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Divergent Pro-Inflammatory Profile of Human Dendritic Cells in Response to Commensal and Pathogenic Bacteria Associated with the Airway Microbiota

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Abstract
Recent studies using culture-independent methods have characterized the human airway microbiota and report microbial communities distinct from other body sites. Changes in these airway bacterial communities appear to be associated with inflammatory lung disease, yet the pro-inflammatory properties of individual bacterial species are unknown. In this study, we compared the immune stimulatory capacity on human monocyte-derived dendritic cells (DCs) of selected airway commensal and pathogenic bacteria predominantly associated with lungs of asthma or COPD patients (pathogenic *Haemophilus* spp. and *Moraxella* spp.), healthy lungs (commensal *Prevotella* spp.) or both (commensal *Veillonella* spp. and *Actinomyces* spp.). All bacteria were found to induce activation of DCs as demonstrated by similar induction of CD83, CD40 and CD86 surface expression. However, asthma and COPD-associated pathogenic bacteria provoked a 3–5 fold higher production of IL-23, IL-12p70 and IL-10 cytokines compared to the commensal bacteria. Based on the differential cytokine production profiles, the studied airway bacteria could be segregated into three groups (*Haemophilus* spp. and *Moraxella* spp. vs. *Prevotella* spp. and *Veillonella* spp. vs. *Actinomyces* spp.) reflecting their pro-inflammatory effects on DCs. Co-culture experiments found that *Prevotella* spp. were able to reduce *Haemophilus influenzae*-induced IL-12p70 in DCs, whereas no effect was observed on IL-23 and IL-10 production. This study demonstrates intrinsic differences in DC stimulating properties of bacteria associated with the airway microbiota.

Introduction

The human body is host to an immense variety of bacterial species living in microbial communities (microbiotas) which may continuously change dependent on acquisition of bacteria encountered in the environment and clearance mediated by the immune system. It is becoming increasingly apparent that the colonizing bacteria are not merely invasive health treats giving rise to infections, but are symbiotes contributing to normal bodily functions through common mutualism [1]. Advances in high-throughput molecular biology have allowed in-depth characterization of microbiotas by abolishing the traditional laborious methods of bacteria identification by cultivation [2]. Instead, bacteria within microbial communities can be identified and quantified highly specific based on genetic composition. Due to the predictable importance of bacteria in the intestine, several studies have focused on characterizing the gut microbiota and addressing changes associated with disease, including autoimmune disease, allergy and obesity. Yet recent focus has turned to the less predictable importance of bacteria in the airway microbiota indicating that microbial communities in the airways consist of bacteria adapted to live in conjunction with the conditions present in this organ [9]. When compared to healthy individuals, airways of asthmatics and COPD patients were recently reported to exhibit disordered microbial airway composition [7–9,11]. Studies indicated that asthmatics and COPD patients were more commonly colonized with pathogenic proteobacteria (*Haemophilus* spp. and *Moraxella* spp.), whereas healthy individuals are more commonly colonized with commensal bacteriodes (*Prevotella* spp.) [10]. Asthma and COPD are diseases giving rise to airway inflammation, and since airway bacteria are likely to interact with the immune system, it is of interest to study the immune stimulating properties of airway-microbes associated with asthma and COPD.
colonizing bacteria to increase our general insight into bacteria-
induced immunity. Furthermore, comparing properties between
commensal and pathogenic strains of the airway microbiota may
contribute to our understanding of disordered microbial commu-
nities observed in airway disease.

Dendritic cells are professional antigen presenting cells that play
a central role in bridging innate and adaptive immunity by
instructing the development of antigen-specific T cell responses
[12]. In the present study we used a well-established model of
human monocyte-derived dendritic cells [13,14] to examine the
immune stimulating properties of three pathogenic and six
commensal bacteria associated with the airway microbiota. The
studied airway bacteria could be segregated into three distinct
groups based on the profile of cytokines produced by DCs. We
propose that the distinct innate DC responses elicited by airway
bacteria may be similar to those reported for commensal and
pathogenic bacteria of the gastro-intestinal tract. Thus, commensal
bacteria of the airways may in a similar manner play a role in
maintaining immune homeostasis and controlling overt airway
inflammation.

