Rothia mucilaginosa is an anti-inflammatory bacterium in the respiratory tract of patients with chronic lung disease

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Shareable abstract (@ERSpublications)
A commensal bacterium of the lower airways, *Rothia mucilaginosa*, inhibits inflammation by NF-κB pathway inactivation. *R. mucilaginosa* abundance inversely correlates with sputum pro-inflammatory markers in chronic lung disease, indicating a beneficial role. https://bit.ly/3lNT9th

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Abstract

Background Chronic airway inflammation is the main driver of pathogenesis in respiratory diseases such as severe asthma, chronic obstructive pulmonary disease, cystic fibrosis (CF) and bronchiectasis. While the role of common pathogens in airway inflammation is widely recognised, the influence of other microbiota members is still poorly understood.

Methods We hypothesised that the lung microbiota contains bacteria with immunomodulatory activity which modulate net levels of immune activation by key respiratory pathogens. Therefore, we assessed the immunomodulatory effect of several members of the lung microbiota frequently reported as present in CF lower respiratory tract samples.

Results We show that *Rothia mucilaginosa*, a common resident of the oral cavity that is also often detectable in the lower airways in chronic disease, has an inhibitory effect on pathogen- or lipopolysaccharide-induced pro-inflammatory responses, *in vitro* (three-dimensional cell culture model) and *in vivo* (mouse model). Furthermore, in a cohort of adults with bronchiectasis, the abundance of *Rothia* species was negatively correlated with pro-inflammatory markers (interleukin (IL)-8 and IL-1β) and matrix metalloproteinase (MMP)-1, MMP-8 and MMP-9 in sputum. Mechanistic studies revealed that *R. mucilaginosa* inhibits NF-κB pathway activation by reducing the phosphorylation of IκBα and consequently the expression of NF-κB target genes.

Conclusions These findings indicate that the presence of *R. mucilaginosa* in the lower airways potentially mitigates inflammation, which could in turn influence the severity and progression of chronic respiratory disorders.

Introduction

Persistent or dysregulated lung inflammation is a hallmark of chronic respiratory diseases such as severe asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and bronchiectasis [1–9]. In

patients with chronic airway disease, the release of pro-inflammatory cytokines by cells of the lung mucosa in response to external triggers (pathogenic microorganisms, cigarette smoke, pollutants or allergens) typically leads to infiltration of innate immune cells such as neutrophils or eosinophils that further exacerbate lung inflammation [5–7, 10, 11]. Persistent inflammation and associated tissue damage impairs lung clearance and facilitates the accumulation of microbes in the lower airways. These microbes represent both recognised respiratory pathogens and oropharyngeal commensal taxa that originate in the upper airways [12].

Colonisation of the lower airways by specific bacterial and fungal species contributes directly to inflammation, tissue damage and lung disease progression [13–15]. These species include *Pseudomonas aeruginosa* and *Staphylococcus aureus* in patients with CF [16–18], *Haemophilus influenzae* and *P. aeruginosa* in patients with bronchiectasis [19], *H. influenzae* in patients with neutrophilic asthma [20], and *Moraxella catarrhalis* in patients with COPD [21]. The presence and abundance of these pathogens is largely viewed as the sole microbial determinant of airway disease. However, in addition to these pathogens, the lower airways of patients with chronic lung disease are also colonised by a much wider microbiota that includes many bacterial taxa not considered to contribute directly to pathogenesis [12, 14, 22–24].

The composition of this wider airway microbiota differs with disease characteristics, severity and patient age [25–27]. However, the potential of microbiota members to influence disease by mediating or moderating the impact of pro-inflammatory external triggers, including airway pathogens, is little understood [28–30].

We hypothesised that the lung microbiota contains bacteria with immunomodulatory activity which modulate net levels of immune activation by key respiratory pathogens, such as *P. aeruginosa*. Therefore, we assessed the immunomodulatory effect of several members of the lung microbiota frequently reported as present in CF lower respiratory tract samples [21, 31, 32]. These species represented recognised pathogens (*S. aureus*), less frequently recovered pathogens (*Achromobacter xylosoxidans* and *Streptococcus anginosus*) and upper respiratory tract commensals thought to be broadly nonpathogenic in this context (*Rothia mucilaginosa* and *Gemella haemolysans*). The latter includes the Gram-positive coccus *R. mucilaginosa*, a common commensal of the oral cavity that is frequently detected in the lungs of patients with chronic lung disease [23, 33–36], and has been shown to be metabolically active and adapted to the CF lung environment [37]. We found that *R. mucilaginosa* exhibits anti-inflammatory activity *in vitro*. We subsequently validated our findings in an animal model of lung inflammation and in a cohort of patients with neutrophilic airways disease. Finally, we identified the host pathway that *R. mucilaginosa* interferes with to exert its anti-inflammatory effect.

Methods

Bacterial species and culturing conditions

Six bacterial species (table 1) commonly isolated from the CF lung were selected for this study, including two pathogens (*P. aeruginosa* and *S. aureus*), two less frequently recovered pathogens (*S. anginosus* and *A. xylosoxidans*) and two bacteria that are not typically considered as pathogens (*R. mucilaginosa* and *G. haemolysans*). Selective media for these species were previously developed [38, 39]. For select experiments we also included additional *Rothia* species, *i.e. Rothia dentocariosa*, *Rothia terrae*, *Rothia amarae* and *Rothia aeria*. All isolates were cultured at 37°C under constant shaking conditions (250 rpm) in Brain Heart Infusion broth (Lab M, Bury, UK) until stationary phase. Most isolates were cultured in aerobic conditions except for *S. anginosus* and *G. haemolysans*, which were cultured in microaerobic conditions (<1% O_2) (Oxoid CampyGen Compact; Thermo Fisher Scientific, Waltham, MA, USA).

Three-dimensional lung epithelial cell culture models

A549 cell line

A previously developed organotypic three-dimensional (3D) cell culture model was used to study the immune response to pro-inflammatory stimuli [40–43]. 3D *in vivo*-like lung epithelial cell culture model systems reflect key aspects of the parental tissue, including 3D architecture, barrier function, apical–basolateral polarity and multicellular complexity [40, 42]. It has been demonstrated that *P. aeruginosa* adhesion, as well as host-secreted cytokine profiles, in 3D cell culture models of lung epithelial cells are more similar to the *in vivo* situation than cells grown as a monolayer on plastic [41]. A 3D model of the human adenocarcinoma alveolar lung epithelial cell line A549 (ATCC CCL-185) was generated in GTSF-2 medium (HyClone, Logan, UT, USA) supplemented with 1.5 $g \cdot L^{-1}$ sodium bicarbonate (Sigma-Aldrich, St Louis, MO, USA), 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA, USA), 2.5 $mg \cdot L^{-1}$ insulin transferrin sodium selenite (Lonza, Basel, Switzerland) and 1% penicillin/streptomycin (Life Technologies) at 37°C under 5% CO_2. 3D A549 cells were cultured for 11–14 days and were transferred to

