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Aggregatibacter is inversely associated with inflammatory mediators in sputa of patients with chronic airway diseases and reduces inflammation in vitro

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Abstract

Background Chronic airway disease (CAD) is characterized by chronic airway inflammation and colonization of the lungs by pro-inflammatory pathogens. However, while various other bacterial species are present in the lower airways, it is not fully understood how they influence inflammation. We aimed to identify novel anti-inflammatory species present in lower airway samples of patients with CAD.

Methods Paired sputum microbiome and inflammatory marker data of adults with CAD across three separate cohorts (Australian asthma and bronchiectasis, Scottish bronchiectasis) was analyzed using Linear discriminant analysis Effect Size (LEfSE) and Spearman correlation analysis to identify species associated with a low inflammatory profile in patients.

Results We identified the genus *Aggregatibacter* as more abundant in patients with lower levels of airway inflammatory markers in two CAD cohorts (Australian asthma and bronchiectasis). In addition, the relative abundance of *Aggregatibacter* was inversely correlated with sputum IL-8 (Australian bronchiectasis) and IL-1β levels (Australian asthma and bronchiectasis). Subsequent in vitro testing, using a physiologically relevant three-dimensional lung epithelial cell model, revealed that *Aggregatibacter* spp. (i.e. *A. actinomycetemcomitans*, *A. aphrophilus*) and their cell-free supernatant exerted anti-inflammatory activity without influencing host cell viability.

Conclusions These findings suggest that *Aggregatibacter* spp. might act to reduce airway inflammation in CAD patients.

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Introduction

Overt inflammation drives disease progression in chronic airway diseases (CAD), such as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), severe asthma, and bronchiectasis [1–4]. One of the contributing factors to the pathogenesis of CAD is an elevated bacterial load in the lungs compared to healthy individuals, with the presence of conventional pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Achromobacter xylosoxidans* [5–10]. However, the airways of those with CAD also harbor other bacterial taxa that are not typically considered as respiratory pathogens [7, 11]. Associations have been reported between the characteristics of this CAD lung microbiome and disease severity, including the frequency of acute exacerbations, the level of airway inflammation, and mortality [12–16].

To gain more knowledge about the role of different members of the lung microbiome during inflammation, we previously isolated bacteria from sputum of people with CF and found that various microbiome members were able to reduce inflammation triggered by pro-inflammatory stimuli relevant for CF and COPD, either alone or as a consortium [17, 18]. Furthermore, recent reports have elucidated that several lung microbiome members are able to suppress inflammation induced by *P. aeruginosa* [19, 20]. Hence, these lung microbiome members might influence disease progression by acting to suppress airway inflammation.

While the above-described culture-dependent approach led to valuable insights on how the lung microbiome could potentially modulate chronic airway inflammation, it is limited to the study of more abundant and easily culturable species [21, 22]. Hence, culture-independent approaches could give complementary insights. In the present study, we aimed to identify potential anti-inflammatory species by comparing the sputum microbiome between individuals with CAD with either high or low levels of airway inflammation. Using this approach, we identified the *Aggregatibacter* genus as more abundant in respiratory samples with the lowest concentrations of pro-inflammatory mediators compared to samples with the highest levels of inflammation. Using a physiologically relevant three-dimensional (3-D) lung epithelial cell culture model, we then demonstrated that *Aggregatibacter* spp. (i.e. *A. actinomycetemcomitans*, *A. aphrophilus*) and their cell-free supernatant have anti-inflammatory activity in vitro.

Materials & methods

Study cohorts

Paired sputum microbiome data and inflammatory mediator levels was available for three adult CAD cohorts: one Australian severe asthma cohort (termed Aus-asthma), one Australian bronchiectasis cohort (termed Aus-bx), and one Scottish bronchiectasis cohort (termed Scot-bx). The Aus-asthma and Aus-bx cohorts were collected as baseline samples as part of a randomized controlled trial (ethics approval ACTRN12609000197235, registration date 20/04/2009, by the Hunter New England human research ethics committee and ACTRN12608000460303, registration date 14/07/2009, by the Mater Health Service human research ethics committee, respectively) [23, 24]. The Scot-bx cohort, comprised of clinically stable bronchiectasis patients, was subjected to a prospective observational study to associate microbiome characteristics with disease severity and long-term health outcomes (ethics approval 12/ES/0059 by the East of Scotland Research Ethics committee) [25]. All studies were conducted in accordance with the amended Declaration of Helsinki. Matching microbiome and inflammatory marker data was available for 101, 62 and 105 individuals from the Aus-asthma, Aus-bx, and Scot-bx cohorts, respectively. The inclusion and exclusion criteria, patient metrics, together with the methodology for sputum collection, inflammatory marker quantification, DNA extraction, 16S rRNA amplicon sequencing and bioinformatic analysis of the three cohorts was extensively described in previous papers [18, 23–27]. Differences in processing, sequencing, and analysis protocols across the three cohorts are listed in Supplementary Table A.

Bacterial strains and culture conditions

Two bacterial species of the genus *Aggregatibacter* (*A. actinomycetemcomitans* CCUG 56172 and *A. aphrophilus* CCUG 56235) and *P. aeruginosa* PAO1 (ATCC15692) were used. Pure cultures of *Aggregratibacter* spp. were grown on Vitox-enriched chocolate agar (Thermo Fisher Scientific, Waltham, MA, US) under microaerobic conditions (3% O_2, 5% CO_2 and 92% N_2) in a hypoxia chamber (Bactrox; Sheldon manufacturing Inc.) at 37 °C for 24 h. *P. aeruginosa* PAO1 was plated on Luria Bertani (LB) agar (Neogen, Lansing, MI, US) and incubated for 24 h at 37 °C under normoxic conditions (i.e. room air). Liquid overnight cultures of all species were made in Brain Heart Infusion broth (BHIB) (Neogen) and cultured at 37 °C under constant shaking (250 rpm) until stationary phase was reached. *Aggregatibacter* spp. were grown under microaerobic conditions ($<1\%$ O_2) (Oxoid CampyGen Compact Sachet; Thermo Fisher Scientific), and *P. aeruginosa* PAO1 was grown under normoxic conditions.

