Chronic obstructive pulmonary disease and asthma-associated Proteobacteria, but not commensal Prevotella spp., promote Toll-like receptor 2-independent lung inflammation and pathology

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COPD and asthma-associated Proteobacteria, but not commensal Prevotella spp., promote TLR2-independent lung inflammation and pathology

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Running title: Lung inflammation in response to common airway bacteria

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Abstract

Recent studies of healthy human airways have revealed colonization by a distinct commensal bacterial microbiota containing Gram-negative *Prevotella* spp. However, the immunological properties of these bacteria in the respiratory system remain unknown. Here we compare the innate respiratory immune response to three Gram-negative commensal *Prevotella* strains (*Prevotella melaninogenica*, *Prevotella nanceiensis* and *Prevotella salivae*) and three Gram-negative pathogenic Proteobacteria known to colonize lungs of COPD and asthma patients (*Haemophilus influenzae* B, non-typeable *Haemophilus influenzae* and *Moraxella catarrhalis*). The commensal *Prevotella* spp. and pathogenic Proteobacteria were found to exhibit intrinsic differences in innate inflammatory capacities on murine lung cells *in vitro*. *In vivo* in mice, non-typeable *Haemophilus influenzae* induced severe TLR2-independent COPD-like inflammation characterized by predominant airway neutrophilia, expression of a neutrophilic cytokine/chemokine profile in lung tissue, and lung immunopathology. In comparison, *Prevotella nanceiensis* induced a diminished neutrophilic airway inflammation and no detectable lung pathology. Interestingly, the inflammatory airway response to the Gram-negative bacteria *Prevotella nanceiensis* was completely TLR2-dependent. These findings demonstrate weak inflammatory properties of Gram-negative airway commensal *Prevotella* spp. that may make colonization by these bacteria tolerable by the respiratory immune system.

Keywords

Respiratory inflammation; commensal microbiota; *Prevotella*; Proteobacteria; COPD; asthma; lung immunopathology.

Abbreviations

BAL: Bronchoalveolar lavage; DC: Dendritic cell; HE: hematoxylin-eosin; H. inf. B: *Haemophilus influenzae* B; H. inf. NT: non-typeable *Haemophilus influenzae*; LPS: Lipopolysaccharide; M. cat.: *Moraxella catarrhalis*; MAMP: Microbial-associated molecular pattern; P. mel.: *Prevotella melaninogenica*; P. nan.: *Prevotella nanceiensis*; P. sal.: *Prevotella salivae*; TLR: Toll-like receptor; WT: Wild-type.

Introduction

The role of commensal bacteria in health and disease is receiving increasing interest with the recognition that the microbiota plays a central role in shaping immune function, metabolism and protection from pathogenic microorganisms (1–3). The human lung has historically been considered sterile due to the absence of cultivable bacteria in bronchial alveolar lavage fluids from healthy individuals (4–6). However, culture-independent molecular methods for bacterial identification have recently been applied to characterize the human airway microbiota (7–11). Gram-negative anaerobic *Prevotella* species were found to be a prevalent bacterial colonizer of healthy airways (7, 11), and reduced frequencies were reported in chronic obstructive pulmonary disease (COPD) and asthma (7). *Prevotella* species are considered commensal bacteria that rarely cause respiratory infections, and only few strains have been reported to be opportunistic in chronic infections, abscesses and anaerobic pneumonia (12–14).

Dysbiosis, a change in microbiota composition detrimental for host health, is associated with altered function of the immune system and inflammatory pathologies (15). It is becoming increasingly appreciated that respiratory bacterial pathogens commonly colonize the lower airways of patients with COPD and asthma (7, 16, 17). Both stable disease and exacerbation episodes in COPD are predominantly associated with the presence of pathogenic Gram-negative Proteobacteria (*Haemophilus influenzae* and *Moraxella catarrhalis*) (18–20). The pathogenic Proteobacteria are believed to take part in disease progression of COPD by enhancing inflammatory processes and tissue degradation in the lower airways (21). Indeed, higher bacterial loads in the airways are associated with increased disease severity, exacerbation frequency, decreased lung function and increased production of inflammatory mediators (22–24). The notion of disease-promoting properties of bacteria is further supported by a recent interventional study showing that prophylactic antibiotic treatment reduced the frequency of bacterial colonization and exacerbations in COPD (25).