TABLE 1 Overview of strains used in this study

Species	Strain designation	Source
Pseudomonas aeruginosa	PAO1 (ATCC 15692)	Wound
	AA2	Early CF isolate [85]
	AA44; AA43	Late CF isolate [85]
	CF127	CF isolate, hyper-biofilm former [86]
Staphylococcus aureus	SP123	Sputum of mechanically ventilated patient [87]
Streptococcus anginosus	LMG14696	Human throat
Achromobacter xylosoxidans	LMG26680	Sputum of CF patient
Gemella haemolysans	LMG18984	Unknown
Rothia mucilaginosa	DSM20746	Throat
	ATCC 49042	Bronchial secretion
	B03V1S1C	Sputum CF patient
	HVOC02-02; HVOC02-03; HVOC03-01; HVOC03-02; HVOC15-01; HVOC23-01; HVOC24-01	Oral cavity healthy volunteer
	PPP1; PPIB2; PPIA2; PPR5A; PPQ3; PPW3A; PPIB1; PPL4C; PPI6A; PPL1A	Sputum asthma patient
	R-36507	Terrestrial microbial mat (Antarctica) [88]
Rothia dentocariosa	HVOC01-01; HVOC05-01; HVOC10-01; HVOC18-02; HVOC26-01; HVOC27-01; HVOC28-01	Oral cavity healthy volunteer
	PPS2A1	Sputum asthma patient
Rothia terrae	HVOC29-01	Oral cavity healthy volunteer
Rothia amarae	R-43211; R-37581; R-38387; R-36663	Terrestrial microbial mat (Antarctica) [88]
Rothia aeria	HVOC04-01; HVOC12-01; HVOC13-01; HVOC14-01	Oral cavity healthy volunteer

CF: cystic fibrosis.

48-well plates at a concentration of 2.5×10^5 cells per well containing fresh serum-free GTSF-2 medium on the day of the infection.

IB-3 and S9 cell lines

The aforementioned 3D cell culture model was optimised for use with IB-3 and S9 cell lines [44]. The IB-3 cell line is a bronchial epithelial cell line heterogeneous for F508del (F508del/W1282X). The S9 cell line originates from the IB-3 cell line and is stably transduced with wild-type CF transmembrane conductance regulator (CFTR). LHC-8 medium without gentamicin (Life Technologies) supplemented with 5% FBS and 1% penicillin/streptomycin was used to culture both cell lines. The 3D models of IB-3 and S9 cells were generated as described for the 3D A549 model, except that a cell:bead ratio of 4:1 was used (compared with 2:1 for the 3D A549 model).

In vitro *infection studies*

Prior to infection, bacterial cultures were centrifuged and resuspended in serum-free host cell culture medium. Cells were infected with single bacterial cultures for 4 h at a multiplicity of infection (MOI) of 10:1, unless otherwise indicated. Additionally, cells were infected with *P. aeruginosa* together with each one of the CF lung microbiota members for 4 h at a 10:1 ratio, resulting in an MOI of 20:1. When indicated, 3D cell aggregates were exposed to other pro-inflammatory stimuli, *i.e. S. aureus* (MOI 10:1), lipopolysaccharide (LPS) (100 $\mu g\cdot mL^{-1}$) (Sigma-Aldrich), rhamnolipid (100 $\mu g\cdot mL^{-1}$) (Sigma-Aldrich) and H_2O_2 (1 mM), together with *R. mucilaginosa* for 4 h. For longer term experiments, 3D cell aggregates were exposed to 100 $\mu g\cdot mL^{-1}$ LPS alone or in combination with *R. mucilaginosa* for 24 h at an MOI of 1:1. For experiments with cell-free supernatant of *R. mucilaginosa*, cultures were centrifuged and filtered through a 0.22 µm PET filter membrane (GE Healthcare, Chicago, IL, USA). To obtain lysed cell suspension, *R. mucilaginosa* cultures were subjected to a cold/heat cycle twice (*i.e.* 5 min ice followed by 5 min at 85°C). Viability of lysed cells was checked by plating on nutrient agar, confirming no culturable bacteria. 3D A549 cells were exposed for 4 h to *P. aeruginosa* PAO1 (MOI 10:1) with or without *R. mucilaginosa* supernatant (100 µL cell-free supernatant and 150 µL GTSF-2 cell culture medium without FBS), lysed cells (100 µL lysed cell suspension initially containing 2.5×10^6 cells, which is equivalent to an MOI of 10:1 and 150 µL cell culture medium) or live cells as described earlier (MOI 10:1).

Cytotoxicity assays

After infection of the 3D cells with various bacteria or exposure to other pro-inflammatory stimuli (LPS, rhamnolipid and H_2O_2), cell viability was assessed using the annexin V/propidium iodide (Life Technologies) and lactate dehydrogenase (Sigma-Aldrich) assays, according to the manufacturer's instructions.

Bacterial cell adherence analysis

Bacterial adherence to the host cells was determined as described previously [42]. Previously developed selective media were used when host cells were co-exposed to *P. aeruginosa* and *R. mucilaginosa*, *i.e.* Luria–Bertani agar supplemented with 1.25 mg·mL^{-1} triclosan to select for *P. aeruginosa*, and nutrient agar supplemented with 5 mg·mL^{-1} mupirocin and 10 mg·mL^{-1} colistin sulfate to select for *R. mucilaginosa* [38].

Evaluation of interleukin-8 degradation

Recombinant interleukin (IL)-8 standard (BioLegend, San Diego, CA, USA) at a concentration of 125 pg·mL^{-1} was added to *R. mucilaginosa* culture in GTSF-2 medium without serum (5×10^7 CFU·mL^{-1} corresponding to an MOI of 10:1) or to control medium. The IL-8 concentration was measured after 0, 15, 30, 60 and 90 min using ELISA as described in the section on inflammatory marker quantification.

In vivo *mouse model*

12-week-old female BALB/c ByJRj mice (Janvier Labs, Le Genest-Saint-Isle, France) were managed in accordance with the guidelines provided by the European Directive for Laboratory Animal Care (Directive 2010/63/EU of the European Parliament). The laboratory Animal Ethics Committee of the University of Antwerp (Antwerp, Belgium) authorised and approved all animal experiments in this study (file 2019-90). Intratracheal administration of *R. mucilaginosa* embedded in alginate beads in the presence or absence of LPS was done using a protocol described by Moser *et al.* [45], with adaptations described in the supplementary material. Embedding bacteria in alginate is a technique developed to retain a sufficient microbial load in the lungs over an extended period of time without immunosuppression [46]. In a preliminary experiment using *R. mucilaginosa* cell suspension for intratracheal administration, consistent colonisation could not be obtained (supplementary material). Moreover, for *R. mucilaginosa*, the method used to prepare alginate beads removed bacterial clumps >0.25 mm, thereby limiting the risk of blocking the airways of the mice. Hence, embedding of *R. mucilaginosa* in alginate beads was used for all experiments. For preparation of the *R. mucilaginosa*-containing alginate beads, three independent cultures were pooled. Mice were instilled with 10 µg per 50 µL LPS with or without *R. mucilaginosa* (either *R. mucilaginosa* DSM20746 or the CF isolate *R. mucilaginosa* B03V1S1C at the maximal dose that could be obtained in the alginate beads, *i.e.* $\sim1\times10^5$ CFU per mouse) embedded in alginate beads for 48 h. Mice were observed during this 48 h for fur quality, posture, state of activity and respiratory symptoms, and were weighed every 24 h. Mice were euthanised by cervical dislocation 48 h post-infection. The left lung was homogenised and used for determination of the microbial load (by plate counting) as well as for cytokine quantification. The spleen and medial lobe of the liver were collected and homogenised to check for dissemination of the instilled bacteria (by plate counting). The right lung was collected in 1 mL of 4% PFA for histological analysis. Sections for histological analysis stained by haematoxylin/eosin were examined blindly and scored as previously described using an EVOS FL Auto microscope (Life Technologies) [47].

