Supplemental figures and legends

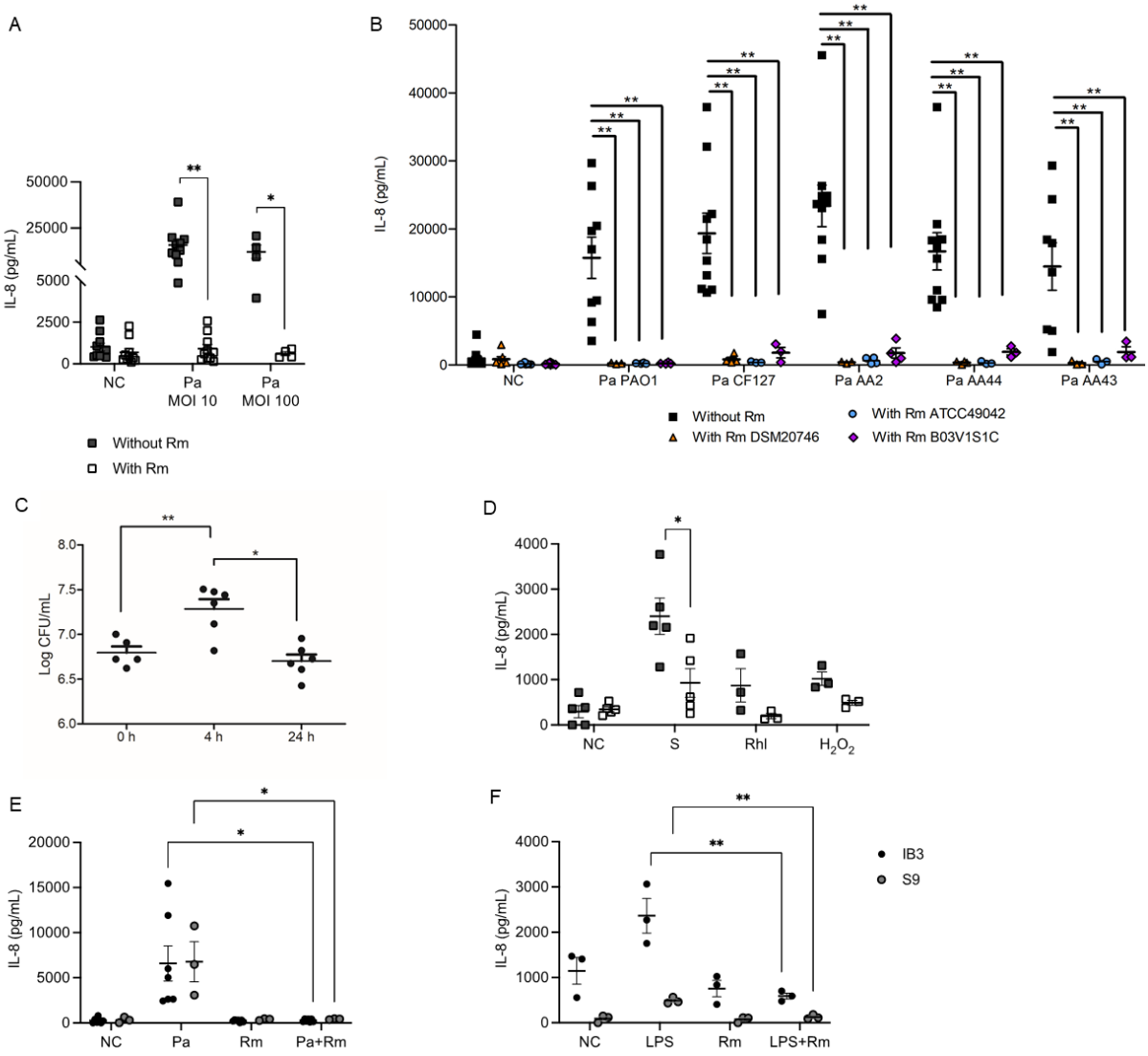

Figure S1. Influence of different *R. mucilaginosa* strains on the pro-inflammatory response of 3-D A549 cells to various *P. aeruginosa* strains and dosages. (A) IL-8 production by 3-D A549 cells after 4h exposure to single *P. aeruginosa* PAO1 culture at various MOI or to co-cultures of *P. aeruginosa* PAO1 with *R. mucilaginosa* DSM20746 at MOI 10:1. (B) IL-8 production by 3-D A549 cells after 4h exposure to various *P. aeruginosa* strains (PAO1, CF127, AA2, AA44, AA43) in single or co-cultures with various strains of *R. mucilaginosa* (DSM20746, ATCC49042, B03V1S1C) at MOI 10:1. (C) CFU/mL of *R. mucilaginosa* DSM20746 at the start of the exposure experiment (inoculum) and after 4h or 24h incubation with 3-D