A role for pathogenic Proteobacteria in asthma pathology remains controversial and few studies are found in the literature. Increased airway colonization by pathogenic Proteobacteria is associated with

stable asthma in children and adults (7), exacerbation episodes in childhood (26) and increased risk of developing asthma (17). We recently reported abnormal bacterial immune responses in infants later developing asthma proposing that asthmatics exhibit decreased bacterial control and that divergent bacterial immune responses within the lung may contribute to asthma pathology in some disease endotypes (27).

Studies using murine models of COPD demonstrated that *Haemophilus influenzae* enhances airway inflammation induced by cigarette smoke exposure (28, 29). Furthermore, mice challenged in the airways with *Haemophilus influenzae* lysates develop inflammatory features of COPD, including production of pro-inflammatory mediators TNFα, IL-6 and IL-1β, airway neutrophilia and lung tissue pathology (30).

Commensal *Prevotella* species are frequent colonizers of the lower and upper airways; however little is known about the inflammatory properties of these bacterial species in the respiratory system. Since pathogenic Proteobacteria are recognized co-drivers of chronic airway inflammation in COPD and have been studied in relevant murine models of the disease, we here compared these to the commensal *Prevotella* spp. We demonstrate weak innate stimulatory properties of Gram-negative commensal *Prevotella* spp. that may make colonization by these bacteria tolerable by the respiratory immune system. On the contrary, the innate stimulatory capacity of pathogenic Proteobacteria likely allow these bacteria to be specific co-drivers of disease progression in COPD and asthma patients.

Materials and Methods

Mice

Wild-type C57BL/6J mice were obtained from Taconic (Silkeborg, Denmark). TLR2 deficient mice on the C57BL/6J background were obtained from Jackson Laboratories (Bar Harbor, ME) and bred in-house. Mice were kept under specific pathogen free conditions, according to national guidelines for experimental animal housing and under the daily care by animal technicians. All experiments were carried out using sex and age-matched mice. The experimental protocols were approved by the Danish Animal Ethics Committee (permission number: 2007/561-1266).

Bacteria growth and preparation

Haemophilus influenzae B (KAK510), *Haemophilus influenzae* NT (KAK509) and *Moraxella catarrhalis* F48 (KAK508) reference strains were kindly provided by Karen A. Krogfelt and Jørgen Skov Jensen, Statens Serum Institut, Copenhagen, Denmark. *Prevotella melaninogenica* (DSM7089), *Prevotella nanceiensis* (DSM19126) and *Prevotella salivae* (DSM15606) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. *Haemophilus* and *Moraxella* strains were grown on chocolate agar plates (Statens Serum Institut) under 37°C microaerobic (5% CO_2) conditions. *Prevotella* strains were grown on anaerobic agar plates (Statens Serum Institut) under 30°C anaerobic conditions. All strains were resuspended from plates with uniform growth and washed once in PBS. Bacteria were resuspended in PBS to OD 1 and UV-irradiated for 45 minutes. UV killing were confirmed by plating. Dry weight of bacterial suspensions in PBS was determined on 3x1 ml portions after freeze-drying (subtracted by weight of PBS). Bacterial suspensions were frozen and stored at -80°C.

Colony forming units (CFU) were determined in bacterial suspensions before UV-irradiation using serial dilution. CFUs pr. bacterial dry weight were as follows: 7.81 x 10^5 CFU/ug (H. inf. B), 4.91 x 10^5 CFU/ug (H. inf. NT), 2.45 x 10^5 CFU/ug (M. cat.), 0.26 x 10^5 CFU/ug (P. mel.), 0.13 x 105 CFU/ug (P. nan.) and 0.15 x 10^5 CFU/ug (P. sal.).