Quantification of Rothia *species in respiratory samples*

Archived, induced sputum samples were obtained from 85 adults with confirmed bronchiectasis and with a history of two or more infective exacerbations (associated with bacterial infection based on the requirement for supplemental systemic antibiotic therapy) in the preceding year, recruited as part of the BLESS randomised controlled trial [48]. Information on the patient cohort (including inclusion/exclusion criteria and sputum induction procedure) is provided in the supplementary methods. The study was approved by the Mater Health Service (South Brisbane, Australia) Human Research Ethics Committee and informed consent was obtained for all participants. Induced sputum was collected at baseline, prior to any intervention, and all patients had a sputum neutrophil abundance of ⩾70% (as a percentage of total sputum cells). All patients had a chronic lower airway infection. The relative abundance of *Rothia* species was measured in 79 out of 85 samples using 16S rRNA gene amplicon sequencing as described previously [49] and as detailed in the supplementary methods. Sequence data are available at the National Center for Biotechnology Information Sequence Read Archive with accession number SRA128000. *R. mucilaginosa* absolute abundance was measured by quantitative PCR (qPCR) in 82 out of 85 samples using the Qiagen Microbial DNA qPCR Assay for *R. mucilaginosa* (BPID00297A; Qiagen, Hilden, Germany). Gene copy numbers were determined by comparing with a standard curve as described previously [50].

Inflammatory marker quantification

For *in vitro* experiments, IL-8 secretion was measured in the cell culture supernatant by a Human IL-8 ELISA MAX Standard assay (BioLegend). For *in vivo* animal experiments, macrophage inflammatory protein (MIP)-2 secretion was measured in lung homogenate by a MIP-2 Mouse ELISA kit (LabNed, Amstelveen, The Netherlands). All other cytokines for *in vitro* cell culture and *in vivo* animal experiments were quantified by Bioplex Multiplex assays (Bio-Rad, Hercules, CA, USA). Inflammatory markers were measured in the sputum of bronchiectasis patients using ELISA (BD Biosciences, San Jose, CA, USA) for IL-8 and IL-1β, and using Magnetic Luminex Performance Assay multiplex kits (R&D Systems, Minneapolis, MN, USA) for matrix metalloproteinase (MMP)-1, MMP-8 and MMP-9 as described previously [51].

Quantitative reverse transcriptase-PCR

3D A549 cells were infected with *P. aeruginosa* PAO1 or a combination of *P. aeruginosa* PAO1 and *R. mucilaginosa* DSM20746 as described earlier. After 4 h of infection, 3D A549 cells were washed three times with Hank's Balanced Salt Solution and RNAprotect Reagent (Qiagen) was added. Total cellular RNA was extracted using the Aurum Total RNA Mini Kit (Bio-Rad) and reverse transcribed into cDNA using the iScript advanced cDNA synthesis kit (Bio-Rad). The expression of various genes involved in inflammation was quantified using the "Bacterial infections in normal airways" PrimePCR panel (Bio-Rad) and SsoAdvanced SYBR Green supermix (Bio-Rad). qPCR experiments were performed on a Bio-Rad CFX96 Real-Time System C1000 Thermal Cycler. *TBP*, *GAPDH* and *HPRT1* were used as reference genes. Genes with an insufficient expression level were excluded from further analysis.

Analysis of NF-κB activation

A 3D epithelial cell model of NF-κB–luciferase-transfected A549 cells (BPS Bioscience, San Diego, CA, USA) was developed as described for the 3D A549 model. Cells were exposed for 4 h to single or co-cultures of *P. aeruginosa* and *R. mucilaginosa* (or its cell-free supernatant) as described earlier. Likewise, screening of NF-κB activation by LPS (100 $\mu g \cdot mL^{-1}$) with or without 36 isolates representing five different *Rothia* species (table 1) was performed. When indicated, the effect of *Rothia* species (*R. mucilaginosa* DSM20146, *R. dentocariosa* HVOC18-02, *R. aeria* HVOC13-01 and *R. terrae* HVOC29-01) on NF-κB pathway activation by LPS (100 $\mu g \cdot mL^{-1}$) was evaluated under microaerobic conditions (3% O_2, 5% CO_2 and 92% N_2) in a BACTROX-2 Hypoxia Chamber (SHEL LAB, Cornelius, OR, USA). The One-Step Luciferase Assay System (BPS Bioscience) was used to quantify NF-κB pathway activation and luminescence was measured by an EnVision luminometer (PerkinElmer, Waltham, MA, USA).

Western blot analysis

The Western blot experiments were done as described previously [52]. Mouse monoclonal antibodies, at concentrations recommended by the manufacturer, were used to detect A20 (Santa Cruz Biotechnology, Dallas, TX, USA), NF-κB p65 (Santa Cruz Biotechnology) and phosphorylated (p)-IκBα (Cell Signaling Technology, Danvers, MA, USA) and a goat polyclonal antibody was used to detect IκBα (Santa Cruz Biotechnology). As secondary antibody, an anti-mouse IgG horseradish peroxidase (HRP)-linked antibody (Santa Cruz Biotechnology) or anti-goat IgG HRP-linked antibody (Santa Cruz Biotechnology) was used.

Statistical analysis

All *in vitro* experiments were carried out at least in biological triplicates ($n \geqslant 3$). *In vitro* infection experiments contained two technical replicates for assessment of the viability, quantification of CFU by plating and quantification of cytokines. For animal experiments, MIP-2, CFU, disease symptoms in the animals, colonisation and dissemination were determined for two independent experiments with *R. mucilaginosa* DSM20746. Validation of the *in vivo* data with another *R. mucilaginosa* strain (B03V1S1C) and quantification of additional cytokines as well as histological analysis were done for one experiment for both strains.

Statistical analysis was performed using SPSS version 24.0 (IBM, Armonk, NY, USA). The Shapiro–Wilk test was applied to evaluate normality of the data. For normally distributed data, differences between the means were assessed by an independent samples t-test or ANOVA followed by Dunnett's *post hoc* analysis. Nonnormally distributed data were further analysed by a Kruskal–Wallis nonparametric test or Mann–Whitney test. Statistical significance is assumed at $p<0.05$. For correlation analysis, between *Rothia* species abundance (relative or absolute) and cytokine/MMP concentrations, correlation coefficients and p-values were determined by either Pearson's test (r) for parametric distributions or Spearman's test (r_s) for nonparametric distributions.

Results

R. mucilaginosa inhibits the production of pro-inflammatory cytokines by lung epithelial cells in vitro

Exposure of an *in vivo*-like 3D alveolar epithelial model to *P. aeruginosa* PAO1 (MOI 30:1) induced high IL-8 production, while low or moderate IL-8 production was observed following exposure to *S. aureus* SP123, *S. anginosus* LMG14696, *A. xylosoxidans* LMG26680, *G. haemolysans* LMG18984 and *Rothia mucilaginosa* DSM20746 (figure 1a). When 3D A549 cells were co-exposed to *P. aeruginosa* and one of each of the five other bacterial species, *R. mucilaginosa* completely abolished the *P. aeruginosa*-induced IL-8 response (figure 1b). *R. mucilaginosa* exposure also lowered the IL-8 response induced by *P. aeruginosa* at an MOI of 100:1 and 10:1 (supplementary figure S1a). The effect of *R. mucilaginosa* DSM20746 on *P. aeruginosa*-induced IL-8 production was confirmed with two other strains of *R. mucilaginosa* (ATCC 49042 and a CF sputum isolate B03V1S1C) (supplementary figure S1b). The three *R. mucilaginosa* strains also reduced the IL-8 response induced by various *P. aeruginosa* CF sputum isolates (supplementary figure S1b). Reduction in IL-8 response by *R. mucilaginosa* was confirmed using LPS (100 $\mu g \cdot mL^{-1}$) as the pro-inflammatory stimulus, both for short-term (4 h) and long-term (24 h) exposure (figure 1c). Under the experimental conditions tested, a similar number of *R. mucilaginosa* CFU was observed between the initial inoculum (0 h) and the total CFU that associated with the host cells and was present in the surrounding liquid at 4 and 24 h, indicating that bacteria remained viable throughout the experiment (supplementary figure S1c). After 4 h, a slight increase in total *R. mucilaginosa* CFU was observed compared with the inoculum (supplementary figure S1c).