Three-dimensional lung epithelial cell culture model

A previously developed organotypic 3-D lung cell culture model of the A549 alveolar epithelial cell line (ATCC CCL185) or the NF-κB–luciferase-transfected A549 recombinant stable cell line (BPS Bioscience, San Diego, CA, US) was used that exhibits in vivo-like phenotypic

and functional properties of alveolar epithelial cells, including barrier function (localized expression of tight junctional markers), apical and basolateral polarity, and responds to infection in ways that are relevant to the infection process in vivo, including cytokine secretion [18, 28–30]. While the A549 cell line is derived from an adenocarcinoma and lacks many in vivo characteristics when grown as 2-D monolayers, when cultured in 3-D these cells downregulate cancer-specific markers (pan-keratin, cytokeratin 7 and vimentin) [29]. Briefly, the 3-D cell culture model was generated by growing monolayers of either cell line in T75 flasks at 37 °C, 5% CO_2. Cells were grown in GTSF-2 medium (HyClone, Logan, UT, US) supplemented with 1.5 g/L sodium bicarbonate (Sigma-Aldrich), 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA, US), 2.5 mg/L insulin transferrin sodium selenite (Lonza, Basel, Switzerland), and 1% penicillin-streptomycin (Sigma-Aldrich). When confluency was reached, 2×10^6 viable cells were transferred into a rotating wall vessel (RWV) bioreactor together with 0.25 g collagen I-coated microcarrier beads (Cytodex-3 microcarrier beads, Sigma-Aldrich) and cultured for 11–14 days with medium change on day 6 and 8. After 11 days, cell medium was changed to GTSF-2 medium without FBS and antibiotics. For host-microbe interaction experiments, 2.5×10^5 cells/well in GTSF-2 medium without FBS and antibiotics were transferred to a 96 well plate.

In vitro host-microbe interaction studies

Bacterial cultures were centrifuged (5000 rpm, 8 min) and resuspended in GTSF-2 without FBS before bringing them in contact with host cells. For experiments with cell-free supernatant of *Aggregatibacter* spp., cultures were centrifuged and subsequently filtered (0.22 µm). Next, 3-D cells were incubated with *Aggregatibacter* spp. at various targeted multiplicity of infection (MOI) ranging from MOI 50 to MOI 0.78 or 100 µL cell-free supernatant (in a total volume of 250 µL), in the presence or absence of pro-inflammatory stimuli (100 µg/mL lipopolysaccharide (LPS) (Sigma-Aldrich) or *P. aeruginosa* PAO1 at an MOI 10). Next, cultures were incubated under microaerobic conditions (3% O_2, 5% CO_2 and 92% N_2) in a hypoxia chamber (Bactrox; Sheldon manufacturing Inc.) for 4 h at 37 °C. After incubation, NF-κB pathway activation, cytokine measurement, cytotoxicity, and bacterial association to epithelial cells were assessed as described below.

NF-κB luciferase assay

3-D NF-κB–luciferase-transfected A549 cells were incubated with *Aggregatibacter* spp. or their cell-free supernatant and/or pro-inflammatory stimuli (LPS or *P. aeruginosa* PAO1) for 4 h. Afterwards, the One-Step Luciferase Assay System (BPS Bioscience) was used to determine the activation of the NF-κB-pathway, according to the manufacturer's protocol. Luminescence was measured with an EnVision microplate reader (Perkin Elmer, Waltham, MA, US). Luminescence values were expressed as a percentage of the signal obtained when cells were incubated with LPS or *P. aeruginosa* PAO1 only (for experiments with *Aggregatibacter* spp.) or BHIB with LPS or *P. aeruginosa* PAO1 (for experiments with cell-free supernatant of *Aggregatibacter* spp.). *Aggregatibacter* spp. were considered as anti-inflammatory when they significantly reduced the LPS or PAO1 triggered NF-κB-pathway activation at a certain MOI by 50% or more, without activating the NF-κB-pathway by itself (i.e. no significant difference between the uninfected control and cells cultured with *Aggregatibacter* sp. alone at a certain MOI) [17]. The minimal MOI (mMOI) is defined as the lowest MOI with anti-inflammatory activity [17].

Western blot

Western blot analysis of 3-D A549 cells incubated with *Aggregatibacter* spp. and/or pro-inflammatory stimulus (LPS, 100 µg/mL) was performed as described previously [18]. Mouse monoclonal antibodies were used to analyze levels of NFκB p65 (Santa Cruz Biotechnology, Inc), p-IκB-α (Cell signaling) and a goat polyclonal antibody was used to detect IκB-α (Santa Cruz Biotechnology, Inc) at concentrations recommended by the manufacturer. The following secondary antibodies were used: anti-mouse IgG HRP-linked Ab (Santa Cruz Biotechnology, Inc) or anti-goat IgG HRP-linked Ab (Santa Cruz Biotechnology, Inc). Western blot images are provided in the supplementary materials (Supplementary file_Western blot image).

Cytokine measurement

After incubation of 3-D A549 cells with *Aggregatibacter* spp. or their cell-free supernatant and/ or pro-inflammatory stimuli (LPS or *P. aeruginosa* PAO1), host cell spent medium was collected to quantify the concentration of secreted IL-8, IL-6, monocyte chemoattractant protein 1 (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF). The concentration of IL-8, IL-6, MCP-1 and GM-CSF was determined using the Human ELISA MAX Standard or Deluxe Set (BioLegend, San Diego, CA, US) according to the manufacturer's instructions. Values (pg/mL) were expressed as a percentage of the signal obtained when cells were incubated with LPS or *P. aeruginosa* PAO1 only (for experiments with *Aggregatibacter* spp.) or BHIB with LPS or *P. aeruginosa* PAO1 (for experiments with cell-free supernatant of *Aggregatibacter* spp.). *Aggregatibacter* spp. were considered as anti-inflammatory when they significantly reduced the LPS or PAO1 triggered cytokine production at a certain

MOI by 50% or more, without activating cytokine production by itself (i.e. no significant difference between the uninfected control and cells co-cultured with *Aggregatibacter* sp. alone at a certain MOI) [17]. The mMOI is defined as the lowest MOI with anti-inflammatory activity [17].