Primary lung cell preparation and stimulation

Mice were anesthetized using Ketamine/Xylazine (10/0.5 mg pr. 50 g body weight; Intervet, Boxmeer, Holland) and euthanized by heart puncture blood removal. PBS was perfused through the heart to remove systemic blood. Lungs were excised, cut into 1-2 mm pieces and incubated for 1h at 37°C in PBS (Lonza, Basel, Switzerland) supplemented with 30U/ml *Clostridium histolyticum* collagenase type II and 150 ug/ml DNAase (Sigma-Aldrich, Copenhagen, Denmark). The tissues were passed through a 70 μm cell strainer (BD Bioscience) to obtain a single cell suspension of lung cells. Cells from lungs of 3-5 mice were pooled and separated into lung constituent cells (CD45-) and lung leukocytes (CD45+) using a CD45+ magnetic cell sorting kit (MACS; MiltenyiBiotec, Bergisch Gladbach, Germany) according to manufacturer's recommendations. Purity was confirmed by flow cytometry. Cells were resuspended in complete RPMI 1640 medium (Lonza, Basel, Switzerland; supplemented with 2 mM L-glutamine (Cambrex, East Rutherford, NJ), 100 U/ml penicillin/streptomycin (Lonza) and 10 % FCS (Lonza)) and plated in 96-well plates ($2*10^5$ cells/well). In experiments with mixed CD45- and CD45+ lung cells a ratio of 1:1 was used.

Primary lung cells were stimulated with 50 μg/ml of bacterial preparations supplemented with 50 μg/ml gentamycin (Sigma-Aldrich) to ensure no bacterial outgrowth. LPS (100 ng/ml) and medium alone were included to serve as a positive and negative control, respectively. All stimulations were done in triplicates. Supernatants were harvested after 24 hours and stored at -80°C until cytokine analyses.

Acute airway inflammation model

Mice were lightly sedated using Ketamine/Xylazine (5 mg/0.25 mg pr. 50 g body weight). While kept in a vertical position, 25 μl bacterial suspension or vehicle (PBS) alone was inhaled through each nostril giving a final dose of 22.5 μg bacteria in 50 μl (450 μg/ml). Mice were kept vertical for 1 minute following inhalation.

24 hours after bacterial inhalation, the mice were anesthetized using Ketamine/Xylazine (10 mg/0.5 mg pr. 50g body weight) and euthanized by heart puncture. Bronchoalveolar lavage (BAL) cells were collected by prefusing lungs with 800 μl PBS five times. Right lung was snap frozen in liquid nitrogen and stored at -80°C until measurement of tissue cytokines. Left lung was embedded in Tissue-tek OCT Compound (Sakura, Tokyo, Japan) and stored at -80°C until histochemical analysis.

Tissue and supernatant cytokine measurements

Measurement of cytokine production in tissue was performed as previously described.(31) Frozen lung tissue were homogenized in a mortar with 500 ul/mg tissue PBS contain 0.1% Tween-20 (v/v) and protease inhibitor cocktail (Complete, Roche, 1 tablet/50 ml PBS). Tissue homogenates were frozen in liquid nitrogen, thawed, sonicated for 30s and centrifuged to remove debris. Lysates were stored at -80°C until cytokine analysis by ELISA.

Cytokines were measured in lung tissue homogenates and supernatants from cell cultures using commercial ELISA kits (IL-5, IL-4 and IL-13 from eBioscience, San Diego, CA; MIP-2α (IL-8), TSLP, TNFα, IL-10, IFNγ, IL-17, CCL20, CXCL16, IL-1β, IL-6 and IL-10 from RnD Systems, Minneapolis, MN) according to manufacturer's recommendations.