R. mucilaginosa also significantly reduced IL-8 levels promoted by the Gram-positive pathogen *S. aureus* (MOI 10:1) in 3D A549 cells (supplementary figure S1d). The anti-inflammatory effect of *R. mucilaginosa* was confirmed in a 3D model of CF bronchial epithelial cells (IB-3 cell line) and in the CFTR corrected control (S9 cell line) (supplementary figure S1e and f).

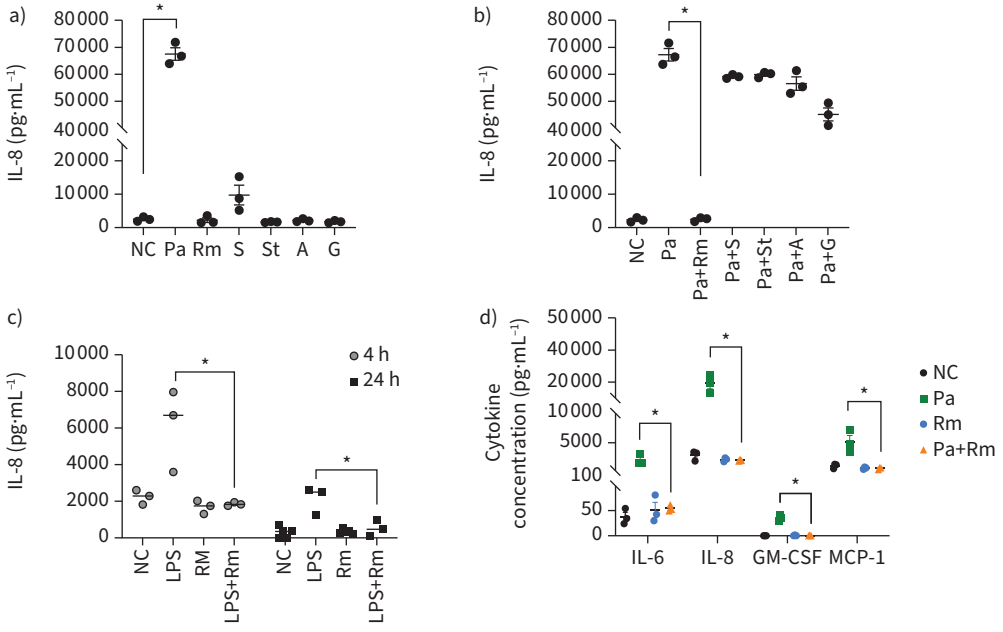

FIGURE 1 Three-dimensional (3D) lung epithelial responses to pro-inflammatory stimuli in the presence and absence of members of the lung microbiota: *Pseudomonas aeruginosa* PAO1 (Pa), *Rothia mucilaginosa* DSM20746 (Rm), *Staphylococcus aureus* SP123 (S), *Streptococcus anginosus* LMG14696 (St), *Achromobacter xylosoxidans* LMG26680 (A) and *Gemella haemolysans* LMG18984 (G). a, b) Interleukin (IL)-8 production by 3D A549 cells after 4 h exposure to a) single bacterial cultures or b) co-cultures of various lung microbiota members with *P. aeruginosa* PAO1 at an MOI of 30:1. c) IL-8 production by 3D A549 cells after 4 or 24 h exposure to 100 $\mu g \cdot mL^{-1}$ lipopolysaccharide (LPS) alone or in co-culture with *R. mucilaginosa* at an MOI 10:1 (4 h) or 1:1 (24 h). d) IL-6, IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF) and monocyte chemoattractant protein (MCP)-1 production of 3D A549 cells after 4 h exposure to *P. aeruginosa* alone or in co-culture with *R. mucilaginosa* at an MOI of 10:1. NC: negative control (uninfected 3D epithelial cells in serum-free medium). Data represent mean±SEM or mean, n⩾3. *: p<0.05; **: p<0.001.

In addition, *R. mucilaginosa* reduced levels of other *P. aeruginosa*-induced pro-inflammatory cytokines (IL-6, IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF) and monocyte chemoattractant protein (MCP)-1) (figure 1d). Exposure to *P. aeruginosa*, *R. mucilaginosa* or the combination showed normal morphology based on microscopic analysis and cells retained >80% viability after infection (supplementary figure S2).

R. mucilaginosa *lowers the LPS-induced pro-inflammatory response in an* in vivo *mouse model*

Next, we evaluated the *in vivo* anti-inflammatory effect of *R. mucilaginosa* by measuring mouse lung inflammatory cytokine levels following LPS instillation or LPS and *R. mucilaginosa* (either DSM20746 and B03V1S1C strains) co-exposure (figure 2a). After 48 h of co-exposure, LPS-induced MIP-2