A549 cells, using an MOI of 10:1. The total CFU/mL, i.e. associated with the 3-D A549 cells and in the surrounding liquid, is presented. (D) IL-8 production by 3-D A549 cells after 4h exposure to various pro-inflammatory stimuli (*S. aureus,* 100 µg/mL LPS, 100 µg/mL rhamnolipid (Rhl), 1 mM H_2O_2) alone or in co-culture with *R. mucilaginosa* at an MOI of 10:1. IL-8 production by 3-D bronchial epithelial CF cells (IB-3) or healthy (CFTR-corrected) 3-D bronchial epithelial cells (S9) after 4h infection with *P. aeruginosa* PAO1 in combination with *R. mucilaginosa* at MOI 10:1 (E) or after 24h exposure to 100 µg/mL LPS in combination with *R. mucilaginosa* at MOI 1:1 (F). Data represent the mean IL-8 concentration (pg/mL) ± SEM, n≥3, *p<0.05, ** p < 0.01

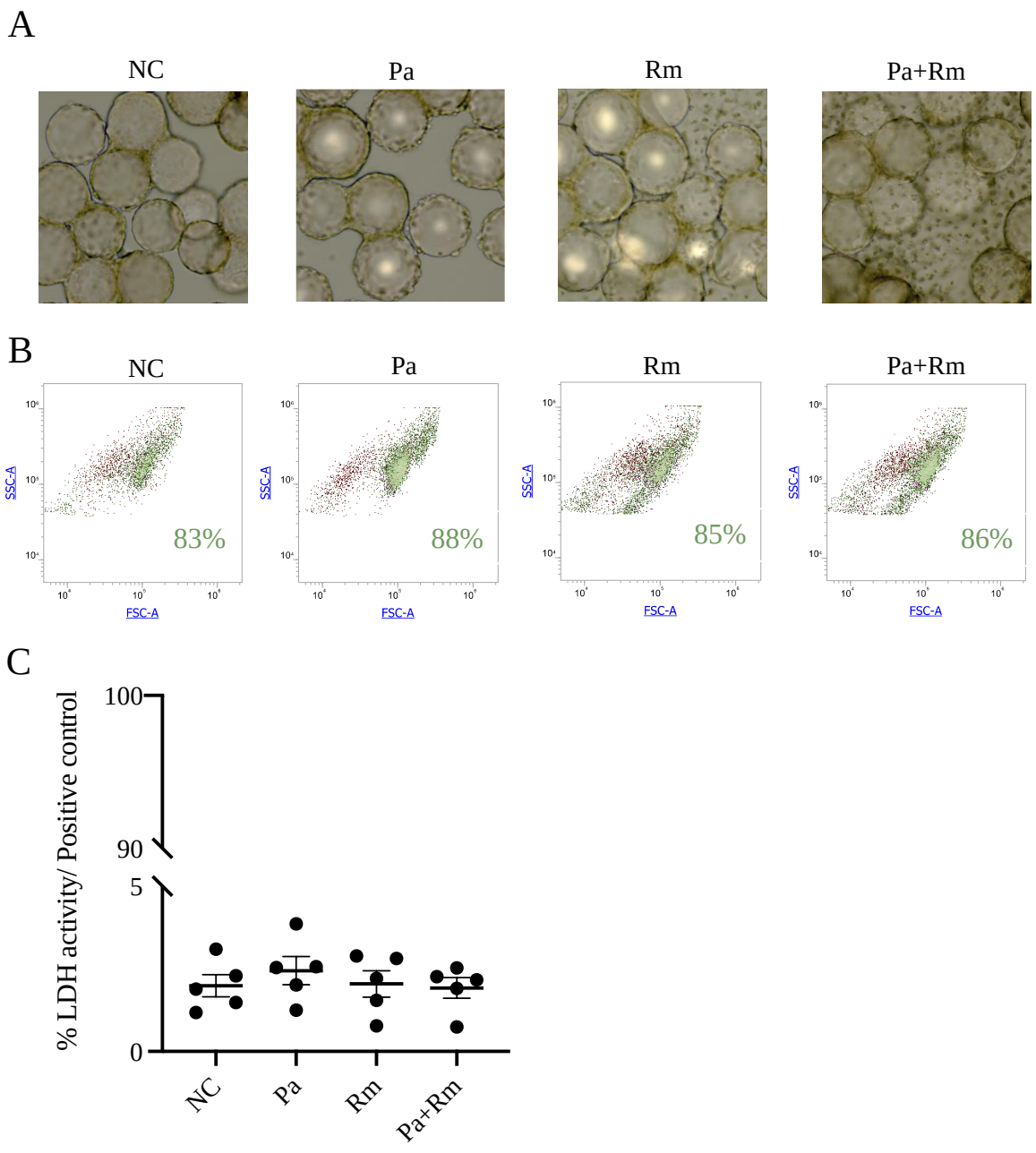

Figure S2. 3-D lung epithelial cell viability. (A) Light microscopy view (276X) of 3-D A549 epithelial cells. Large clusters of *R. mucilaginosa* cells are visible in single (Rm) and mixed (Pa+Rm) culture. (B) Annexin V/PI flow cytometry assay results. Viable cells are displayed in green, dead cells are displayed in red. (C) Percentage LDH activity versus positive control (i.e. lysed 3-D A549 cells). NC = negative control, uninfected 3-D A549 cells in serum-free GTSF-2 medium; Pa = 3-D A549 cells infected for 4 hours with *P. aeruginosa* PAO1; Rm = 3-D A549 cells infected for 4 hours with *R. mucilaginosa* DSM20746; Pa+Rm =3-D A549 cells infected for 4 hours with a co-culture of *P. aeruginosa* PAO1 and *R. mucilaginosa* DSM20746.