Cytotoxicity assay

After incubation of 3-D A549 cells with *Aggregatibacter* spp. or their cell-free supernatant and/ or pro-inflammatory stimuli (LPS or *P. aeruginosa* PAO1), cell viability was assessed using the lactate dehydrogenase (LDH) detection kit (Sigma-Aldrich). Since bacterial interference with LDH activity was previously reported, the intracellular LDH assay was used [31]. Cells were washed twice with Hank's Balanced Salt Solution (HBSS, Life Technologies), and cell lysate was obtained by vigorous pipetting with 1% Triton X-100 (Sigma-Aldrich). Next, the manufacturer's protocol was applied to determine LDH activity, and cell viability was expressed as a percentage of the lysed uninfected control. *Aggregatibacter* spp. were considered as cytotoxic for the host when host cell viability was lower than 80% and a statistically significant difference was obtained compared to the uninfected control ($p<0.05$) [32, 33].

Host association assay

Adherence of *Aggregatibacter* spp. to host cells in the presence or absence of LPS or *P. aeruginosa* PAO1 was determined as described previously [30]. Briefly, after incubation 3-D epithelial cell aggregates were transferred to a new 96-well plate, washed twice with HBSS (Life Technologies) and lysed using 1% Triton X-100 (Sigma-Aldrich). Host cell-associated *Aggregatibacter* spp. were quantified by plating on Vitox-enriched chocolate agar (Thermo Fisher) followed by microaerobic incubation, while host-associated *P. aeruginosa* PAO1 was quantified by plating on LB agar under aerobic conditions. For cultures containing both *Aggregatibacter* spp. and *P. aeruginosa* PAO1, lysate was plated on Vitox-enriched chocolate agar (Thermo Fisher) under anaerobic conditions (enabling selective growth of *Aggregatibacter* spp.) and on LB agar under aerobic conditions (enabling selective growth of *P. aeruginosa* PAO1).

Statistical analysis

The Linear discriminant analysis Effect Size (LEfSe) analysis was performed using version 1.1.1. The LEfSe algorithm uses class comparison, biological consistency tests, and effect size estimation to identify metagenomic biomarkers [34]. In that way, differences between two or more biological conditions can be discovered. Default LEfSe parameters were applied, which included an LDA cut-off of 2 and one-against-all strategy. For each cohort, patient samples below or above the 25th (Q1) and 75th (Q3) percentiles (based on inflammatory marker concentrations) were assessed. These 25th and 75th percentiles were obtained by ordering patients samples based on their sputum inflammatory marker data (i.e. IL-8, IL-1β, TNF-α or elastase concentrations). Hence, a low and high inflammation group was designated for every cohort and for every inflammatory marker for comparison in the LEfSe analysis.

Other statistical analyses were performed using IBM SPSS Statistics (Version 28). All experiments were done at least in biological triplicate. The Shapiro-Wilk test was used to evaluate normal distribution of the data. For normally distributed data, a one-sample t-test (NF-κB luciferase assay, cytokine measurement and LDH assay) or an independent sample t-test (bacterial host association assay) was applied. When data was not normally distributed, data analysis was performed using a Wilcoxon signed-rank test (NF-κB luciferase assay, cytokine measurement and LDH assay) or a Mann-Whitney U test (bacterial host association assay). Benjamini-Hochberg correction was applied to control for multiple testing, and statistical significance was considered at $p<0.05$ after correction [35]. For Western blot band intensity statistical analysis, a one-way ANOVA was used for normally distributed data. For data that was not normally distributed, the Kruskal-Wallis one-way ANOVA was used. Post-hoc analysis was done with the Bonferroni and Dunnet methods. For correlation analyses between the relative abundance of *Aggregatibacter* spp. and sputum inflammatory marker concentrations, Spearman's test (r_s) were applied to determine the correlation coefficients and p-values, and statistical significance was considered at $p<0.05$.

Results

Multiple bacterial taxa inversely associate with inflammation in patient sputa

To identify lung microbiome members that could be associated with a lower inflammatory profile in patients with CAD, LEfSe was applied for each study cohort (i.e. Aus-bx, Aus-asthma, Scot-bx) and each sputum inflammatory marker (i.e. IL-8, IL-1β, TNF-α, elastase) (Fig. 1). Various genera (i.e. 32 in total) were more abundant in patients with a lower inflammatory profile (25th percentile) in one or more of the cohorts tested (e.g. *Aggregatibacter*, *Gemella*, *Lactobacillus*, *Prevotella*, *Rothia*, *Streptococcus*, *Veillonella*). Other genera were only associated with a high inflammatory profile (75th percentile) in at least one of the tested cohorts (i.e. *Cardiobacterium*, *Eikenella*, *Pseudomonas*). Contradictory results were obtained for some genera as they were more abundant in the low or high inflammation group depending on the tested cohort or inflammatory marker (i.e. *Actinomyces*,