Results

We selected three pathogenic and six commensal strains
associated with healthy and diseased airway microbiosa (Table 1)
for a comparative analysis. The pathogenic proteobacteria
associated with asthma and COPD were Haemophilus influenzae
and Moraxella spp. (subspecies unknown) [10]. We included
Haemophilus influenzae B (H. inf. B) and non-typeable Haemophilus
influenzae (H. inf. NT) as both are common airway pathogens giving
rise to infections, but structurally different as non-typeable
Haemophilus influenzae has no capsule and is predominantly
associated with the respiratory tract [15,16]. Moraxella catarrhalis
(M. cat.) was included as the most common pathogenic Moraxella
strain [17]. The commensal bacteroidetes Prevotella melaninogenica
(P. mecl.), Prevotella nanceiensis (P. nan.) and Prevotella salivae (P. sal.)
were studied as representatives of bacteria associated with healthy
airways [10]. The firmicutes Veillonella dispar (V. disp.) was enrolled
as it is the most predominant airway commensal present in both
healthy and diseased airways [10]. Lastly, commensal Actinomyces
graevenitzii (A. grae.) and Actinomyces oris (A. oris) were included as
less common members of the actinobacteria phylum associated
with both healthy and diseased airways [10]. All the bacteria
designated as commensal are rarely reported to cause infectious
disease compared to the common pathogenic bacteria and are
here thus considered as commensals.

Various airway bacteria induce similar dendritic cell
maturation

Dendritic cells patrol peripheral tissues sampling the environ-
ment to sense invading microorganisms by recognizing microbial-
associated molecular patterns or danger signals derived from
tissues. Upon encounter with immunogenic components DCs
acquire a mature phenotype enabling migration to nearby lymph
nodes, crosstalk with T cells, and initiation of adaptive immune
responses [12,14]. CD83 is a well established differentiation
marker used as a general indicator of DC maturation/activation in
vivo [18,19]. Furthermore, mature DCs upregulate the co-
stimulatory markers CD40 and CD86 of importance for initiation
and propagation of T cell activation via DC/T cell crosstalk. Here
we used the CD83, CD40 and CD86 markers to address the
presence of immune activating entities in both pathogenic and
commensal bacteria. It was found, that all analyzed bacteria
induced expression of these markers (figure 1) when compared to
immature DC (medium). Lipopolysaccharide (LPS) recognized to
promote DC maturation via TLR4 served as a positive control.
The level of CD83, CD40 and CD86 expression between each
bacterium were comparable suggesting that the bacteria had
similar capability to induce DC maturation.

Differential cytokine production by airway bacteria in
dendritic cells

Mature dendritic cells produce cytokines that mediate inflam-
mation and instruct development of antigen-specific helper T cells.
IL-12p70 and IL-23 cytokines play a central role in mediating
development or proliferation of Th1 and Th17 cells, respectively.
Furthermore, these cytokines drive inflammation by stimulating
production of the pro-inflammatory cytokines IFNγ (Th1) and IL-
17 (Th17) by T cells leading to recruitment and activation of pro-
inflammatory immune cells [20,21]. On the contrary, IL-10
exhibit anti-inflammatory properties by inhibiting production of
pro-inflammatory cytokines by various immune cells, including T
cells, macrophages and epithelial cells [22]. In order to investigate
the potential pro-inflammatory and anti-inflammatory properties
of the pathogenic and commensal airway-associated bacteria, we
analyzed the production of IL-23, IL-12p70 and IL-10 by the
stimulated DCs. For each bacterium, we found that the average
level of IL-23 and IL-10 cytokine production by DCs was
generally 2–3 fold higher than the level of IL-12p70 (Table 2).

Due to donor-specific variation, DC cytokine production was
normalized to the average response of the three pathogenic
bacteria, as these consistently induced similar and the highest
cytokine production levels within each donor. Figure 2 show
normalized cytokine production by DCs in response to the
pathogenic and commensal bacteria. It was found that bacteria
within the same family (Haemophilus spp., Prevotella spp. and
Actinomyces spp.) and the corresponding phyla (proteobacteria,
bacteriodes, actinobacteria) induced similar levels of IL-23, IL-
12p70 and IL-10. In general, pathogenic proteobacteria produced
3–5 fold higher levels of IL-23, IL-12p70 and IL-10 compared to
the commensal bacteria. Lowest were the production of IL-23 and
IL-12p70 by Actinomyces spp., which induced levels comparable to
that of immature DCs.