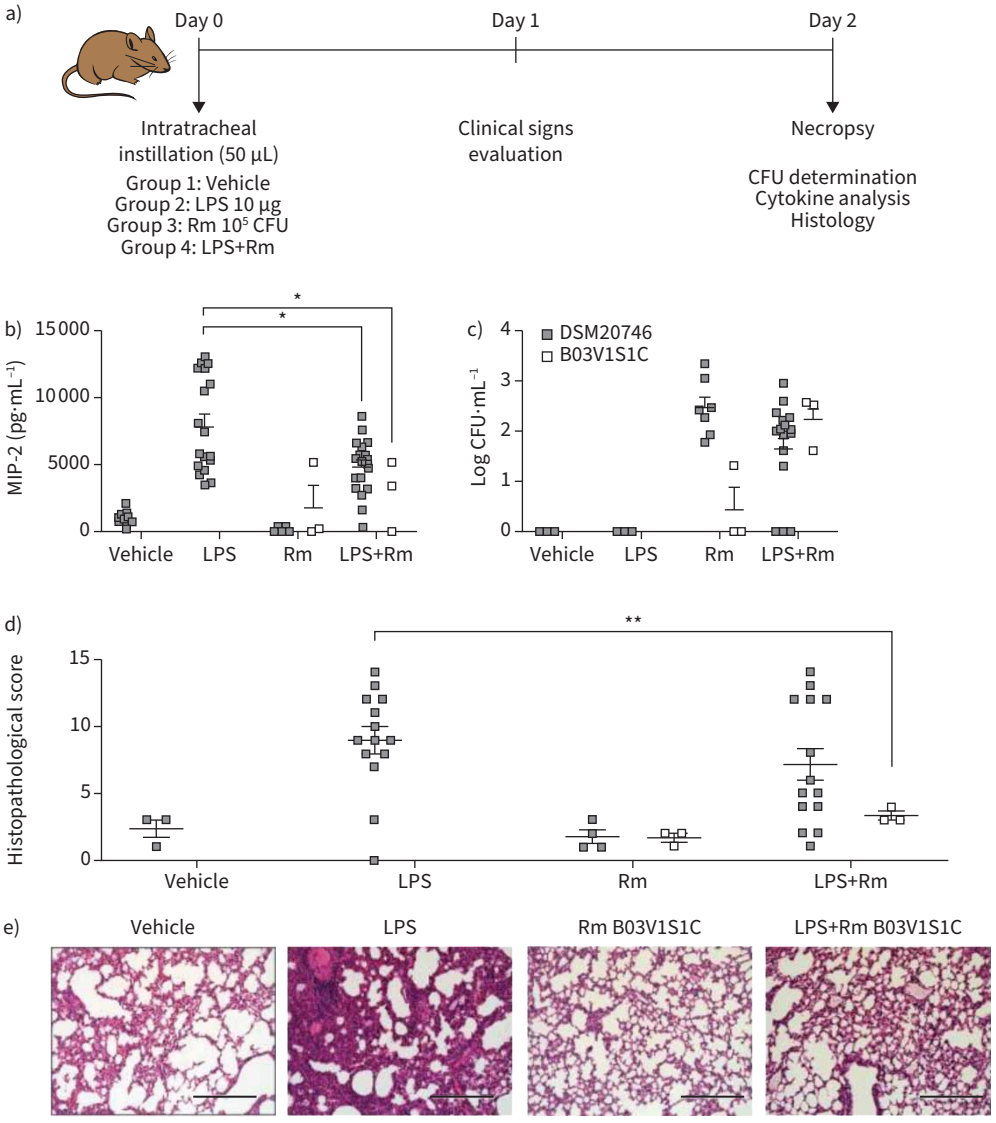

FIGURE 2 Influence of *Rothia mucilaginosa* (Rm) on the *in vivo* responses to lipopolysaccharide (LPS). a) Timeline of animal infection and necropsy, b) macrophage inflammatory protein (MIP)-2 concentration (measured by ELISA), and c) number of CFU·mL^{-1} in mice lung homogenates after 48 h exposure to vehicle (n=7), LPS (n=18), *R. mucilaginosa* suspension (n=9 for *R. mucilaginosa* DSM20746 and n=3 for *R. mucilaginosa* B03V1S1C) or a combination of LPS+*R. mucilaginosa* (n=18 for *R. mucilaginosa* DSM20746 and n=3 for *R. mucilaginosa* B03V1S1C). d) Lung histopathological score on haematoxylin/eosin-stained sections of the right lung. e) Lung haematoxylin/eosin-stained sections of vehicle, LPS, *R. mucilaginosa* B03V1S1C and LPS+*R. mucilaginosa* B03V1S1C instilled mice. Scale bar: 200 µm. Vehicle: sterile alginate beads; LPS: 10 µg per 50 µL. The data presented is from two independent animal experiments for strain DSM20746 and one experiment for strain B03V1S1C. Data represent mean±SEM, n⩾3. *: p<0.05; **: p<0.01.

(homologue for human IL-8) was reduced by 40% by strain DSM20746 (figure 2b). The results obtained were validated using another *R. mucilaginosa* strain (B03V1S1C), which demonstrated a reduction of LPS-induced MIP-2 production of 70% (figure 2b). Levels of other cytokines (MCP-1, IL-6, IL-1α, IL-5, IL-10 and GM-CSF) were also significantly lower in mice exposed to LPS and *R. mucilaginosa* B03V1S1C compared with LPS alone (supplementary figure S3). The lungs of *R. mucilaginosa*-infected mice contained a similar number of bacteria ($CFU \cdot mL^{-1}$) in the presence and absence of LPS (figure 2c). No bacteria were detected in the liver and spleen, suggesting no dissemination, and mice showed an overall normal fur quality, posture, body weight and no respiratory symptoms in all test conditions. Haematoxylin/eosin-stained lung sections of LPS-instilled mice showed significantly more pronounced tissue damage compared with lung sections of mice co-exposed to LPS and *R. mucilaginosa* B03V1S1C, and the same trend for *R. mucilaginosa* DSM20746 was observed (figure 2d and e).

Rothia *species inhibit NF-κB activation in epithelial cells*

To decipher the mode of action of *R. mucilaginosa*, the expression of genes that are part of major pro-inflammatory pathways was evaluated in 3D A549 cells exposed to *P. aeruginosa* or co-exposed to *P. aeruginosa* and *R. mucilaginosa*. *R. mucilaginosa* co-exposure significantly downregulated the expression of genes encoding IL-8 (*IL8*), IL-6 (*IL6*) and pro-IL-1β (*IL1B*) (figure 3) caused by *P. aeruginosa*. In addition, *R. mucilaginosa* significantly downregulated *NFKB1* expression (figure 3). Regulation of *NFKBIA*, *NFKBIE* and *REL* genes showed a downward trend that further indicates an effect of *R. mucilaginosa* on the NF-κB pathway.

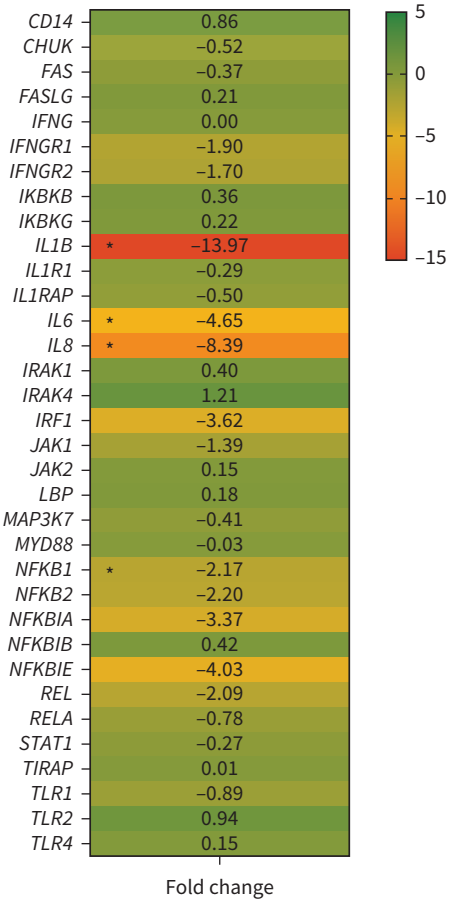

FIGURE 3 Differential expression of genes involved in inflammation by three-dimensional (3D) A549 alveolar epithelial cells exposed to *Pseudomonas aeruginosa versus P. aeruginosa* in combination with *Rothia mucilaginosa*. Quantitative PCR analysis showing average fold changes in mRNA levels of 3D A549 cells stimulated by *P. aeruginosa* PAO1 *versus* a co-culture of *P. aeruginosa* and *R. mucilaginosa*. n=3. *: statistically significant ($p<0.05$) fold change.

The influence of *R. mucilaginosa* on activation of the NF-κB pathway was confirmed in a NF-κB–luciferase reporter 3D A549 cell model, showing a significant reduction of NF-κB activity by co-culturing *R. mucilaginosa* with *P. aeruginosa* compared with *P. aeruginosa* single exposure (figure 4a). The inhibition of the NF-κB pathway by *R. mucilaginosa* was also shown using LPS as a pro-inflammatory stimulus and was observed for 36 isolates representing five different *Rothia* species from clinical and environmental sources (supplementary figure S4a and b). The anti-inflammatory effect of different *Rothia* species on NF-κB pathway activation by LPS was also observed under microaerobic conditions (supplementary figure S4c), which can be encountered by microorganisms in the mucus of patients with chronic lung disease such as CF [53]. The minimal effective dose of *R. mucilaginosa* to inhibit *P. aeruginosa*-induced inflammation was an MOI of at least 5:1 for all doses of *P. aeruginosa* tested (up to MOI 100:1) (supplementary figure S5a). We also tested higher doses of *R. mucilaginosa* (MOI 100:1 and 1000:1) and the anti-inflammatory effect was confirmed (supplementary figure S5b).