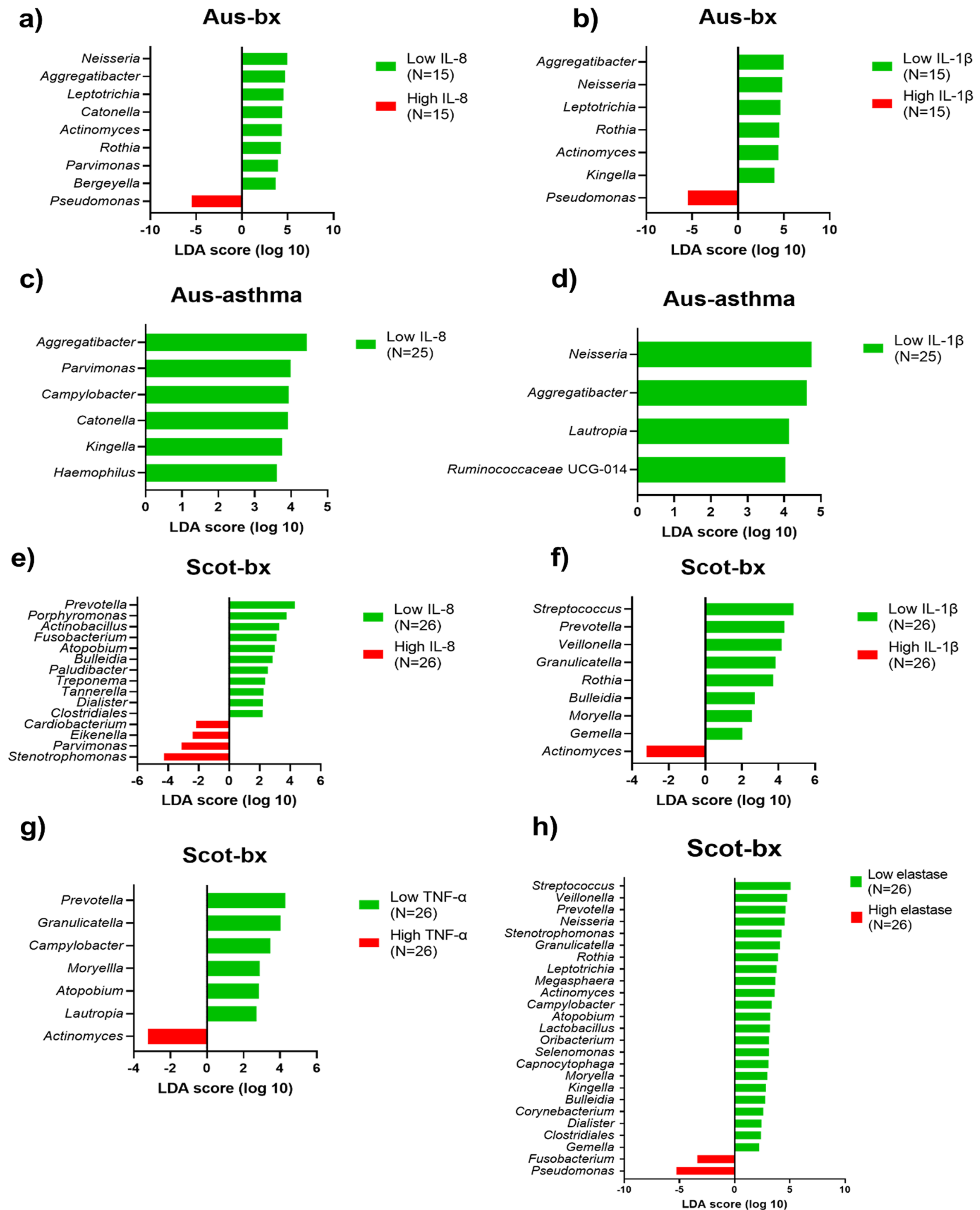

Fig. 1 LEfSe analysis of lung microbiome members in the low (25th percentile, Q1) and high inflammation subgroups (75th percentile, Q3) of **a**) the Aus-bx cohort based on IL-8, **b**) the Aus-bx cohort based on IL-1β, **c**) the Aus-asthma cohort based on IL-8, and **d**) the Aus-asthma cohort based on IL-1β, **e**) the Scot-bx cohort based on IL-8, **f**) the Scot-bx cohort based on IL-1β, **g**) the Scot-bx cohort based on TNF-α, **h**) the Scot-bx cohort based on elastase. The LEfSe plots show the microbial genera with significant differences between the low inflammation group (green) and high inflammation group (red). The lengths of the bar column represents the LDA score

Fusobacterium, *Parvimonas*, *Stenotrophomonas*). Interestingly, the genus *Aggregatibacter* – together with *Rothia*, *Neisseria* and *Prevotella* – was most frequently associated with a lower inflammatory profile in CAD patients, across all eight comparisons (Supplementary Figure A). We focused on *Aggregatibacter* for further study since (i) prior research already described the anti-inflammatory activity of *Rothia* and *Prevotella* [17–20, 36] and the association of *Neisseria* with exacerbations in bronchiectasis patients as well as cytotoxicity in 3-D lung epithelial cells [17, 37], and (ii) *Aggregatibacter* was consistently associated with low inflammation across two different cohorts (Aus-bx and Aus-asthma).

Next, an alternative approach was used to investigate the observed relationship between *Aggregatibacter* relative abundance and inflammatory marker concentrations. Using Spearman rank correlation, the relative abundance of *Aggregatibacter* was also found to be inversely correlated with sputum IL-8 and IL-1β in the Aus-bx cohort, whereas in the Aus-asthma cohort, a significant negative correlation was observed for IL-1β. Also for the Scot-bx cohort, a non-significant trend towards a negative association between *Aggregatibacter* relative abundance and elastase was observed (r_s=-0.178, p=0.07) (Fig. 2).

Aggregatibacter was detected in 72/101 (71%), 24/62 (39%), and 42/105 (40%) individuals for the Aus-asthma, Aus-bx and Scot-bx cohorts, respectively. The mean relative abundance of *Aggregatibacter* was 14% (±15%), 11% (±18%) and 0.03% (±0.09%) for the analyzed samples of the Aus-asthma, Aus-bx and Scot-bx cohorts, respectively (Supplementary Figure B). To understand whether the high relative abundance of *Aggregatibacter* in certain cohorts could be attributed to saliva contamination, squamous epithelial cell levels (available for the Aus-asthma cohort) were considered in the analysis as high levels of squamous epithelial cells (>20%) are indicators of saliva contamination of the lower airway sample [38, 39]. Hence, LEfSe analysis of the Aus-asthma cohort was performed again, but this time after exclusion of 13 samples with >20% squamous epithelial cells (Supplementary Figure C). *Aggregatibacter* was still present in the low inflammation group of this cohort and for all tested inflammatory markers, suggesting that the high relative abundance of *Aggregatibacter* in the Aus-asthma cohort was most likely not due to saliva contamination.