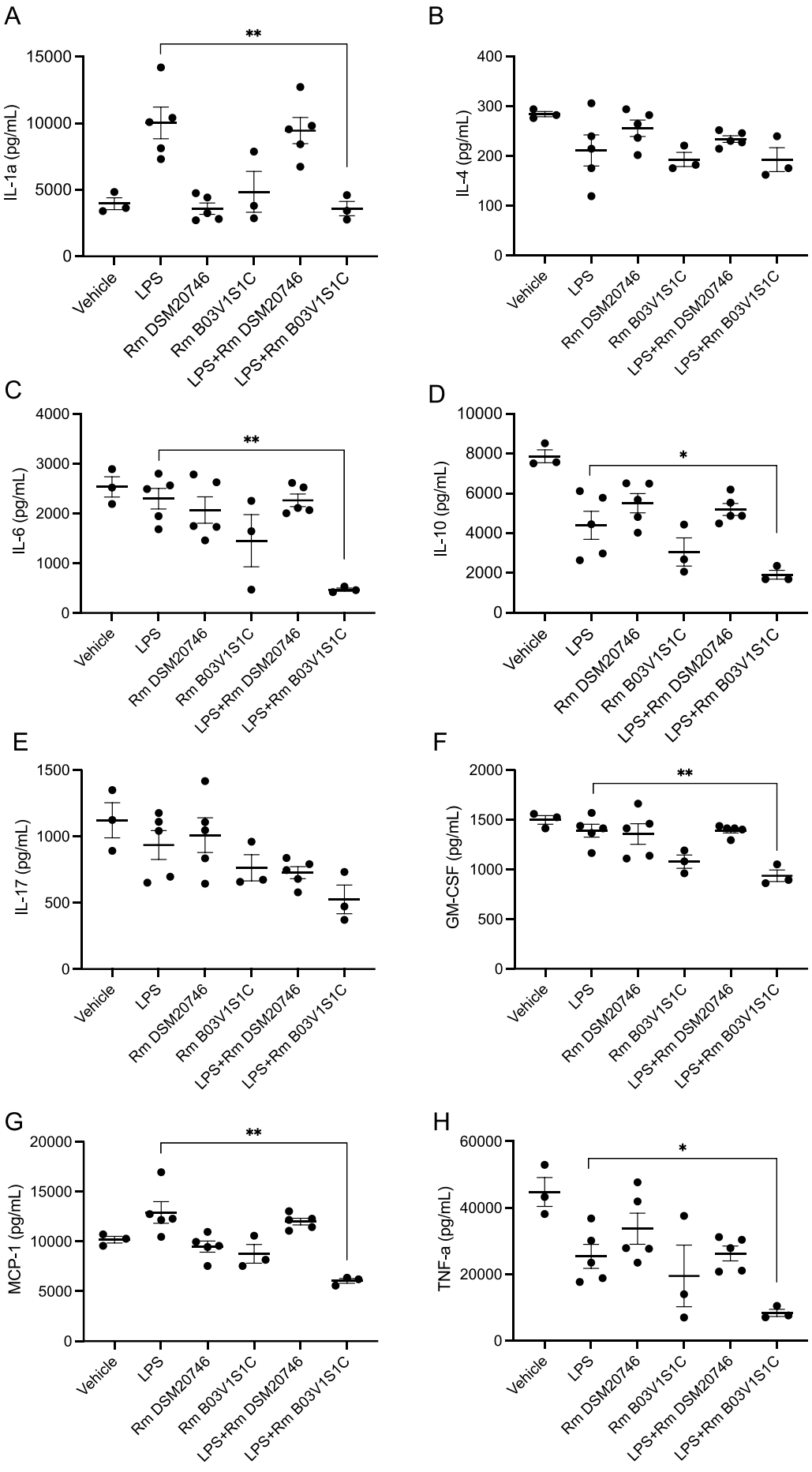
A
IL-1a (pg/mL)
15000
10000
5000
0
**
B
IL-4 (pg/mL)
400
300
200
100
0
C
IL-6 (pg/mL)
4000
3000
2000
1000
0
**
D
IL-10 (pg/mL)
10000
8000
6000
4000
2000
0
*
E
IL-17 (pg/mL)
1500
1000
500
0
F
GM-CSF (pg/mL)
2000
1500
1000
500
0
**
G
MCP-1 (pg/mL)
20000
15000
10000
5000
0
**
H
TNF-a (pg/mL)
60000
40000
20000
0
*
Vehicle
LPS
Rm DSM20746
Rm B03V1S1C
LPS+Rm DSM20746
LPS+Rm B03V1S1C

Figure S3. Influence of *R. mucilaginosa* on the *in vivo* cytokine production in response to LPS. Cytokine concentrations (measured by Bioplex) in mice lung homogenates after 48 h exposure to sterile beads (vehicle) (n=3), LPS (n=14), *R. mucilaginosa* suspension (n=5 for *R. mucilaginosa* DSM20746 and n=3 for *R. mucilaginosa* B03V1S1C) or a combination of LPS and *R. mucilaginosa* (n=14 for *R. mucilaginosa* DSM20746 and n=3 for *R. mucilaginosa* B03V1S1C). Data represent the mean cytokine concentration (pg/mL) ± SEM, n≥3, *p<0.05, **p<0.01. Vehicle = sterile alginate beads; LPS = 10µg/50µL; DSM20746 = *R. mucilaginosa* DSM20746; B03V1S1C = *R. mucilaginosa* B03V1S1C. Data in this figure were obtained in a single animal experiment.