Segregation of airway bacteria into functional subgroups
based on their inflammatory properties

By use of a principal component analysis (PCA), it was possible
to separate the bacteria species into three functionally distinct
groups based on the levels of IL-23, IL-12p70 and IL-10 induced
in DCs (figure 3). The PC1 and PC2 of the PCA score plot shown
in figure 3 were found to describe more than 95% of the variance
between the bacteria-induced DC responses. As indicated by the
loading plot, the differences in levels of all cytokines (IL-23, IL-
12p70 and IL-10) were the main factor responsible for
discriminating the bacteria along PC1. The PCA analysis
indicated that the bacteria could be divided into three groups:
Highly stimulatory bacteria (Group I; Haemophilus spp. and
Moraxella spp.), intermediate stimulatory bacteria (Group II;
Prevotella spp. and Veillonella spp.), and weakly stimulatory bacteria
(Group III; Actinomyces spp.). Using multivariate ANOVA it was
found that the cytokine production profile were statistically
significantly different between the identified groups (Group I vs.
Group II: p<5.8 * 10^-12; Group I vs. Group III: p<2.5*10^-10,
Group II vs. Group III: p<0.000034). These results imply that it is
possible to classify bacteria of the airway microbiosa into distinct
groups based on their functional immune profiles in DC that
reflect properties of being associated with asthma and COPD, or healthy lungs.

**Prevotella** strains reduce *Haemophilus*-induced IL-12p70 production by DC

When present in tissues, bacteria with distinct functional characteristics are likely to interact simultaneously with the immune system affecting the overall response. This has been studied and demonstrated in relation to gut bacteria that modulated immune responses to other bacteria or MAMPs [23]. Yet it remains unknown if bacterial species associated with the airway microbiota demonstrate similar properties. Since *Haemophilus influenzae* is the pathogen most strongly associated with asthma and COPD [10], we analyzed the ability of three different *Prevotella* strains to modulate *Haemophilus*-induced IL-23, IL-12p70 and IL-10 production in DCs. It was found, that all studied *Prevotella* strains could partially reduce *Haemophilus*-induced IL-12p70 production by DCs, whereas no statistically significant effect on IL-23 and IL-10 production was apparent (figure 4). This indicates that commensal bacteria of the airways, similarly to the gut, may exhibit properties that enable modulation of the immune responses to specific pathogenic bacteria.

**Discussion**

The commensal microbiota of the gastro-intestinal tract has demonstrated importance for metabolism, maturation of the immune system and protection from invasive microorganisms [24]. Furthermore, studies indicate that changes in the gut microbial composition are associated with inflammatory bowel disease [25,26], with a protective role of specific commensal bacteria [27,28]. Only recently has the existence of a commensal microbiota in the lower airways been appreciated and characterized [8–10]. The potential physiological role of these commensal airway bacteria remains to be established. To date, no studies have been reported addressing or comparing the immunological properties of the airway-associated commensal bacteria included in this study. Some studies have focused on the immunological mechanisms elicited by epithelial cells, macrophages and T cells in response to the pathogenic bacteria *Haemophilus influenzae* or *Moraxella catarrhalis* [29–34]. However, the response by DCs to these central respiratory pathogens remains to be clarified.

We found that all airway pathogenic and commensal bacteria included in our study induced similar maturation of DCs. This demonstrates that all the bacteria exhibit innate activating properties that may enable bacteria-exposed DCs to prime T cell responses. On the contrary to DC maturation, the bacteria induced varied levels of the T cell-polarizing cytokines IL-23, IL-12p70 and IL-10. This indicates that the bacteria may elicit different immunological processes in vivo due to the development of distinct helper T cell responses. The bacteria-specific differences in DC cytokine profiles are likely a result of the strain-specific composition of microbial-associated molecular patterns (MAMPs) that stimulate several innate receptors, including toll-like receptors (TLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs). Most strikingly is the absence of IL-23 and IL-12p70 in DCs stimulated with the commensal Gram-positive *Actinomyces* spp. when compared to the remaining airway bacteria that are all Gram-negative in origin. This difference is most likely based on the fact that cell membranes of Gram-positive bacteria lack LPS, contrarily to those of Gram-negative bacteria. LPS, especially in the presence of other MAMPs, is an effective inducer of IL-12p70 production in DCs via TLR4 [35]. Indeed, Gram-negative bacteria in general have been shown to induce more potent innate immune responses as compared to Gram-positive bacteria in a TLR4-dependent manner [36].

Within the group of Gram-negative bacteria (*Haemophilus* spp., *Moraxella* spp., *Prevotella* spp. and *Veillonella* spp.), we observed significantly higher levels of IL-23, IL-12 and IL-10 from DCs in response to the pathogenic bacteria species, *Haemophilus* spp. and *Moraxella* spp. The reason for the differences in DC cytokine-inducing properties amongst the Gram-negative airway bacteria could be the structure of LPS, which have been reported to vary amongst Gram-negative bacteria [37]. Particularly the structure of lipid A within LPS seems predominantly important for the biological activity. Notably, the classical LPS of E. coli is composed