In vitro evaluation of the anti-inflammatory activity of *Aggregatibacter* spp

Since LEfSe and Spearman correlation analyses of different CAD cohorts revealed an association between the abundance of *Aggregatibacter* spp. and a low inflammatory profile, we explored if this genus has anti-inflammatory properties in vitro. To this end, a physiologically relevant 3-D lung epithelial cell culture model was exposed to *Aggregatibacter* spp. alone or in the presence of LPS at different MOIs. Inflammation was evaluated based on NF-κB-pathway activation and IL-8 production. Both tested *Aggregatibacter* spp. did not induce inflammation in 3-D lung epithelial cells (Fig. 3). *A. actinomycetemcomitans* reduced LPS-triggered inflammation at a MOI of 12.5 (mMOI) and at all higher MOIs based on both IL-8 production and NF-κB-pathway activation. For *A. aphrophilus* a mMOI of 3.13 was needed to reduce NF-κB-pathway activation, whereas a mMOI of 50 decreased IL-8 production. Results on the inhibition of NF-κB-pathway activation by *Aggregatibacter* spp. were validated by Western blot analysis of several NF-κB-pathway mediators (i.e. p65, IκB-α and p-IκB-α) for *A. actinomycetemcomitans* (tested at MOI 50). In the presence of the pro-inflammatory stimulus LPS, *A. actinomycetemcomitans* significantly decreased the levels of p65 and showed a consistent but non-significant trend in decreasing IκB-α levels as well, confirming the involvement of the NF-κB-pathway in the anti-inflammatory effect of *Aggregatibacter* spp. (Fig. 3). Finally, the cytotoxicity of *Aggregatibacter* spp. was evaluated as well as its association to 3-D lung epithelial cells. Both species were non-cytotoxic and no significant differences in adherence to the host cells with and without LPS were observed (Supplementary Figure D).

Aggregatibacter spp. reduce various pro-inflammatory cytokines triggered by LPS or *P. aeruginosa* PAO1 in a 3-D lung epithelial cell model

Since both *Aggregatibacter* spp. reduced LPS-triggered NF-κB pathway activation and IL-8 production without cytotoxicity in the 3-D cell culture model, we evaluated if the release of other pro-inflammatory cytokines (i.e. IL-6, MCP-1 and GM-CSF) was diminished as well. In addition, we evaluated anti-inflammatory activity when 3-D cells were stimulated with the common respiratory pathogen *P. aeruginosa* (based on the release of IL-8, IL-6, MCP-1, GM-CSF and NF-κB pathway activation). This evaluation was done at two different MOIs (i.e. 50 and 12.5). Both *A. actinomycetemcomitans* and *A. aphrophilus* at a MOI of 50 reduced the production of IL-8, IL-6, MCP-1 and NF-κB pathway activation when either LPS or *P. aeruginosa* were used as pro-inflammatory stimulus (Fig. 4). *A. actinomycetemcomitans* (MOI 50) also reduced GM-CSF production after stimulation with LPS or *P. aeruginosa*. A lower MOI (12.5) of both species did not reduce *P. aeruginosa*-triggered inflammation by >50% (i.e. defined threshold for anti-inflammatory activity) for neither NF-κB pathway activation or cytokine production. However, when using LPS as a trigger, the lower MOI of *A. actinomycetemcomitans* (12.5) had anti-inflammatory activity based on all measured outcomes (i.e. IL-8, IL-6, MCP-1, GM-CSF, NF-κB