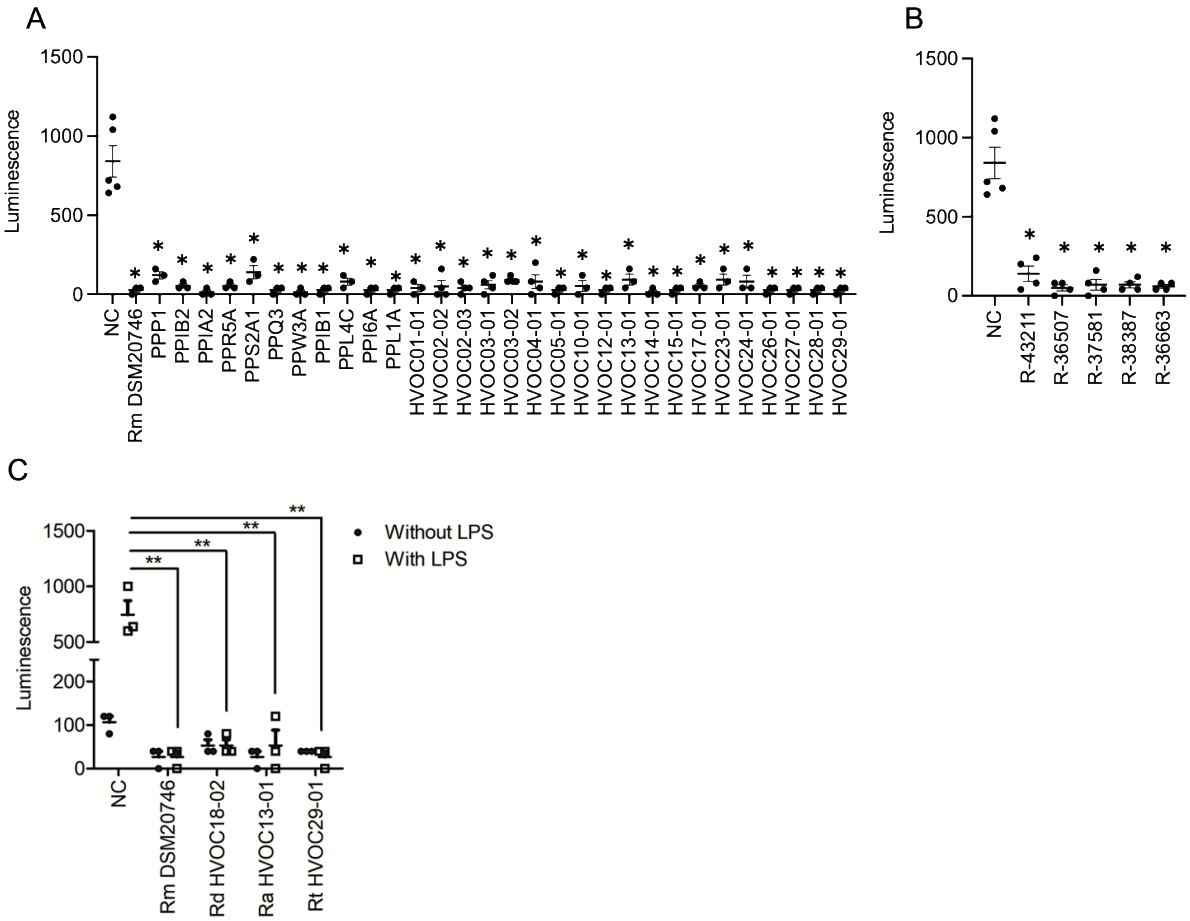

Figure S4. Anti-inflammatory effect of various *Rothia* species. Quantification of NF-κB activation by (A) *P. aeruginosa* PAO1 alone or in co-culture with various clinical or (B) environmental *Rothia* species or (C) by LPS alone or in co-culture with various *Rothi*a species under microaerobic conditions (measured by luminescence of a 3-D A549 reporter cell line). Data represent the mean luminescence ± SEM, n≥3, *p<0.05, **p<0.01 compared to *P. aeruginosa* PAO1 (A, B) or LPS (C).