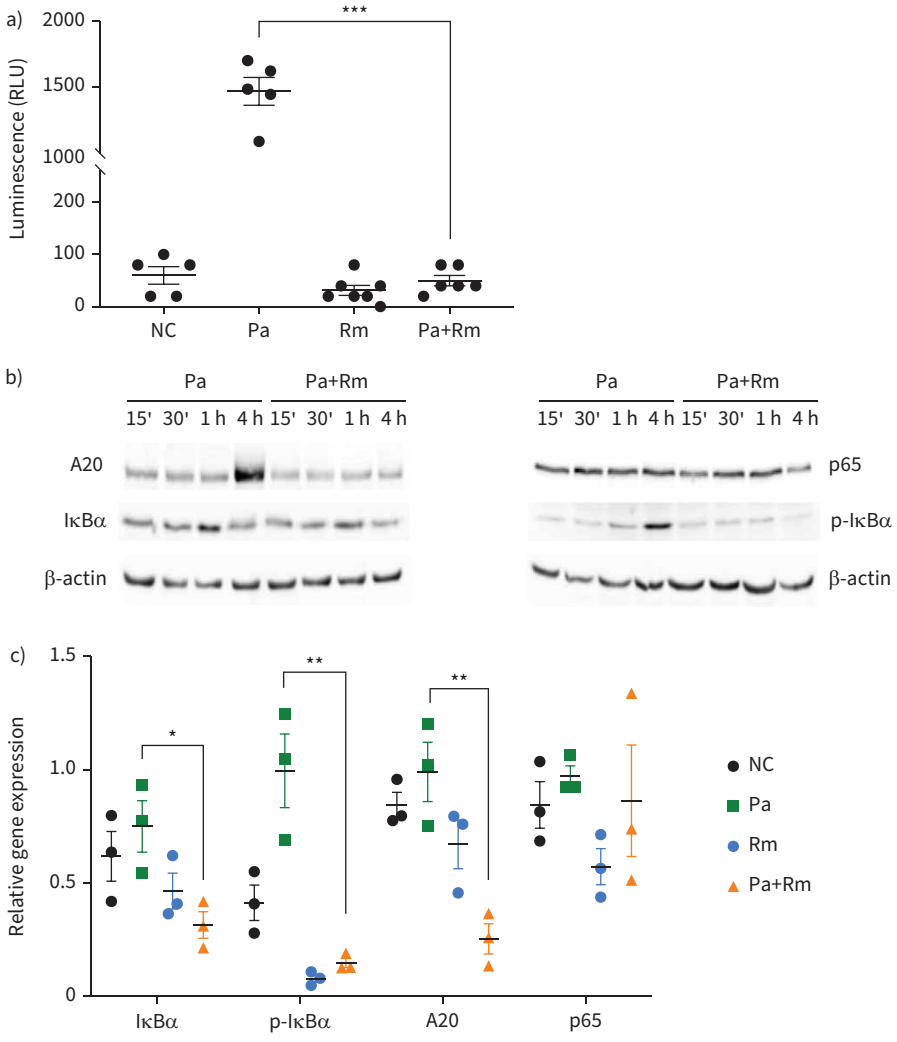

FIGURE 4 Effect of *Rothia mucilaginosa* (Rm) on NF-κB pathway activation by *Pseudomonas aeruginosa* (Pa). a) Activation of the NF-κB pathway measured *via* luminescence of three-dimensional (3D) NF-κB reporter A549 cells. 3D cells were exposed for 4 h to *P. aeruginosa* PAO1 alone or in co-culture with *R. mucilaginosa* DSM20746. b) Semiquantitative determination (by Western blotting) of proteins (*i.e.* A20, IκBα, p65 and phosphorylated (p)-IκBα) produced by 3D A549 cells stimulated with *P. aeruginosa* PAO1 with or without *R. mucilaginosa* DSM20746 for 15 min (15′), 30 min (30′), 1 h and 4 h. c) Band intensity (normalised to β-actin) of Western blot at 4 h of 3D A549 cells stimulated with *P. aeruginosa* PAO1 with or without *R. mucilaginosa* DSM20746. RLU: relative light units; NC: negative control (uninfected 3D NF-κB reporter A549 cells in serum-free GTSF-2 medium). Data represent mean±SEM, n=3. *: p<0.05; **: p<0.01; ***: p<0.001.

We subsequently performed Western blot analysis to investigate at which stage *R. mucilaginosa* affects NF-κB signalling. After 4 h co-culture of *P. aeruginosa* and *R. mucilaginosa*, *R. mucilaginosa* prevented *P. aeruginosa*-induced IκBα phosphorylation (figure 4b and c). This suggests that *R. mucilaginosa* affects NF-κB signalling upstream or at the level of the IκBα kinase complex (IKK). In line with this, the expression of both IκBα and A20, two NF-κB target genes, was greatly reduced in the co-culture samples (figure 4c).

Finally, we excluded degradation of IL-8 or inhibition of *P. aeruginosa* adhesion to 3D A549 epithelial cells by *R. mucilaginosa* as alternative explanations for the observed anti-inflammatory effect (supplementary figure S6).

R. mucilaginosa *supernatant inhibits IL-8 production and NF-κB pathway activation in response to pro-inflammatory stimuli in 3D lung epithelial cells*

Exposure of 3D A549 cells to *P. aeruginosa* PAO1 (MOI 10:1) in the presence of *R. mucilaginosa* cell-free supernatant, but not lysed cells, strongly reduced IL-8 production (figure 5a). We confirmed that *R. mucilaginosa* DSM20746 cell-free supernatant fully abolished LPS-stimulated NF-κB pathway activation (figure 5b).

Inverse relationship between airway inflammation and Rothia *species in patients with neutrophilic airways disease*

Two culture-independent approaches were applied to measure *Rothia* species in induced sputum samples from patients with bronchiectasis, a neutrophilic airways disease. The first approach (measuring *R. mucilaginosa* absolute load) indicated that *R. mucilaginosa* load significantly inversely correlated with levels of IL-8 (figure 6a) and IL-1β (figure 6b), as well as with those of MMP-1 (figure 6c) and MMP-8 (figure 6d). There were no significant correlations between *R. mucilaginosa* and MMP-9 (figure 6e) or neutrophil percentage in sputum (figure 6f). In the second approach, we determined the relative abundance of *Rothia* and found it also inversely correlated with levels of IL-8, IL-1β, MMP-1 and MMP-8. The correlation between *Rothia* relative abundance and MMP-9 was also significant. There was again no correlation between *Rothia* abundance and neutrophil percentage (supplementary figure S7).