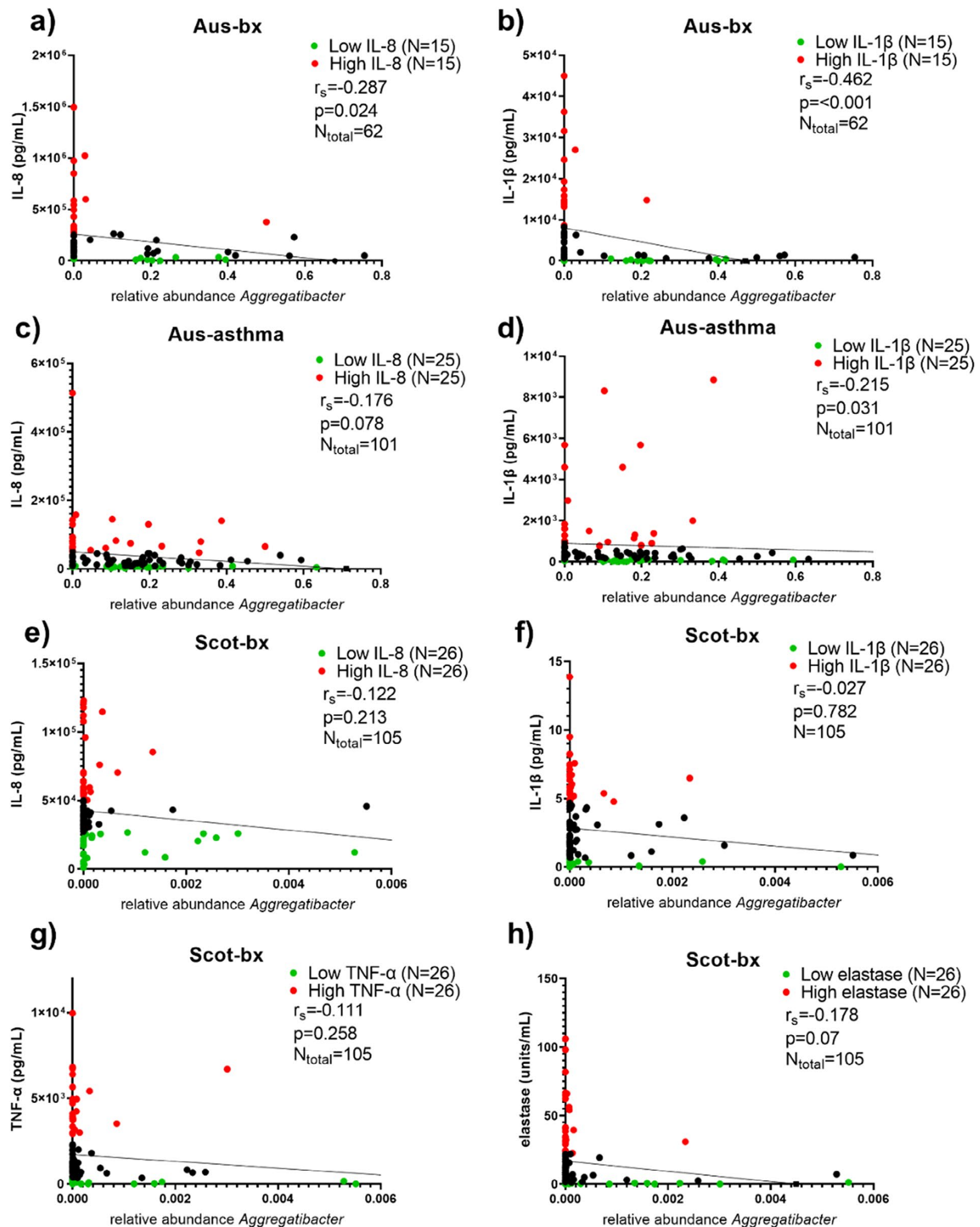

Fig. 2 Correlation of the relative abundance of *Aggregatibacter* with the concentration of pro-inflammatory cytokines in sputum samples of bronchiectasis patients from the Aus-bx cohort [**a**) IL-8, **b**) IL-1β], and adults with asthma from the Aus-asthma cohort [**c**) IL-8, **d**) IL-1β], and bronchiectasis patients from the Scot-bx cohort [**e**) IL-8, **f**) IL-1β, **g**) TNF-α, **h**) elastase]. On the vertical axis the sputum concentration (pg/mL) of the pro-inflammatory mediator is shown (IL-8, IL-1β, TNF-α, elastase), on the horizontal axis the relative abundance of *Aggregatibacter* is depicted. Data points represent individual sputum samples, and in correspondence with Fig. 1, individual sputum samples that were included in the high or low inflammation group are marked in red or green, respectively. Correlation coefficients (r_s) and p-values were calculated by Spearman's test