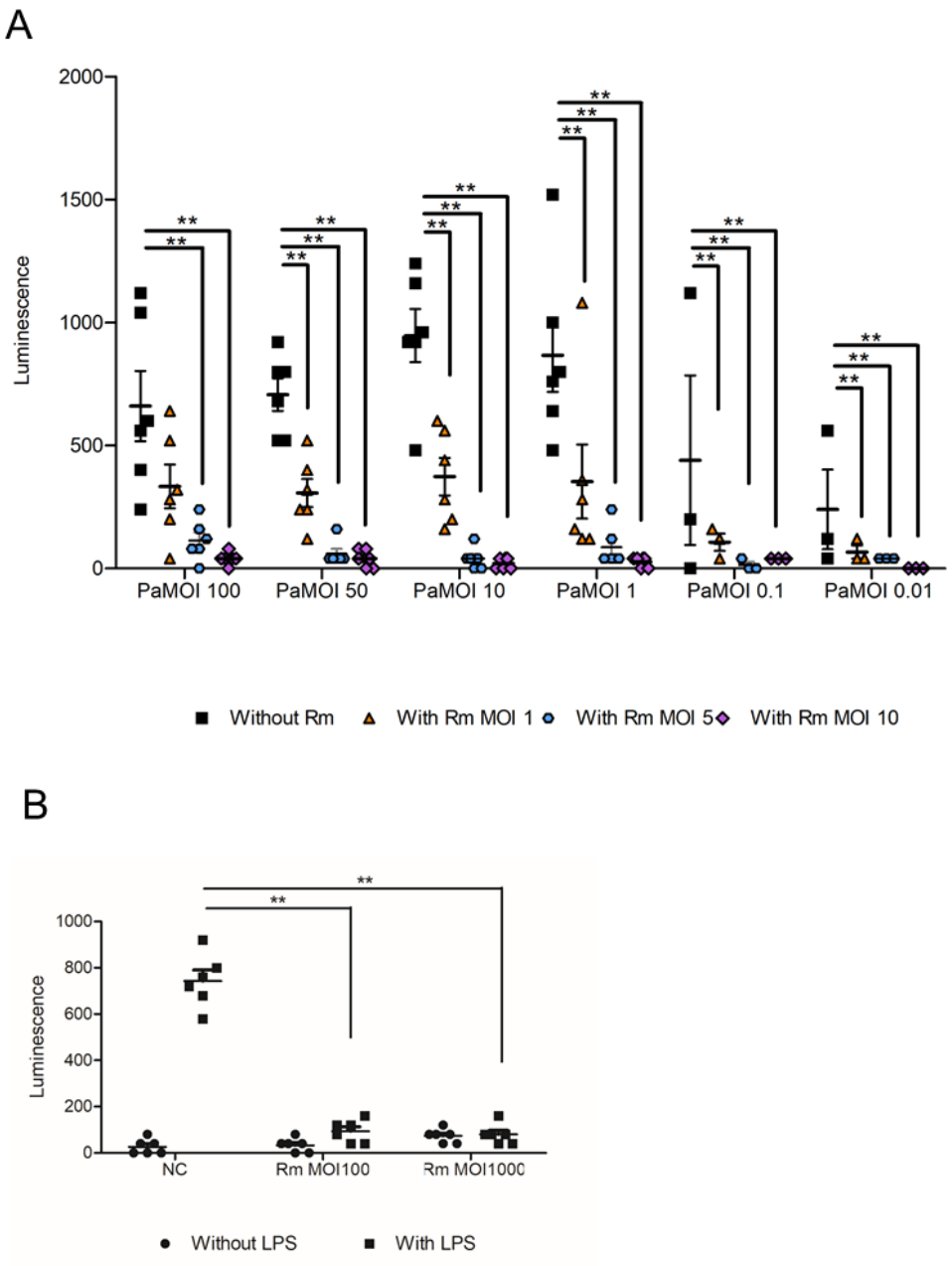

Figure S5. Minimal effective dose of *R. mucilaginosa*. Quantification of NF-κB activation in 3-D A549 cells (measured by luminescence of a 3-D A549 reporter cell line) after exposure to (A) various MOI of *P. aeruginosa* PAO1 alone or in co-culture with various MOI of *R. mucilaginosa* DSM20746, (B) various MOI of *R. mucilaginosa* DSM20746 with or without LPS. Pa = 3-D A549 cells infected for 4 hours with *P. aeruginosa* PAO1; Rm = 3-D A549 cells infected for 4 hours with *R. mucilaginosa* DSM20746; Data represent the mean luminescence ± SEM, n≥3, *p<0.05, **p<0.01.