Flow cytometry

The composition of BAL cells was analyzed by flow cytometry as previously described(32) using the following anti-mouse fluorochrome-conjugated antibodies: Anti-CD45/eFlour450, anti-CD11b/FITC, anti-CD11c/APC (eBioscience), anti-Siglec-F/PE (BD Bioscience), anti-Gr-1/APC-Cy7 and anti-mF4/80/PE-Cy7 (Biolegend). Staining was performed in PBS containing 1 % FCS, 0.1 % sodium azide and Fc-block (BD Bioscience) for 30 minutes at 4°C. Cells were analyzed on a BD FACSCanto™ II system running FACSdiva 6.0 software (BD Biosciences, San Jose, CA). Immune cells were identified among CD45+ leukocytes as follows: Eosinophils (CD11c-, Siglec-F+), lymphocytes (CD11b-CD11c-), macrophages (F4/80+Gr-1-), neutrophils

(F4/80-Gr-1+), monocytes (F4/80-Gr-1-CD11bintCD11c-), CD11b+ dendritic cells (CD11b+ DCs; F4/80-Gr-1-CD11b+CD11c+) and CD11b-/low dendritic cells (CD11b-/low DCs; F4/80-Gr-1-CD11bintCD11c+). Flow cytometry data was analyzed using FlowJo 7.6.5 (Tree Star, Ashland, OR).

Histochemical staining

Tissue-tek OCT Compound embedded lung tissues were thawed overnight in 4 % (v/v) para-formaldehyde/PBS at 4^{o}C. Tissues were embedded in paraffin and cut into 5 µm sections. Sections were stained by Mayer's haematoxylin and eosin (Pioneer research chemicals, Colchester, UK) and evaluated by microscopy. Two pictures were taken representing areas with most and least inflammation in each lung section. Both pictures were used to grade lung pathology giving an average score. Pathology scores were given on a relative scale (1 – 6) judging integrity of the bronchial epithelium, peri-bronchial/arterial inflammation, as well as alveolar integrity and inflammation. Pathology scoring was performed in a blinded manner.

Data analysis and statistics

Statistical analysis was performed using GraphPad PRISM 5.01 (GraphPad Software, La Jolla, CA). Differences in BAL cell composition, cytokines levels in lung tissue and pathology scores were analyzed by two-way ANOVA and post-hoc by Bonferroni's multiple comparison test. Cytokine production in lung tissue was reported after subtracting mean levels measured in tissue from PBS treated mice (background). Student's one-sample t-test was applied to test if a cytokine was induced in the lung tissue after bacterial challenge (testing if mean level different from 0 ng/g lung tissue). Differences in the capacity of pathogenic Proteobacteria and *Prevotella* spp to induce cytokine production in primary lung cells *in vitro* was analyzed in a compartmentalized manner using Student's t-test comparing data from the three bacteria in each phylum. Bar charts show mean and standard error of the mean (SEM). P-values below 0.05 were considered statistically significant. P-values are indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Results

Lung commensal *Prevotella* spp exhibit distinct inflammatory properties compared to COPD and asthma-associated Proteobacteria