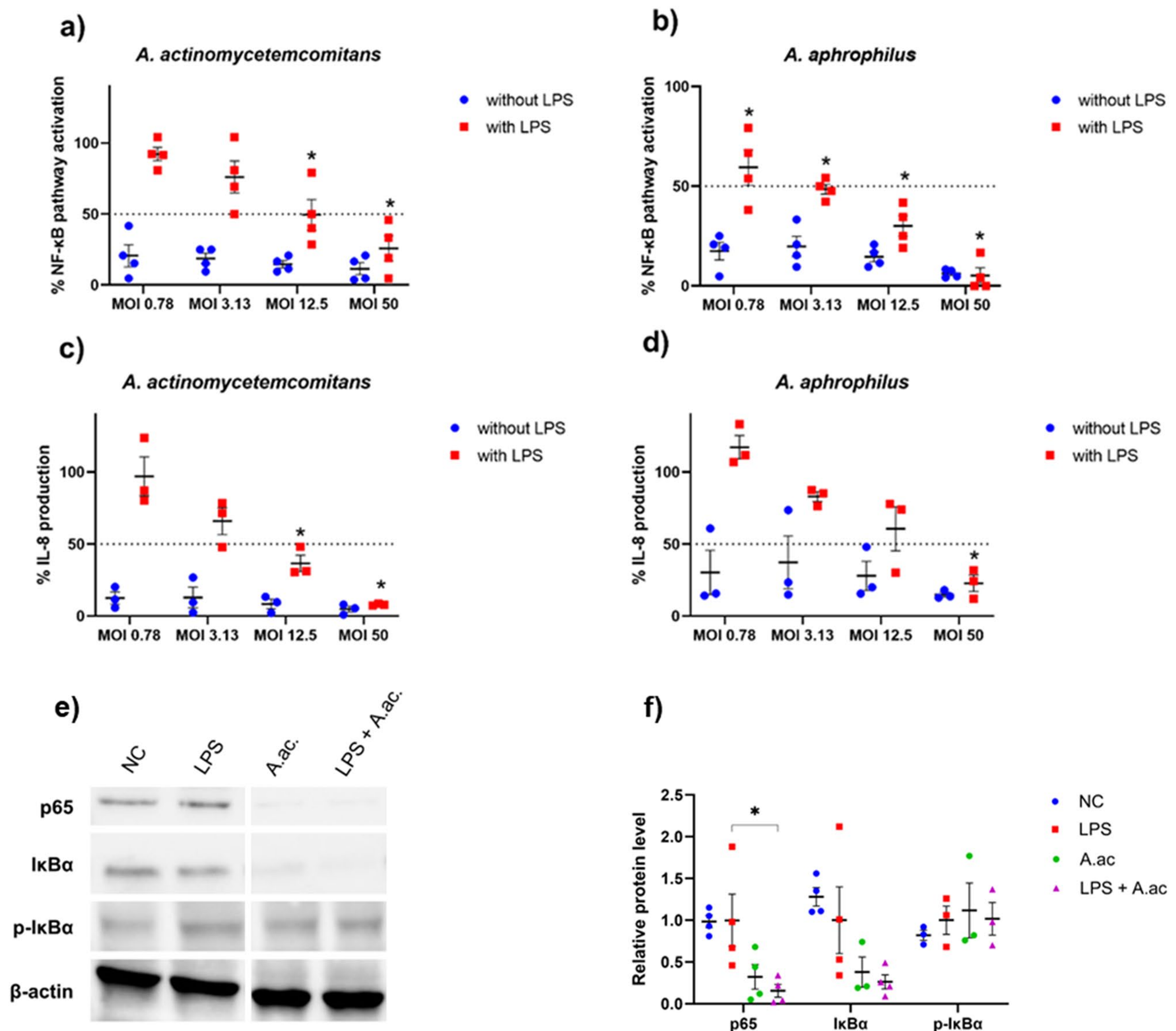

Fig. 3 Determination of the minimal effective MOI (mMOI) of **a**) *A. actinomycetemcomitans* and **b**) *A. aphrophilus* based on NF-κB pathway activation and determination of the mMOI of **c**) *A. actinomycetemcomitans* and **d**) *A. aphrophilus* based on IL-8 production in 3-D lung epithelial cells. On the vertical axis % NF-kB pathway activation / IL-8 production compared to LPS-stimulated cells is shown, on the horizontal axis the tested MOI is depicted. The mMOI was defined as the lowest MOI with anti-inflammatory activity (i.e. < 50% NF-kB pathway activation / IL-8 production compared to LPS-stimulated cells as visualized by a dotted line, $p < 0,05$). **e**) Western blot analysis of proteins (i.e. IκBα, p65 and phosphorylated (p)-IκBα) produced by 3-D A549 cells stimulated with LPS in the presence or absence of *A. actinomycetemcomitans* (targeted MOI 50:1) for 4 h (representative replicate is shown, image contains cropped blot – the full original blot is shown in Supplementary file_Western blot image). **f**) Band intensity (normalised to β-actin) of Western blot at 4 h of 3-D A549 cells stimulated with LPS with/without *A. actinomycetemcomitans*. Results are expressed as a percentage of the positive control (i.e. LPS). NC: negative control (untreated 3-D A549 cells in serum-free GTSF-2 medium), A.ac. *A. actinomycetemcomitans*. Data represent mean ± SEM, * $p < 0.05$, $n \geq 3$

pathway activation), whereas *A. aphrophilus* could only reduce MCP-1 secretion and activation of the NF-κB pathway. No significant production of IL-8, IL-6, and MCP-1 or NF-κB pathway activation was observed upon exposure of *Aggregatibacter* spp. to 3-D lung epithelial cells (Supplementary Figure E). Only *A. aphrophilus* significantly induced GM-CSF production at both MOIs. Finally, cytotoxicity and bacterial association to 3-D lung epithelial cells incubated with *Aggregatibacter* spp. and *P. aeruginosa* PAO1 was assessed (Supplementary Figure F). No cytotoxicity was observed, and adhesion to the host cells by either *Aggregatibacter* or *P. aeruginosa* PAO1 was not influenced by culturing these species together.

Supernatant of *Aggregatibacter* spp. has anti-inflammatory activity

Next, we evaluated whether the cell-free supernatant of *Aggregatibacter* spp. could reduce LPS- and/or

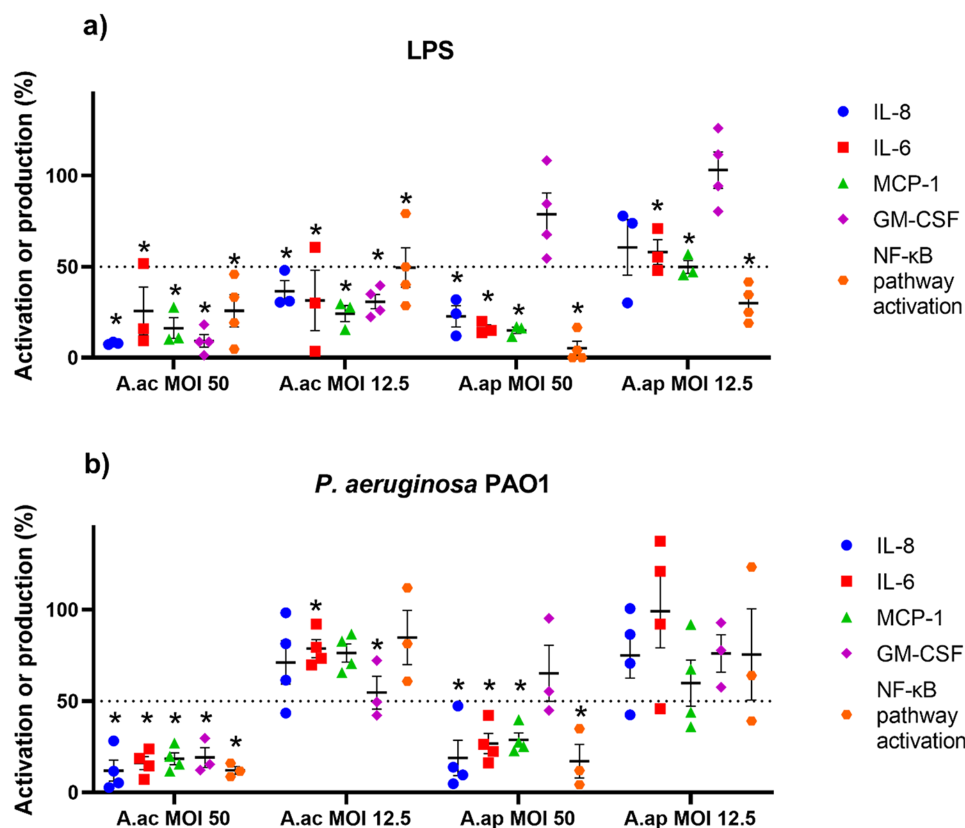

Fig. 4 Effect of *A. actinomycetemcomitans* (A.ac) and *A. aphrophilus* (A.ap) at MOI 50 and MOI 12.5 on the production of IL-8, IL-6, MCP-1, GM-CSF and NF-κB pathway activation by 3-D lung epithelial cells with **a**) LPS and **b**) *P. aeruginosa* PAO1 as pro-inflammatory stimulus. On the vertical axis % activation (NF-kB pathway) / production of cytokines (IL-8, IL-6, MCP-1, GM-CSF) compared to cells stimulated with LPS or *P. aeruginosa* only is shown. On the horizontal axis, the tested MOI is depicted. Anti-inflammatory effects were defined as a significant reduction of NF-kB pathway activation / cytokine production compared to cells stimulated with only LPS / *P. aeruginosa* as visualized by a dotted line, $p<0.05$. * $p<0.05$, $n \geq 3$

PAO1-triggered inflammation in the 3-D lung cell culture model (Fig. 5). Anti-inflammatory activity was again assessed based on different inflammatory outcomes (i.e. IL-8, IL-6, MCP-1, GM-CSF and NF-κB pathway activation). When 3-D cells were stimulated with LPS, all inflammatory outcomes were significantly reduced by at least 50% (i.e. defined threshold for anti-inflammatory activity) for both *Aggregatibacter* spp. ($p<0.05$). Only for *A. actinomycetemcomitans*, the reduction in IL-8 production and NF-κB pathway activation was 44% and 49%, respectively ($p<0.05$). Similar results were obtained when *P. aeruginosa* PAO1 was used as pro-inflammatory stimulus, except for GM-CSF production which was not significantly inhibited by either *Aggregatibacter* spp. In the absence of a pro-inflammatory stimulus, the supernatant of both *A. actinomycetemcomitans* or *A. aphrophilus* did not significantly induce cytokine production or NF-κB pathway activation (Supplementary Figure G). No cytotoxicity of the cell-free supernatant was detected (Supplementary Figure H).

