchiectasis. These studies will help define personalized microbiomes for individual patients with resistant infections in bronchiectasis.

In summary, our study for the first time, to our knowledge, shows growth of an oral commensal from lower airways of patients with bronchiectasis. In a murine model, we have shown that *S. mucilaginosus* can increase the pathogenicity of *P. aeruginosa* by induction of COX-2. Lastly, our study indicates a role for COX-2 inhibition as an adjunctive therapy in patients with bronchiectasis. Our study has fundamentally important implications and provides a new insight into the bacteriology and personalized microbiome of patients with bronchiectasis, which may help us understand the pathogenesis and progression of this orphan disease.

Disclosures

The authors have no financial conflicts of interest.

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