Three bacterial strains representing the COPD and asthma-associated Proteobacteria, as well as three *Prevotella* spp associated with healthy lungs were chosen for an initial analysis of innate inflammatory properties on primary murine lung cells. *Haemophilus influenzae* and *Moraxella catarrhalis* (M. cat.) were selected as the key members of pathogenic Proteobacteria bacteria associated with COPD and asthma. It was decided to include the *Haemophilus influenzae* B (H. inf. B) and non-typeable *Haemophilus influenzae* (H. inf. NT) strains, as these common strains are structurally different due to the presence or absence of bacterial capsule, respectively (33, 34). *Prevotella melaninogenica* (P. mel.), *Prevotella nanceiensis* (P. nan.) and *Prevotella salivae* (P. sal.) were included as representative commensal *Prevotella* spp predominantly associated with healthy airways (7, 11). The innate inflammatory capacities of the Proteobacteria and *Prevotella* spp were addressed by stimulating primary CD45+ and CD45- cells isolated from murine lungs representing lung leukocytes and non-immune cells, respectively. The production of key acute-phase cytokines MIP-2α (IL-8) and TNFα known to be involved in COPD and asthma pathology were analyzed in supernatants (35). IL-10 was analyzed to address potential anti-inflammatory aspects of the bacterial response. TSLP production was addressed as a cytokine produced exclusively by epithelial cells, and involved in priming of type-2 immune responses (36). In addition, cytokines IL-5, IL-4 and IL-13 were analyzed to assess potential type-2 immune responses induced by the bacteria. Co-cultures of CD45+ and CD45- cells (1:1) were performed to examine possible cytokine-enhancing cross talk between these cellular compartments.

We observed significant differences between the cytokine profiles induced in primary murine lung cells by the COPD and asthma-associated Proteobacteria and the commensal *Prevotella* spp (figure 1A). *Prevotella* spp generally induced lower cytokine production compared to Proteobacteria, but the observation was dependent on the cytokine investigated and the cellular compartment. In CD45+ lung cells,

Prevotella spp induced lower TNFα production compared to the three Proteobacteria. Additionally, TNFα, MIP-2α (IL-8) and TSLP were lower for *Prevotella* spp in the CD45- compartment. These cytokines and IL-10 were also produced less in response to *Pretovella* spp in 1:1 co-cultures of CD45+ and CD45- cells isolated from murine lungs (figure 1A). Interestingly, measurements of MIP-2α (IL-8) production indicated a synergistic effect between CD45+ and CD45- cells giving rise to generally higher MIP-2α (IL-8) levels in co-cultures. It is observed that production of some cytokines is restricted to a particular compartment. Expectedly, TSLP was only produced in CD45- lung cells, as this is an epithelial-derived cytokine (36). IL-5 production was absent in the CD45- compartment indicating this cytokine is only produced by lung immune cells. Furthermore, MIP-2α (IL-8), TNFα and IL-10 were generally lower in the non-immune CD45- lung cell compartment. IL-4 and IL-13 production in response to the bacteria could not be detected in bacteria-exposed CD45+ or CD45- lung cells (data not shown).

The observed differences in innate immune stimulatory properties between the three COPD and asthma-associated Proteobacteria and the commensal *Prevotella* spp could be due to differences in concentrations of specific innate ligands (microbe-associated molecular patterns, MAMPs) shared by the bacteria. To test this, we stimulated primary lung cells isolated from mice with increasing concentrations of the three Proteobacteria and *Prevotella* spp. TNFα and TSLP were measured as representative cytokines with observed differences in production induced by the two groups of bacteria in the CD45+ and CD45- lung cells compartments, respectively. The cytokine response to increasing concentrations of bacteria from the Proteobacteria and *Prevotella* spp fitted well to a one-site saturation binding equation (figure 1B). There was a significant difference in the maximum capacity of the Proteobacteria and *Prevotella* spp to induce innate stimulation in lung cells (TNFα from CD45+ lung cells, Bmax 1378 pg/ml vs. 1021 pg/ml, $p < 0.0001$; TLSP from CD45- lung cells, Bmax 53.2 pg/ml vs. 30.5 pg/ml, $p < 0.0001$). These findings demonstrate intrinsic innate stimulatory divergence between the three COPD and asthma-associated Proteobacteria and commensal *Prevotella* spp likely due to differences in composition of microbial-associated molecular patterns (MAMPs) with alternating capacity to activate innate immune receptors.