| Table 1. Phyla and features of the bacterial strains analyzed. |
|---------------------------------------------------------------|
| **Bacterial strain** | **Phylum**    | **Gram** | **Feature**                                                                 | **Ref.** |
| *Haemophilus influenzae B*                                    | Proteobacteria | Neg      | Pathogenic bacteria found in the airway microbiota of asthma and COPD patients. Associated with development of asthma in children. | [10,59] |
| *Haemophilus influenzae NT*                                   | Proteobacteria | Neg      | Pathogenic bacteria present in the airway microbiota of asthma and COPD patients. Associated with development of asthma in children. | [10,59] |
| *Moraxella catarrhalis*                                       | Proteobacteria | Neg      | Pathogenic bacteria found in the airway microbiota of asthma and COPD patients. Associated with development of asthma in children. | [10,59] |
| *Prevotella melaninogenica*                                   | Bacteroidetes  | Neg      | Commensal bacteria associated with the airway microbiota of healthy individuals. | [10]     |
| *Prevotella nanceiensis*                                      | Bacteroidetes  | Neg      | Commensal bacteria associated with the airway microbiota of healthy individuals. | [10]     |
| *Prevotella salviae*                                          | Bacteroidetes  | Neg      | Commensal bacteria associated with the airway microbiota of healthy individuals. | [10]     |
| *Veillonella dispar*                                           | Firmicutes     | Neg      | The most predominant commensal bacteria associated with the airway microbiota. | [10]     |
| *Actinomyces graevenitzii*                                     | Actinobacteria | Pos      | Less predominant commensal bacteria associated with the airway microbiota. | [10]     |
| *Actinomyces oris*                                            | Actinobacteria | Pos      | Less predominant commensal bacteria associated with the airway microbiota. | [10]     |

*NT: Non-typeable.
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|          | CD83 | CD40 | CD86 |
|----------|------|------|------|
| Medium   | 17   | 183  | 27   |
| LPS      | 102  | 786  | 229  |
| H. inf. B| 80   | 617  | 239  |
| H. inf. NT| 91  | 753  | 248  |
| M. cat.  | 75   | 753  | 221  |
| P. mel.  | 118  | 685  | 236  |
| P. nan.  | 106  | 639  | 181  |
| P. sal.  | 100  | 748  | 169  |
| V. disp. | 91   | 576  | 175  |
| A. grae. | 95   | 430  | 193  |
| A. oris. | 96   | 607  | 233  |

**Proteobacteria**

- H. inf. B
- H. inf. NT
- M. cat.
- P. mel.
- P. nan.
- P. sal.
- V. disp.
- A. grae.
- A. oris.

**Bacteroidetes**

- Medium
- LPS

**Firmicutes**

- Medium
- LPS

**Actinobacteria**

- Medium
- LPS
of hexa-acylated lipid A and seems to be the most biologically potent LPS structure [38]. However, some Gram-negative bacteria are reported to contain less potent atypical LPS composed of lipid A with shorter or fewer acyl chains [39–41]. LPS derived from Haemophilus influenzae and Moraxella catarrhalis has been reported to contain hexa- and hepta-acylated lipid A, respectively [42,43]. No studies have been reported on LPS of the Prevotella or Veillonella species included in our study; yet LPS from other species within these genera have been analyzed. LPS of the oral commensal Prevotella intermedia contain penta-acylated lipid A and demonstrate about 10-fold reduced potency compared to LPS from E. coli [44]. Similarly, approximately 10-fold reduced potency in LPS has been reported from the gut commensal Veillonella parvula suggesting the absence of an optimal number of acyl chains in lipid A for propagation of a strong pro-inflammatory signal [45]. Combined these studies indicate, that the difference observed in this study between Gram-negative pathogenic and commensal bacteria of the airways may be due to varying potencies within their LPS components. However it should be noticed, that the pathogenic and commensal bacteria in this study are of different genera and other MAMPs within the bacteria could play a role in DC modulation via simultaneous engaging of receptors with pro- or anti-inflammatory activity.

In this study, we used monocyte-derived DCs to examine the bacteria-DC interaction as the quantity of DCs available per donor allowed us to screen for effects of several airway bacteria within the same donor. It should be emphasized that DCs of the airways, the cellular subset that will interact with airway microbes under in situ conditions, might respond differently than monocyte-derived DCs to the microbes. However, as DCs of the conducting airways and monocyte-derived DCs propagate from the common myeloid progenitor [46], it is likely that they share common response patterns. Airway DCs are known to sample environmental antigens in the airway lumen, and will likely be exposed to the collective bacterial ecosystem of the airway tract. It is therefore also possible that airway commensal bacteria may affect the overall DC response to potentially pathogenic bacteria. For this reason we studied how commensal Prevotella spp., or other components present within this bacteria spp., inhibit the ability of Haemophilus influenzae LPS to elicit complete TLR4 signaling hence leading to the reduction in IL-12p70 production by DCs. Yet, the presence of several TLR2 and NLR ligands within the complex bacteria may well allow for full level expression of IL-23. The biological relevance of IL-12p70 modulation in DCs by commensal bacteria remains elusive. However, it can be speculated that a change in the IL-23/IL-12 balance will translate into a predominant type-17 mediated responses by the DCs as IL-23 is the key mediator of proliferation and cytokine production by Th17 cells [52]. The modulation could enhance Th17 cell mediated immunity, which