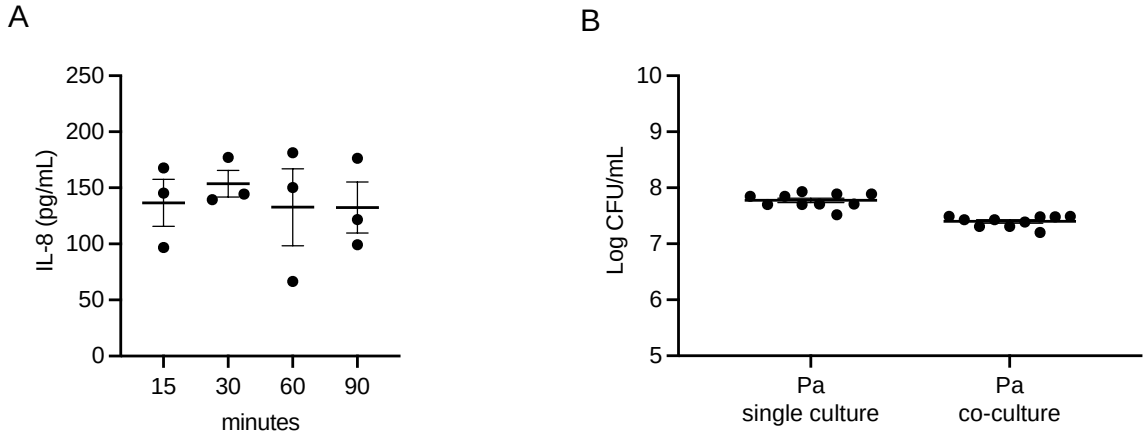

Figure S6. Effect of *R. mucilaginosa* on degradation of IL-8 and PAO1 adhesion to epithelial cells. (A) Concentration (pg/mL) of IL-8 over time after treatment with *R. mucilaginosa* DSM20746. (B) Adhesion (log CFU/mL) of *P. aeruginosa* PAO1 after 4h infection in single culture or in co-culture with *R. mucilaginosa* DSM20746. Data represent the mean IL-8 concentration (pg/mL) and mean Log CFU/mL ± SEM, n≥3.

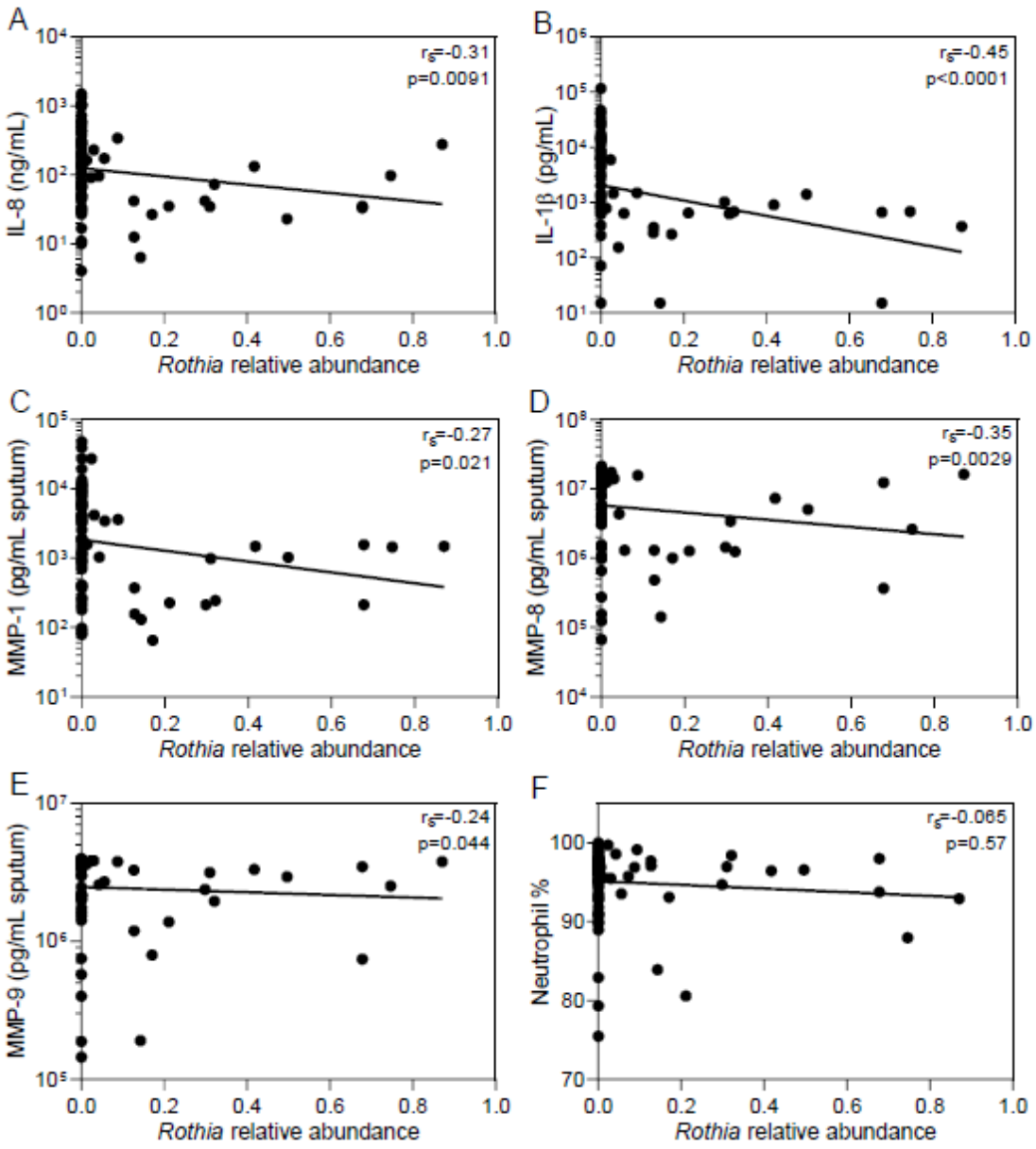

Figure S7. Correlation of relative abundance of *R. mucilaginosa* with pro-inflammatory parameters in induced sputum samples from bronchiectasis patients. (A) IL-8 (ng/mL), (B) IL-1β (pg/mL), (C) MMP-1 (pg/mL), (D) MMP-8 (pg/mL), (E) MMP-9 (pg/mL, (F) % neutrophils. Dots represent individual induced sputum samples. Correlation coefficients and p-values were calculated on ranked values and determined using a Spearman's correlation analysis.