COPD and asthma-associated non-typeable *Haemophilus influenzae*, but not *Prevotella nanceiensis*, potently induce airway inflammation and pathology in mice

Airway challenge with *Haemophilus influenzae* has been shown to induce inflammatory aspects of COPD in lungs of mice, including production of acute phase cytokines TNFα, IL-6 and IL-1β, airway neutrophilia and tissue pathology (30). We sought to investigate how the intrinsic differences in immune stimulatory capacity between the three Proteobacteria and *Prevotella* spp may translate to the presentation of COPD- and/or asthma-like airway inflammation *in vivo*. We challenged mice with *Haemophilus influenzae* (H. inf. NT) and *Prevotella nanceiensis* (P. nan.) as representative strains of the two groups of bacteria. Airways challenged with H. inf. NT induced predominant recruitment of neutrophils as demonstrated by flow cytometry analysis of BAL cells (figure 2A and 2B). No statistically significant recruitment of eosinophils, lymphocytes, macrophages, monocytes or dendritic cell subsets could be observed. We analyzed a panel of acute-phase cytokines and chemokines (MIP-2α (IL-8), IL-6, IL-1β, TNFα) known to be associated with airway inflammation (35, 37). Furthermore, the analysis included cytokines and chemokines associated with type-17 (IL-17, CCL20) responses, type-1 (IFNγ) responses, type-2 (IL-5, TSLP) responses, immune regulation (IL-10) and recruitment of T cells to the lung (CXCL16) (38). The airway neutrophilia induced by H. inf. NT was accompanied by production of CCL20, CXCL16, IL-1β, IL-6 and MIP-2α (IL-8), and to lesser extent IFNγ and TNFα in lung tissue (significant induction by Student's one-sample t-test; figure 2C). The induced cytokines and chemokines relate primarily to type-17 inflammation and neutrophil recruitment; however induction of the prototype type-17 cytokine IL-17, as well as IL-10, TSLP and IL-5 were not detectable in the lung tissue. Immunohistochemical analysis revealed that the airway inflammation mediated by H. inf. NT was accompanied by lung pathology characterized by massive peri-bronchial inflammation, recruitment of immune cells and destruction of alveolar integrity (figure 2D and 2E).

P. nan. was found to induce some airway neutrophil recruitment which was 3-4 fold lower than the recruitment mediated by H. inf. NT (figure 2B). The decreased airway inflammation by P. nan. was associated with significant decrease in the acute-phase cytokine IL-1β, and the neutrophilic chemokines CCL20 and MIP-2α (IL-8) as compared to H. inf. NT (figure 2C). No lung tissue pathology could be detected following airway challenge with P. nan. (figure 2D and 2E). These findings suggest that Prevotella spp associated with healthy lungs may be intrinsically tolerated by the respiratory immune system, whereas pathogenic Proteobacteria exhibit distinct properties that can mediate COPD-like inflammatory features.

A different role for TLR2 in airway inflammation mediated by non-typeable *Haemophilus Influenzae* and *Prevotella nanceiensis*

Toll-like receptors (TLRs) play an important role in mediating inflammation by recognizing conserved MAMPs. TLR4 is the receptor for LPS, a molecule classical viewed as the most potent MAMP found in Gram-negative bacteria. Activation of TLR4 and TLR2 has been reported to account for up to 90 % of the inflammatory response to common pathogenic Gram-negative bacteria (39). Both the Proteobateria and *Prevotella* spp analyzed in this study are Gram-negative and contain LPS with the potential to activate TLR4. However, the two groups of bacteria may have different TLR4 activating properties. This is exemplified by, the gut commensal Gram-negative *Bacteroides fragilis* of the same phylum as the *Prevotella* spp (Bacteroidetes), which have been shown to activate TLR2, but not TLR4 (40). It is possible to address the contribution of TLR2 in TLR2 KO mice, and hence indirectly examine the contribution from other ligands, such as LPS (TLR4). The role of TLR2 in the airway inflammatory response to *Haemophilus influenzae* (H. inf. NT) and *Prevotella nanceiensis* (P. nan.) was compared using WT and TLR2-/- mice. We found TLR2 to be dispensable for acute airway neutrophilia and lung tissue pathology mediated by H. inf. NT (figure 3A and 3C). However, MIP-2α (IL-8) production in the lung in response to H. inf. NT was diminished in TLR2-deficient mice (figure 3B), indicating that MIP-2α (IL-8) is not essential for full expression of airway neutrophilia.