Table 2. Average cytokine production by DCs among all donors in response to bacteria or control stimuli.

| Bacterial strain | Phylum            | IL-23 (pg/ml) | IL-12p70 (pg/ml) | IL-10 (pg/ml) |
|------------------|-------------------|---------------|-----------------|--------------|
| Control          | n/a               | 262           | 73              | 144          |
| LPS              | n/a               | 1829          | 1090            | 5500         |
| Haemophilus influenzae B | Proteobacteria | 11450          | 4424            | 12452        |
| Haemophilus influenzae NT* | Proteobacteria | 14108          | 5710            | 14797        |
| Moraxella catarrhalis | Proteobacteria | 13256          | 5507            | 14176        |
| Prevotella melaninogenica | Bacteroidetes | 2862           | 2684            | 6436         |
| Prevotella nanceiensis | Bacteroidetes | 2976           | 1751            | 7378         |
| Prevotella salivae | Bacteroidetes | 5267           | 2011            | 9669         |
| Veillonella dispar | Firmicutes        | 5331          | 2450            | 5167         |
| Actinomyces graevenitzi | Actinobacteria   | 156            | 81              | 1849         |
| Actinomyces oris  | Actinobacteria    | 147            | 36              | 1649         |

*NT: Non-typeable.

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has been shown to play a central role in clearance of pathogenic airway bacteria [53,54].

The present study was primarily initiated due to the observation that commensal Prevotella spp. were absent, whereas pathogenic Haemophilus spp. and Moraxella spp. colonized the airways of asthmatics and COPD patients [10]. This finding suggests a divergent role of airway bacteria in chronic inflammatory airway diseases with a protective or modulator role in disease development. The association between pathogenic proteobacteria airway bacteria and COPD has been investigated for several decades giving rise to some controversy [55]. Yet, it is now established that pathogenic airway bacteria are associated with acute disease exacerbations, which leads to significant morbidity and mortality within this patient group [56,57]. Pathogenic proteobacteria has more recently been associated with asthma in case-control studies [10], during exacerbation attacks [58], and reported as a risk factor for asthma development in children [59]. Interestingly, COPD, asthma exacerbations and some asthma phenotypes are associated with neutrophilic airway inflammation [60,61,56]. Th17 cells producing IL-17 are central mediators of neutrophilic inflammation and activation in tissues, shown to play a role in clearance of Gram-negative extracellular pulmonary pathogens [21]. This inflammatory pathway are primed and driven by IL-23 production by dendritic cells. In this study we found that pathogenic airway bacteria were potent inducers of IL-23 and IL-12p70 in DCs suggesting the development of bacteria-specific Th17 and Th1 cells in vivo. Studies in humans have demonstrated the development of a Th1 response to non-typeable Haemophilus

Figure 2. Cytokine production by dendritic cells stimulated with pathogenic and commensal airway bacteria. IL-23, IL-12p70 and IL-10 cytokine production measured in DC culture supernatants following 24 h stimulation with medium, LPS, Haemophilus influenzae B (H. inf. B), non-typeable Haemophilus influenzae (H. inf. NT), Moraxella catarrhalis (M. cat.), Prevotella melaninogenica (P. mel.), Prevotella nanceiensis (P. nan.), Prevotella salivae (P. sal.), Veillonella dispar (V. disp.), Actinomyces graevenitzii (A. grae.) or Actinomyces oris (A. oris.). Labels within each group represent different donors (n = 3–9). Cytokine production measurements were normalized to account for donor-specific variation. *p<0.05, **p<0.01, ***p<0.001. doi:10.1371/journal.pone.0031976.g002