Discussion

We report that common members of the lung microbiota belonging to the genus *Rothia* exert anti-inflammatory properties *in vitro* and that for at least one (*R. mucilaginosa*) this effect is also apparent *in vivo*. Being frequently part of the oral microbiota and only rarely causing lung infection [54, 55], *R. mucilaginosa* is often considered part of salivary contamination of samples from the lower airways. However, comparison of the microbiome of the oral cavity with the lung microbiome suggests that *R. mucilaginosa* is not a contaminant [37, 56, 57]. The core lung microbiome varies interindividually, yet *Rothia* species are detected in COPD and asthmatic patients, in paediatric and adult individuals with CF

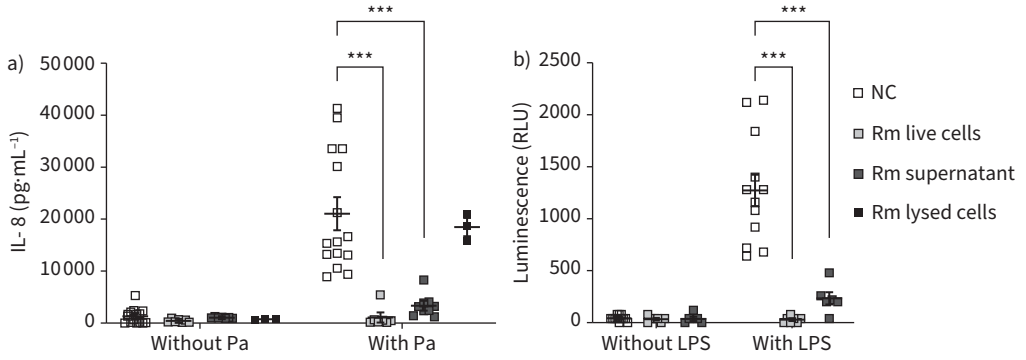

FIGURE 5 The anti-inflammatory effect of *Rothia mucilaginosa* (Rm) is mediated by its cell-free supernatant. a) Interleukin (IL)-8 production by three-dimensional (3D) A549 epithelial cells after exposure to *P. aeruginosa* PAO1 (Pa) for 4 h, with or without *R. mucilaginosa* DSM20746 live cells, lysed cells or cell-free supernatant. b) Quantification of NF-κB pathway activation after 4 h exposure to lipopolysaccharide (LPS) (100 μg·mL^{-1}) with or without *R. mucilaginosa* DSM20746 cell-free supernatant. The multiplicity of infection was 10:1 in all experiments. RLU: relative light units; NC: negative control (uninfected 3D epithelial cells). Data represent mean±SEM, n⩾3. ***: p<0.001.

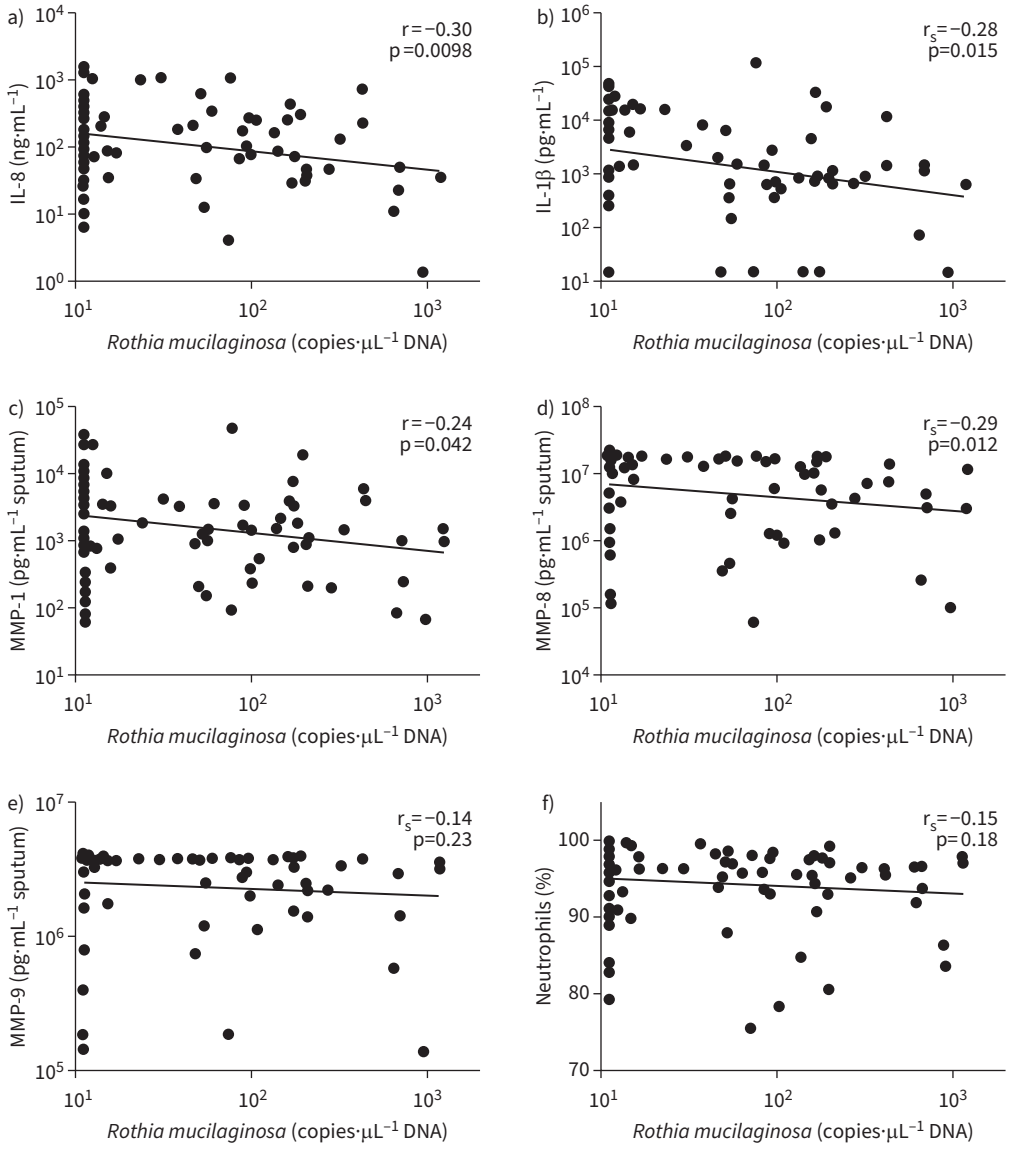

FIGURE 6 Correlation of absolute load of *Rothia mucilaginosa* with pro-inflammatory parameters in induced sputum samples from bronchiectasis patients: a) interleukin (IL)-8, b) IL-1β, c) matrix metalloproteinase (MMP)-1, d) MMP-8, e) MMP-9 and f) neutrophils. Data points represent individual induced sputum samples. Correlation coefficients and p-values were calculated on ranked values when Spearman's test was used (r_s).

[23, 33, 35, 36, 58], and also in the lower airways of healthy individuals [59]. Nevertheless, little is known about its role in chronic lung disease [24, 60].

The presence of bacteria in the lower airways in chronic lung disease is conventionally considered as a contributor to the pathophysiology [13–15]. Results obtained in the present study strongly suggest that the presence of *R. mucilaginosa* in the airways is beneficial as it not only inhibited the production of *P. aeruginosa*-induced pro-inflammatory cytokines, it also showed an inhibitory effect on the IL-8 response induced by LPS and by another important respiratory pathogen, *S. aureus*. The anti-inflammatory effect was confirmed for a large collection of *Rothia* species and was also observed under microaerobic conditions. Since *Rothia* species are facultative anaerobes, it is likely that these microorganisms will colonise niches with low oxygen levels found in the lung mucus of patients with chronic lung disease [53]. Nevertheless, a minimal dose of *Rothia* was needed to obtain an anti-inflammatory effect *in vitro* for all doses of *P. aeruginosa* tested, suggesting a dose-dependent effect. The dose used most frequently throughout our study (MOI 10:1) is justified as a model for patients with chronic lung disease since a total

bacterial MOI of ∼100:1 has been reported for CF patients [61], and the relative abundance of *Rothia* varies between 1% and 40% [62, 63]. Also, in the BLESS cohort that was analysed in our study, the mean relative abundance of *Rothia* was 9%, supporting the physiological relevance of the dose used. Nevertheless, we cannot rule out that at some point, high loads of *Rothia* may result in a net pro-inflammatory effect in the lung environment, regardless of its anti-inflammatory properties. Very high doses of *R. mucilaginosa* (up to MOI 1000:1) still exerted an anti-inflammatory effect *in vitro*; therefore, further research is needed to evaluate the load of *Rothia* that can be tolerated *in vivo* without causing adverse effects.