Airway inflammation mediated by P. nan. was found to be completely dependent on TLR2. Cellular recruitment in airways was absent (no difference when compared to PBS-treated group) and none of the analyzed cytokines were found to be induced (tested by Student's one-sample t-test) in response to P. nan. in TLR2-deficient mice (figure 3A and 3B). Expectedly, also TLR2-deficient mice showed no lung pathology in response to P. nan. (figure 3C). These findings indicate that the innate stimulatory potential of *Prevotella* spp is mainly related to TLR2 activation, whereas pathogenic Proteobacteria stimulate via other immune receptors that can fully express COPD-like inflammatory features.

Discussion

The present study demonstrates intrinsic differences in innate stimulatory and airway inflammatory properties between pathogenic Proteobacteria associated with chronic inflammatory airway disease (COPD and asthma) and commensal *Prevotella* spp associated with healthy lungs.

Bacteria contain several conserved compounds including the MAMPs that cause immune activation via innate receptors. LPS is a well known ubiquitous cell membrane constituent of all Gram-negative bacteria and is classically viewed as a potent MAMP for innate immune activation via TLR4. Studies have shown that TLR4 and TLR2 account for approximate 90 % of the pro-inflammatory innate response of human leucocytes to common pathogenic Gram-negative bacteria (39). Here we found that airway inflammation driven by *Prevotella* spp was completely dependent on TLR2. This finding suggests that *Prevotella* spp do not contain immunostimulatory LPS that can mediate airway inflammation. Indeed, the difference in inflammatory potential between the Gram-negative COPD and asthma-associated Proteobacteria and commensal *Prevotella* spp can likely be ascribed to differences in LPS structures (41). It is known that tetra- and penta-acylated LPS structures retain lower stimulatory activity on TLR4 than hepta- and hexa-acylated LPS (42, 43). The prototypic hexa-acylated LPS of *E. coli* seems to be the most biologically potent structure, whereas LPS with fewer and shorter acyl-chains have lower capacity to stimulate TLR4 (44–46). *Haemophilus influenzae* and *Moraxella catarrhalis* has been reported to contain hexa- and hepta-acylated LPS, respectively (47, 48). On the contrary, *Prevotella* spp and other members of the Bacteroidetes phylum have been reported to contain penta-acylated LPS, which have implications for recognition of the bacteria by TLR4. LPS isolated from the oral commensal *Prevotella intermedia* demonstrate approximate 10-fold reduced potency to induce IL-6 in murine macrophages as compared to *E. coli* LPS (49). Furthermore, the gut commensal *Bacteroides fragilis* contain penta-acylated LPS (50), and this bacterium has been found to stimulate TLR2 but not TLR4 (40). The presence of immune inactive LPS in all *Prevotella* spp is supported by a recent bioinformatic-based analysis performed by our group. We analyzed all sequenced bacterial genomes publicly available and studied the genes involved in LPS synthesis. We found

that the *LpxM* gene encoding the enzyme (KDO)2-(lauroyl)-lipid IVA acyltransferase needed to add a 6th acyl chain to lipid A (important for TLR4 immune activity) is absent in all members of the Bacteroidetes phylum including *Prevotella* spp, but present in gamma-Proteobacteria such as *H. influenzae* (Brix *et al*., manuscript in revision).