Figure 3. Principal component analysis of dendritic cell cytokine profiles in response to pathogenic and commensal airway bacteria. Principal component analysis reveals clustering of DC responses to airway bacteria. Three significantly different groups are distinguished by the production of IL-23, IL-12p70 and IL-10 cytokines. I: Pathogenic Gram-negative Haemophilus influenzae B (H. inf. B), non-typeable Haemophilus influenzae (H. inf. NT) and Moraxella catarrhalis (M. cat.); II: Commensal Gram-negative Prevotella melaninogenica (P. mel.), Prevotella nanceiensis (P. nan.), Prevotella salivae (P. sal.) and Veillonella dispar (V. disp.); III: Commensal Gram-positive Actinomyces graevenitzii (A. grae.) and Actinomyces oris (A. oris.). Responses to each bacterium were based on 3–6 different donors. Shaded areas represent the 67% confidence area within the three bacteria groups. doi:10.1371/journal.pone.0031976.g003
Dendritic cell responses to airway bacteria

**Materials and Methods**

**Bacteria growth and preparation**

*Haemophilus influenzae* B (KAK510), *Haemophilus influenzae* NT (KAK509) and *Moraxella catarrhalis* F48 (KAK508) reference stains were kindly provided by Karen Krogfelt and Jørgen Skov Jensen, Statens Serum Institut, Copenhagen, Denmark. *Prevotella melanogenica* (DSM7089), *Prevotella nanceiensis* (DSM19126), *Prevotella salivae* (DSM15606), *Veillonella dispar* (DSM20735), *Actinomyces gnavenitzii* (DSM15540), *Actinomyces oris* (DSM23056) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. *Haemophilus influenzae* and *Moraxella* strains were grown on chocolate agar plates (Statens Serum Institut) under 37°C microaerobic (5% CO2) conditions. *Prevotella* strains were grown on anaerobic agar plates (Statens Serum Institut) under 30°C anaerobic conditions. *Veillonella dispar* was grown on anaerobic agar plates under 37°C anaerobic conditions. *Actinomyces* strains were grown on chocolate agar plates under 30°C aerobic 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 was confirmed by plating. Dry weights of bacteria suspensions in PBS were determined on 3×1 ml portions after freeze-drying (subtracted by weight of PBS). Bacterial suspensions were frozen and stored at −80°C.

**Dendritic cell stimulation and analysis**

Dendritic cells were stimulated by replacing medium with complete RPMI 1640 medium containing stimuli supplemented with 50 μg/ml gentamycin (Sigma-Aldrich, Copenhagen, Denmark) to ensure no bacterial outgrowth. In preliminary experiments, the gentamycin at 50 μg/ml was found not to affect LPS-induced activation of DCs. All stimulations were done in triplicates, and a concentration of 50 μg/ml was used for all bacterial stimulations. In experiments with mixtures of two bacteria, the concentration of each bacteria was 50 μg/ml. LPS (100 ng/ml) and medium alone were included to serve as a positive and negative control, respectively.

![Image](58x24 to 76x41)

![Image](58x552 to 439x730)

**Figure 4. Modulation of *Haemophilus*-induced cytokine production in dendritic cells by *Prevotella* species.** IL-23, IL-12p70 and IL-10 cytokine production measured in DC culture supernatants 24 h following stimulation with non-typeable *Haemophilus influenzae* (H. inf. NT) in combination with *Prevotella melanogenica* (P. mel.), *Prevotella nanceiensis* (P. nan.) or *Prevotella salivae* (P. sal.). The effect of *Prevotella* species on *Haemophilus*-induced cytokine production was calculated relative to *Haemophilus* stimulation alone for each donor. The numbers identify individual donors (n = 3–5). *p < 0.05, **p < 0.01.

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eBioscience, San Diego, CA; IL-12p70 and IL-10; R&D Systems, Minneapolis, MN) according to manufacturer’s recommendations. In some experiments, DC surface expression of CD83, CD40 and CD86 were measured by flow cytometry. Briefly, 2 × 10^6 cells were stained with fluoresein isothiocyanate-conjugated anti-hCD83, anti-hCD40 and anti-hCD86 all from BD Pharmingen, San Jose, CA) in PBS containing 1% FCS and 0.1% sodium azide 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) followed by data analysis in FCS Express v4 (De Novo, Los Angeles, CA).

Data analysis and statistics

Univariate statistical analysis was performed using GraphPad PRISM 5.01 (GraphPad Software, La Jolla, CA). Differences in normalized cytokine production were analyzed by one-way ANOVA with Tukey’s multiple comparison test. Pevorcella strains effect on Haemophilius-induced cytokine production in DCs were analyzed using Student’s one sample t-test. Principal component analysis and multivariate ANOVA (MANOVA) analysis used to compare groups of bacteria were performed using the R software package (Foundation for Statistical Computing, Vienna, Austria). The PCA was performed with the procop function that uses Singular Value Decomposition on the covariance matrix for the PCA computations. P-values below 0.05 were considered statistically significant.