R. mucilaginosa was also able to reduce the LPS-triggered production of pro-inflammatory cytokines in lung homogenates of mice, using an alginate bead model. The levels of several cytokines in mice exposed to empty alginate beads were similar to those of LPS-treated mice, which is in agreement with a previous study reporting inflammatory responses in mouse lungs after instillation with sterile alginate beads [64]. Nevertheless, *R. mucilaginosa* caused a clear decline in the inflammatory response of lung tissue regardless of the stimulus and the degree of the anti-inflammatory effect was strain dependent.

Interestingly, previous studies have shown a negative correlation between the relative abundance of *Rothia* species and sputum inflammatory markers such as neutrophil elastase, IL-8 and IL-1β in CF patients [65, 66]. A recent report found the relative abundance of the genus *Rothia* in expectorated sputum to be a positive predictor of lung function in patients with CF [67]. Not only were we able to confirm a negative correlation between *Rothia* species and inflammation for the BLESS cohort at the relative abundance level, but we also showed that the absolute abundance of *R. mucilaginosa* was negatively correlated with inflammatory markers (based on IL-8 and IL-1β levels) and mediators of airway remodelling (MMP-1 and MMP-8 levels). Levels of MMP-1, MMP-8 and MMP-9, which degrade extracellular matrix proteins [68], are increased in the airways of patients with neutrophilic airways disease, are inversely correlated with lung function [51] and contribute to irreversible airway damage [68]. We did not find an association between *Rothia* and neutrophil abundance. This is potentially attributable to relatively low levels of variation in neutrophil percentages between patients (all ⩾70%). Assessment of *Rothia* abundance in other cohorts of neutrophilic airways disease is needed to confirm a possible anti-inflammatory effect of *Rothia* species in patients and to determine the extent to which this might impair the progression of structural lung disease.

While several studies have associated an increased abundance of *Rothia* with positive health outcomes, it was recently demonstrated that the relative abundance of *Rothia* species was higher in a cohort of patients with a range of chronic lung diseases (severe asthma, COPD and bronchiectasis) [69] and in patients with tuberculosis compared with healthy individuals [70]. It remains to be determined whether the increased *Rothia* relative abundance also implies an actual change in the absolute microbial load of that species or merely reflects a decreased abundance of other (more dominant) species. Nevertheless, the study of Hong *et al*. [70] indicated that *R. mucilaginosa* may serve as an anchor species for *Mycobacterium tuberculosis* and 20 other taxa. It is possible that metabolic cross-feeding could alter the co-occurrence of other pathogenic or nonpathogenic species in the lung environment. For *R. mucilaginosa*, it was demonstrated that its metabolic products can cross-feed *P. aeruginosa* [71]. While we did not observe *R. mucilaginosa*-induced growth of *P. aeruginosa* in our study, long-term interspecies interactions are worth exploring further to evaluate whether commensal species such as *R. mucilaginosa* may influence overall community composition, in addition to exerting an anti-inflammatory activity. Furthermore, whether an altered *Rothia* abundance in the described and present studies is a driver of disease/disease markers or a consequence of an altered lung physiology (*e.g.* due to altered airway clearance or differential levels of reactive oxygen species, pro-inflammatory cytokines or mucins) remains a question to be fully addressed. The obtained data in animals suggest that supplementation of *Rothia* in the lungs may diminish the pro-inflammatory response to external stimuli, yet further exploration of the causal relation between *Rothia* abundance and inflammation in patients is needed.

The discovery of *Rothia* as an anti-inflammatory genus in the lung microbiota raises the question whether other nonpathogenic commensals in the respiratory tract, in particular originating from the oral cavity, may have immunomodulatory properties. Recent studies in animals [72] or *in vitro* [73] support this hypothesis. For example, pulmonary aspiration of oral human commensals (*Prevotella melaninogenica*, *Veillonella parvula* and *Streptococcus mitis*) in mice induces a T-helper cell type 17 inflammatory phenotype that diminishes susceptibility to *Streptococcus pneumoniae*. Our data on cytokine production and on NF-κB activation indicate that *R. mucilaginosa* prevents NF-κB signalling, probably at the level of IKK activation. Indeed, IL-8, IL-1β, IL-6 and tumour necrosis factor-α are regulated by NF-κB through binding of pathogen-associated molecular patterns to Toll-like receptors (TLRs) [74]. This is of particular interest since the NF-κB pathway mediates the elevated innate immune response in patients with chronic lung

diseases [75–84]. Cell-free supernatant exhibited anti-inflammatory activity, indicating that *R. mucilaginosa* secretes (an) anti-inflammatory mediator(s) that directly interferes with the NF-κB pathway or induces host mediators that resolve inflammation. Since the anti-inflammatory activity was observed using both the pure TLR4 ligand LPS as well as different microbial species as the pro-inflammatory trigger, we can exclude that antimicrobial activity of *R. mucilaginosa* is causing the effect. Research to identify the immunomodulatory mediator(s) will be necessary to further understand how *Rothia* species modulate the pro-inflammatory response.

Understanding the contribution of potentially beneficial species to net inflammation could enable us to better understand variation in disease severity and progression between patients with chronic lung disease. Prospective studies to assess the clinical utility of *R. mucilaginosa* as a marker of airway inflammation, and the extent to which it may provide a basis for the development of novel therapeutic interventions, are now required.

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Author contributions: A. Crabbé conceptualised the study. A. Crabbé, C. Rigauts and T. Coenye are responsible for the overall study design. J. Aizawa, C. Rigauts, M. Govaerts and L. Ostyn performed the animal experiments. S.L. Taylor and S. Sims performed DNA sequencing, qPCR experiments and data analysis. C. Rigauts and L. Ostyn performed the *in vitro* experiments, and C. Rigauts analysed the data. L. Burr and J.L. Simpson were responsible for clinical sampling. G.B. Rogers, P. Cos, Y. Dondelinger, M. Sze, L. Vereecke, A. Bragonzi, C. Cigana, H. Van Acker and E. Vandeplassche provided critical guidance in the experimental set-up, analysis and/or interpretation of results. C. Rigauts, A. Willems, M.M. Tunney, G.B. Rogers and S.L. Taylor isolated bacterial strains used in this study. C. Rigauts, A. Crabbé, G.B. Rogers, S.L. Taylor and J. Aizawa wrote the manuscript. T. Coenye, P. Cos, M.M. Tunney, A. Willems, Y. Dondelinger, L. Vereecke and C. Cigana revised the manuscript.

Conflict of interest: C. Rigauts declares a patent application related to this work. J. Aizawa has nothing to disclose. S.L. Taylor has nothing to disclose. G.B. Rogers has nothing to disclose. M. Govaerts has nothing to disclose. P. Cos has nothing to disclose. L. Ostyn has nothing to disclose. S. Sims has nothing to disclose. E. Vandeplassche has nothing to disclose. M. Sze has nothing to disclose. Y. Dondelinger has nothing to disclose. L. Vereecke has nothing to disclose. H. Van Acker has nothing to disclose. J.L. Simpson has nothing to disclose. L. Burr has nothing to disclose. A. Willems has nothing to disclose. M.M. Tunney has nothing to disclose. C. Cigana has nothing to disclose. A. Bragonzi has nothing to disclose. T. Coenye declares a patent application related to this work. A. Crabbé declares a patent application related to this work.

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