Supplementary methods

***In vivo* mouse model.** To prepare the inoculum for intratracheal infection, *R. mucilaginosa* cryostock was thawed, incubated in 50 mL falcons with TSB broth for 18 h at 180 rpm and 37 °C. Three independent cultures of 50 mL were pooled to obtained the inoculum. The overnight cultures were centrifuged at 4,750 rpm. The overnight culture was centrifuged at 4,750 rpm. The supernatant was discarded, and 1.2 mL of the pooled pellet was resuspended and embedded in 12 mL of sterile seaweed alginate suspension at 1% filtered through 0.8 µm, 0.45 µm and finally 0.2 µm (Protanal LF 10/60 FT purchased from FMC Biopolymer). The suspension was placed in a 20 mL syringe and forced through a nozzle with a coaxial jet of air blowing to create alginate droplets (Nisco encapsulating unit VarJ30). The alginate droplets were collected in a solution of 0.1 M $CaCl_2$ Tris-HCl buffer (0.1 M, pH 7.0). After 1 h of stirring, the resulting <30 µm alginate beads were washed twice in 0.9% NaCl with 0.1 M $CaCl_2$. The number of bacteria embedded in the alginate beads was determined using plate counts on TSA. Before intratracheal challenge, mice were anesthetized with isoflurane (Halocarbon, Norcross, GA) and all efforts were made to minimize suffering. Anesthetized mice were inoculated with *R. mucilaginosa*-containing alginate beads in 50 µL PBS. The negative control consisted of empty beads produced in the same way as described but without bacteria inside, and the positive control (LPS) was added together with empty alginate beads. To evaluate if colonization of mouse lungs with *R. mucilaginosa* was achievable without being embedded in alginate beads, a preliminary experiment was performed using two 7 week old animals. Bacterial cultures were prepared as described above and a dose of 10^7 CFU/animal (in PBS) was administered intratracheally. Processing of left lungs for CFU determination resulted in colonization of one animal with 5 x 10^3 CFU/mL lung homogenate, while 0 CFU/mL could be recovered for the other animal.

Bronchiectasis cohort

Induced sputum samples were obtained from the Bronchiectasis and Low-dose Erythromycin Study (BLESS)[1], corresponding to baseline samples, prior to the trial intervention. Participants had the following inclusion and exclusion criteria and were considered to have moderate to severe bronchiectasis.

Bronchiectasis subject inclusion criteria

1. Able to provide written informed consent.
2. Confirmed diagnosis of bronchiectasis by HRCT within 3 years.
3. Airways obstruction on spirometry (ratio FEV1/ FVC <0.7) and FEV1 ≥25% predicted.
4. Chronic productive cough with at least 5 mLs sputum production per day.
5. At least two exacerbations of bronchiectasis requiring either oral or intravenous supplemental antibiotic therapy (of at least 7 days on each occasion) in the prior 12 months.
6. Aged 20-85 inclusive.
7. Clinically stable for at least four weeks (defined as no symptoms of exacerbation, no requirement for supplemental antibiotic therapy, and FEV1 within 10% of best recently recorded value where available).

Exclusion criteria

1. Bronchiectasis as a result of CF or focal endobronchial obstruction.
2. Currently active tuberculosis or non-tuberculous mycobacterial (NTM) infection. Subjects with evidence of prior pulmonary NTM infection could be included only if they have completed a course of therapy that is deemed successful on the basis of negative NTM cultures following cessation of therapy. All subjects required a negative NTM culture prior to screening.
3. Any symptoms or signs to suggest recent deterioration in respiratory disease, including exacerbation of pulmonary disease (as previously defined) in the preceding 4 weeks.

4. Any change to medications in the preceding 4 weeks.
5. Prescription of either oral or intravenous antibiotic therapy in the preceding 4 weeks.
6. Cigarette smoking within the preceding 6 months.
7. Any history of malignant arrhythmia (unless in the immediate post-myocardial infarction period and not requiring any regular therapy) or QTc prolongation on baseline ECG.
8. Any of the following within the three (3) months prior to enrolment:
    - Acute MI
    - Acute CVA
    - Major surgery
9. History of any of the following:
    - Active malignancy (excepting non-melanoma skin malignancies that have been treated and considered cured)
    - Listed for transplantation
    - Any other significant active illness likely to affect the patient's survival within 12 months
    - Receiving long-term domiciliary oxygen therapy
10. Allergy to macrolide antibiotics, other than minor, dose-related gastrointestinal intolerance that would not be anticipated to recur with low-dose erythromycin.
11. Any prescription or receipt of long-term macrolide antibiotics, or receipt of a treatment course within 4 weeks.
12. Predominant diagnosis of emphysema (rather than bronchiectasis) on HRCT scan of the chest.
13. Requirement for supplemental oxygen therapy.
14. Inability to complete required study procedures for whatever reason (including 6 minute walk test, hypertonic saline sputum induction).