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Author Contributions

Conceived and designed the experiments: J. M. Larsen SB. Performed the experiments: DBSJ. Analyzed the data: DBSJ J. M. Larsen J. M. Laursen. Contributed reagents/materials/analysis tools: JNS HSM TMB. Wrote the paper: J. M. Larsen SB.

References

1. Sommernburg JL, Angenent LT, Gordon JJ (2004) Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? Nature reviews. Microbiology 5: 369–73.
2. Medini D, Serrato D, Parkhill J, Relman DA, Donati C, et al. (2008) Microbiology in the post-genomic era. Nature reviews. Microbiology 6: 419–30.
3. Grice EA, Segre JA (2011) The skin microbiome. Nature reviews. Microbiology 9: 244–53.
4. Kahn FW, Jones JM (1987) Diagnosing bacterial respiratory infection by bronchoalveolar lavage. The Journal of infectious diseases 155: 862–9.
5. Haemophilus influenzae adherence and invasion. Cellular Microbiology 4: 391–400.
6. Harris JK, De Groote MA, Sagel SD, Zemanick ET, Kapsner R, et al. (2007) Bronchoalveolar lavage for diagnosing acute bacterial pneumonia. The Journal of infectious diseases 195: 855–61.
7. Erb-Downward JR, Thompson DL, Han MK, Freeman CM, McCluskey L, et al. (2011) Analysis of the lung microbiome in the “healthy” smoker and in COPD. PLoS one 6: e16384.
8. Harris JK, De Groote MA, Sagel SD, Zemanick ET, Kapsner R, et al. (2007) Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. Proceedings of the National Academy of Sciences of the United States of America 104: 2529–33.
9. Chassagnon ES, Chen J, Caston-Alien R, Bittinger K, Li H, et al. (2010) Disordered microbial communities in the upper respiratory tract of cigarette smokers. PLoS one 5: e15216.
10. Hilty M, Burke C, Pedro H, Cardenas P, Bush A, et al. (2010) Disordered microbial communities in asthmatic airways. PLoS one 5: e10571.
11. Huang YJ, Nelson CE, Brodie EL, Desantis TZ, Baek MS, et al. (2011) Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally responsive asthma. PloS one 5: e8578.
12. Berenson CS, Murphy TF, Wrona CT, Sethi S, et al. (2005) Outer membrane protein C of non-typeable Haemophilus influenzae porin induces Toll-like receptor 2-mediated cytokine production in human monocytes and mouse macrophages. Infection and immunity 73: 2728–35.
13. King PT, Hutchinson PE, Johnson PD, Holmes PW, Freezer NJ, et al. (2005) Adaptive immunity to non-typeable Haemophilus influenzae. American journal of respiratory and critical care medicine 171: 31–40.
14. Sallusto F, Lanzavecchia A (1994) Efficient presentation of soluble antigen by dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. The Journal of experimental medicine 179: 1109–18.
15. Kapenbergs NL (2003) Dendritic-cell control of pathogen-driven T-cell polarization. Nature reviews. Immunology 3: 904–9.
16. Agrawal S, Agrawal A, Doughty B, Gerwitz A, Blenis J, et al. (2003) Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. Journal of immunology 171: 4981–9.
17. Elson G, Dunn-Sigrist I, Daubeuf B, Pugin J (2007) Contribution of Toll-like receptors to the innate immune response to Gram-negative and Gram-positive bacteria. British journal of haematology 139: 1574–83.
18. Miller SJ, Ernst RK, Bader MW (2005) LPS, TLR4 and infectious disease diversity. Nature reviews. Microbiology 3: 36–46.
19. Leber S, Vanderleyden J, Keersmaecker SCJDe (2010) Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nature reviews. Microbiology 8: 171–84.
20. Caroff M, Karibian D (2003) Structure of bacterial lipopolysaccharides. Carbohydrate research 338: 2431–47.
40. Caroff M, Karibian D, Cavallin JM, Haefliger-Cavallin N (2002) Structural and functional analyses of bacterial lipopolysaccharides. Microbes and infection 4: 915–26.

41. Alhawi M, Stewart J, Erridge C, Patrick S, Poxton IR (2009) Bacteroides fragilis signals through Toll-like receptor (TLR) 2 and not through TLR4. Journal of medical microbiology 58: 1015–22.

42. Schweda EKH, Twelkmeyer B, Li J (2008) Profiling structural elements of short-chain lipopolysaccharide of non-typeable Haemophilus influenzae. Innate immunity 14: 199–211.