In the present report, *Prevotella* spp associated with healthy human lungs was well-tolerated in murine airways by inducing limited neutrophilia, chemokine and cytokine production, as well as provoking no detectable lung immunopathology as compared to pathogenic Proteobacteria. Observational studies reporting lung dysbiosis with increased pathogenic proteobacteria and reduced *Prevotella* spp. in COPD and asthma (7) suggest that the well-tolerated *Prevotella* spp serves a disease-protective role. A protective role of *Prevotella* spp. could be ascribed to 1) a direct modulation of innate immune responses in the lung, 2) induction of functional and developmental changes in the immune system, 3) suppression of Proteobacterial colonization or infection by taking up a ecological niche in the microbiota, and 4) direct killing of Proteobacteria. Interestingly, a recent study in mice reported that the acquisition of a Bacteroidetes-rich lung microbiota during early life was associated with the establishment of tolerance in the lung and suppression of allergic inflammation, whereas Proteobacterial-rich lungs of neonatal mice showed exaggerated responses to allergen (51). Additionally, we have previously reported that *Prevotella* spp. modulates the *in vitro* inflammatory response of human dendritic cells to *Haemophilus influenzae* suggesting that members of the commensal lung microbiota may directly limit the immune response to specific pathogens (41). Additional studies are needed to establish if *Prevotella* spp. exhibit causal disease-protective effects.

We found that the three Proteobacteria associated with COPD and asthma potently mediated airway neutrophilia, inflammatory chemokine/cytokine production and tissue pathology in mice. This is in line with previous studies reporting, that certain Proteobacteria can induce COPD-like inflammatory features and promote COPD disease in murine models (28–30). The results from murine studies support the notion of pathogenic Proteobacteria as potential co-drivers of COPD disease (21–24). We speculate that it is the

specific immune active hexa-acylated structure of LPS in these bacterial strains that gives the bacteria its disease-promoting activity. Indeed, inhalation of LPS in humans mediates production of IL-1β, IL-6 and TNFα, neutrophil recruitment and decreased lung function (FEV1) (52, 53). Based on these findings it could be proposed to examine the applicability of inhaled TLR4 antagonists as an option for treatment of chronic inflammatory airway disease with involvement of pathogenic Proteobacteria.

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JML conceived the study, designed experiments, analyzed data and wrote the manuscript. HSM and TMB performed experiments and analyzed data. CI performed histological staining and scoring. SB supervised the study. All authors provided important intellectual contributions to the study and writing of manuscript.

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Conflict of Interest

The authors have no financial or commercial conflicts of interest to disclose in relation to publication of the manuscript.

Figure legends

FIGURE 1: Inflammatory response of murine lung cells to three airway-related Proteobacteria and *Prevotella* spp.

MIP-2α (IL-8), TNFα, IL-10, TSLP and IL-5 production by primary CD45+, CD45- or mixed CD45+/- (1:1) cells isolated from murine lungs in response to 24h stimulation with medium, LPS, three Proteobacteria or *Prevotella* spp (A). Dose-response titration of Proteobacteria and *Prevotella* spp on primary murine lung cells (B). Data represents one of four (A) and two (B) independent experiments (n = 3, mean + SEM).

FIGURE 2: Airway inflammation following challenge with *Haemophilus influenzae* or *Prevotella nanceiensis* in mice.

Flow cytometry of BAL cells (A), absolute cell numbers in BAL (B), chemokine/cytokine levels in lung tissue homogenates (C), representative HE-stained lung tissue sections (D), and pathology scores (E) obtained from murine lungs 24h after airway challenge with vehicle (PBS), *Haemophilus influenzae* or *Prevotella nanceiensis*. Data represents two independent experiments (n = 8, mean + SEM).

FIGURE 3: The role of TLR2 in the airway inflammatory response to *Haemophilus influenzae* or *Prevotella nanceiensis* in mice.

Absolute cell numbers in BAL (A), chemokine/cytokine levels in lung tissue homogenates (B), and pathology scores (C) obtained from WT or TLR2-/- murine lungs 24h after airway challenge with *Haemophilus influenzae* or *Prevotella nanceiensis*. Data represents two independent experiments (n = 8, mean + SEM).

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