15. Respiratory symptoms (including cough, sputum production, recurrent exacerbations) not predominantly the result of bronchiectasis in the opinion of the PI; where treatable causes for exacerbations existed, these were treated before considering trial enrolment.

Sputum induction procedure

Subjects were instructed to perform their usual chest physiotherapy regime on the morning of the sputum induction procedure. Prior to commencement of hypertonic saline inhalation, any spontaneous sputum expectorated was collected for standard culture. Sputum induction (SI) was performed after inhalation of 400 ug of albuterol, using 4.5% hypertonic saline nebulised from an ultrasonic nebuliser (output >1 mL/ min) for 20 minutes in 4 periods of 5 minutes each, according to the standardised protocol recommended by the European Respiratory Society taskforce [2]. Following mouth-rinsing and expectoration, sputum was collected following each nebulisation period, on each occasion preceded immediately by spirometry. The first sputum sample was refrigerated immediately following collection and frozen at -80 °C within an hour. A cold chain was maintained up until the point of DNA extraction.

Neutrophils in sputum as a percent of total non-squamous cells

Collected sputum was placed on ice immediately and transferred for processing within 60 minutes. Sputum was processed according to the methods of the US Cystic Fibrosis Therapeutics Development Network Standard Operating Procedure. Briefly, an equal volume of sterile 10% dithiothreitol (DTT) (Sputolysin; Calbiochem- Novabiochem Corp., San Diego, CA), was added to the sputum, then incubated in a shaking water bath at 37°C for 5-10 min, and gently mixed using a transfer pipette at 5-min intervals. A further three times the volume of both DTT and phosphate-buffered saline (Dulbecco's; Gibco BRL, Grand Island, NY) was added and the mixture incubated again in the 37°C shaking water bath for another 5-10 min.

Ten microliters of the homogenized sputum samples, mixed with Trypan Blue, was used to calculate total cell counts, using a standard hemacytometer. A further 0.25-0.50 ml of both samples was used to prepare cytospin slides for differential cell counts. After staining the slides with Wright's stain, 300 cells were counted and cell differentials calculated.

Pyrosequencing

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed as reported in a previous publication [3]. Primers: Gray28F 5'-TTTGATCNTGGCTCAG-3' and Gray519r 5'-GTNTTACNGCGGCKGCTG-3') were used to amplify the V1-V3 16S rRNA hypervariable region. Initial generation of the sequencing library involved a onestep PCR of 30 cycles, using a mixture of Hot Start and HotStar high fidelity Taq DNA polymerase. Tag-encoded FLX amplicon pyrosequencing analyses utilized Roche 454 FLX instrument with Titanium reagents, titanium procedures performed at the Research and Testing Laboratory (Lubbock, TX, USA) using RTL protocols (www.researchandtesting.com).

Sequence processing pipeline

The following information is as described in protocol documentation provided by Molecular Research DNA, Texas, USA (www.mrdnalab.com). Custom software written in C# within a MicrosoftH.NET (Microsoft Corp, Seattle, WA, USA) development environment was used for all post sequencing processing. Quality trimmed sequences obtained from the FLX sequencing run were derived directly from FLX sequencing run output files. Tags were extracted from the multi-FASTA file into individual sample-specific files based upon the tag sequence. Tags which did not have 100% homology to the sample designation were not considered. Sequences which were less than 150 bp after quality trimming were not considered. All failed sequence reads, low quality sequence ends and tags and primers were removed. Sequences with

ambiguous base calls, sequences with homopolymers > 6bp were removed, as were any non-bacterial ribosomal sequences and chimeras.

Individual samples were assembled using CAP3 after parsing the tags into individual FASTA files [4]. The ace files generated by CAP3 were then processed to generate a secondary FASTA file containing the tentative consensus (TC) sequences of the assembly along with the number of reads integrated into each consensus. TC were required to have at least 2-fold coverage.
To determine the identity of bacterial species in the remaining sequences, sequences were denoised, assembled into OTU clusters at 97% identity, and queried using a distributed .Net algorithm that utilizes Blastn+ (KrakenBLAST www.krakenblast.com) against a database of high quality 16S rRNA gene bacterial sequences. Using a .NET and C# analysis pipeline the resulting BLASTn+ outputs were compiled, data reduction analysis performed, and sequence identity classification carried out, as described previously [5]. The relative abundance of OTU clusters that align to Rothia were extracted and used for analysis.

References

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2. Paggiaro PL, Chanez P, Holz O, Ind PW, Djukanovic R, Maestrelli P, Sterk PJ. Sputum induction. *The European respiratory journal Supplement* 2002: 37: 3s-8s.

3. Rogers GB, Zain NM, Bruce KD, Burr LD, Chen AC, Rivett DW, McGuckin MA, Serisier DJ. A novel microbiota stratification system predicts future exacerbations in bronchiectasis. *Annals of the American Thoracic Society* 2014: 11(4): 496-503.

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5. Dowd SE, Wolcott RD, Sun Y, McKeehan T, Smith E, Rhoads D. Polymicrobial nature of chronic diabetic foot ulcer biofilm infections determined using bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP). *PloS one* 2008: 3(10): e3326.