43. Masoud H, Perry MB, Richards JC (1994) Characterization of the lipopolysaccharide of Moraxella catarrhalis. Structural analysis of the lipid A from M. catarrhalis serotype A lipopolysaccharide. European journal of biochemistry/FEBS 220: 299–16.

44. Hashimoto M, Asai Y, Jinno T, Umataki K, et al. (2003) Chemical structure and immunobiological activity of lipid A from Prevotella intermedia ATCC 25611 lipopolysaccharide. FEBS letters 543: 98–102.

45. Matera G, Muto V, Vinci M, Zicca E, Abdollahi-Roodsaz S, et al. (2009) Receptor recognition of and immune intracellular pathways for Veillonella parvula lipopolysaccharide. Clinical and vaccine immunology 16: 1804–9.

46. de Heer HJ, Hannad H, Kool M, Lambrecht BN (2005) Dendritic cell subsets and immune regulation in the lung. Seminars in immunology 17: 295–303.

47. Somerville JE, Cassiano L, Bainbridge B, Cunningham MD, Darveau RP (1996) A novel Escherichia coli lipid A mutant that produces an antiinflammatory lipopolysaccharide. The Journal of clinical investigation 97: 359–65.

48. Coats SR, Reife RA, Bainbridge BW, Pham T-T, Darveau RP (2003) Porphyromonas gingivalis lipopolysaccharide antagonizes Escherichia coli lipopolysaccharide at toll-like receptor 4 in human endothelial cells. Infection and immunity 71: 6799–807.

49. Coats SR, Pham T-T, Bainbridge BW, Reife RA, Darveau RP (2005) MD-2 mediates the ability of tetra-acylated and penta-acylated lipopolysaccharides to antagonize Escherichia coli lipopolysaccharide at the TLR4 signaling complex. Journal of immunology 175: 4490–8.

50. Goriely S, Molle C, Nguyen M, Albarani V, Haddou NO, et al. (2006) Interferon regulatory factor 3 is involved in Toll-like receptor 4 (TLR4)- and TLR3-induced IL-12p35 gene activation. Blood 107: 1078–84.

51. Gerota F, Baldani-Guerra B, Lyakh LA, Batoni G, Esin S, et al. (2008) Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells. The Journal of experimental medicine 205: 1447–61.

52. Kastelein RA, Hunter CA, Cua DJ (2007) Discovery and biology of IL-23 and IL-27: related but functionally distinct regulators of inflammation. Annual review of immunology 25: 221–42.

53. Lu Y, Gross J, Bogaert D, Finn A, Bagrade L, et al. (2008) Interleukin-17A mediates acquired immunity to pneumococcal colonization. PLoS pathogens 4: e1000159.

54. Zhang Z, Clarke TB, Weiser JN (2009) Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. The Journal of clinical investigation 119: 999–909.

55. Murphy TF, Sethi S, Niederman MS (2000) The role of bacteria in exacerbations of COPD. A constructive view. Chest 118: 204–9.

56. Sethi S, Evans N, Grant JB, Murphy TF (2002) New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. The New England journal of medicine 347: 465–71.

57. Sethi S, Sethi R, Eschberger K, Lobbins P, Cai X, et al. (2007) Airway bacterial concentrations and exacerbations of chronic obstructive pulmonary disease. American journal of respiratory and critical care medicine 176: 556–61.

58. Bisgaard H, Hermansen MN, Bonnellykke K, Stokholm J, Baty F, et al. (2010) Association of bacteria and viruses with wheezy episodes in young children: prospective birth cohort study. BMJ 341: c4978.

59. Bisgaard H, Hermansen MN, Buchwald F, Loland I, Halkjaer LB, et al. (2007) Childhood asthma after bacterial colonization of the airway in neonates. The New England journal of medicine 357: 1487–95.

60. Gibson PG, Simpson JL, Salton N (2001) Heterogeneity of airway inflammation in persistent asthma: evidence of neutrophilic inflammation and increased sputum interleukin-8. Chest 119: 1329–36.

61. Haldar P, Pavord ID (2007) Noneosinophilic asthma: a distinct clinical and pathologic phenotype. The Journal of allergy and clinical immunology 119: 1043–52; quiz 1053–4.

62. Moghaddam SJ, Clement CG, Garza MM De la, Zou X, Travis EL, et al. (2008) Haemophilus influenzae lyase induces aspects of the chronic obstructive pulmonary disease phenotype. American journal of respiratory cell and molecular biology 38: 629–38.

63. Gaschler GJ, Skurz M, Zavitza CCJ, Lindahl M, Omervik PO, et al. (2009) Bacteria challenge in smoke-exposed mice exacerbates inflammation and skew the inflammatory profile. American journal of respiratory and critical care medicine 179: 666–75.