Discussion

In this study, we identified the genus *Aggregatibacter* to be associated with a less severe inflammatory profile in two out of three CAD study cohorts by using a culture-independent approach. We further confirmed that this genus exhibits anti-inflammatory properties in a physiologically relevant 3-D lung epithelial cell model.

Aggregatibacter is a relatively new genus that was classified in 2006 to encompass *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus* and *Haemophilus segnis* [40]. Recently, *Aggregatibacter kilianii* was added to this genus [41]. *Aggregatibacter* spp. are common members of the oral microbiome, but are also reported in low abundance in the lung microbiome of patients with lung disease [41–50]. *Aggregatibacter* spp. are also known to be involved in infections such as periodontitis, endocarditis or empyema processes [51, 52]. Lung infections with *Aggregatibacter* spp. are rare with some case reports mentioning an *A. actinomycetemcomitans* pulmonary infection [53]. These patients often had periodontal complications, which could contribute to the initiation of the lung infection [53–56].

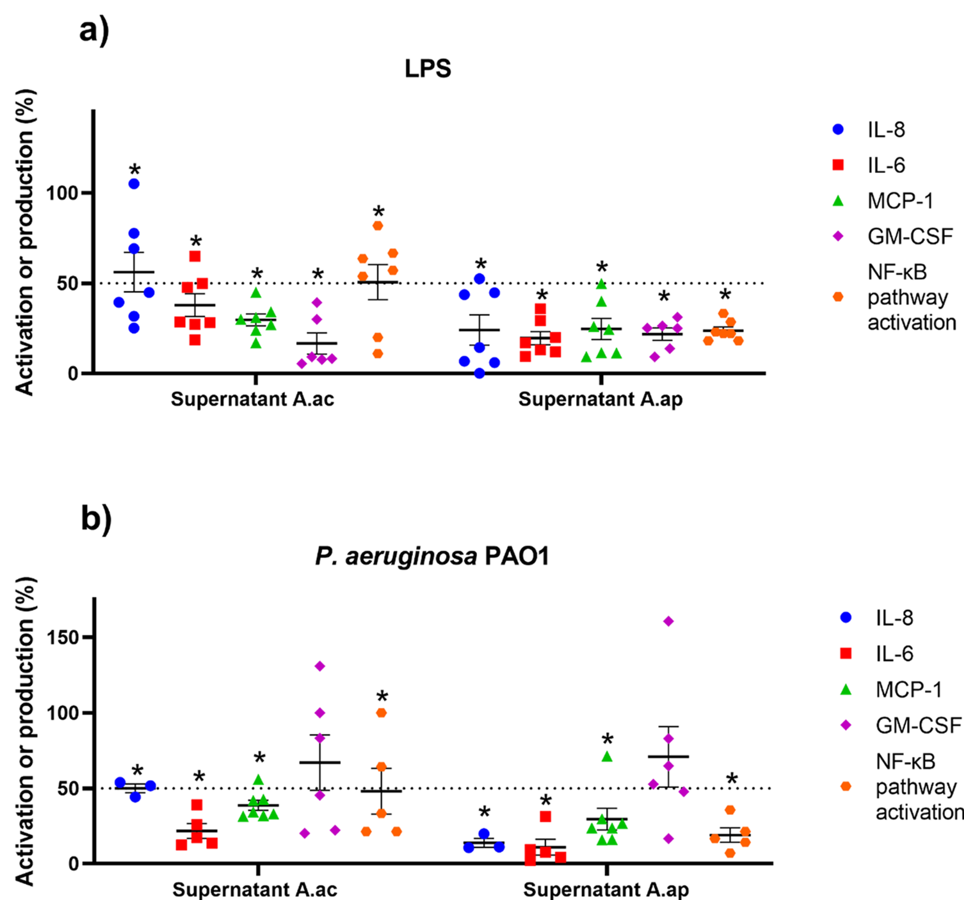

Fig. 5 Effect of the cell-free supernatant of *A. actinomycetemcomitans* (A.ac) and *A. aphrophilus* (A.ap) on the production of IL-8, IL-6, MCP-1, GM-CSF and NF-κB pathway activation by 3-D lung epithelial cells with **a**) LPS and **b**) *P. aeruginosa* PAO1 as pro-inflammatory stimulus. On the vertical axis % activation (NF-kB pathway) / production (IL-8, IL-6, MCP-1, GM-CSF) compared to cells stimulated with LPS or *P. aeruginosa* only is shown. On the horizontal axis, the tested conditions are depicted. Anti-inflammatory effects were defined as a significant reduction of NF-kB pathway activation / cytokine production compared to cells stimulated with only LPS / *P. aeruginosa* as visualized by a dotted line, $p < 0.05$. * $p < 0.05$, $n \geq 3$

Aggregatibacter was associated with a lower inflammatory profile in the Aus-asthma and Aus-bx cohorts based on both LEfSe and Spearman's correlation analyses. However, LEfSe results from the Scot-bx cohort did not identify *Aggregatibacter* as a discriminator between low and high inflammation groups for all studied inflammatory mediators. A possible reason for this discrepancy is the difference in the mean relative abundance of *Aggregatibacter* between the cohorts. The Aus-asthma and Aus-bx cohorts had a higher mean relative abundance [14.4% (±14.5%) and 10.6% (±17.6%) respectively] compared to the Scot-bx cohort [0.03% (±0.09%)]. The difference in relative abundance between cohorts could potentially be explained by the DNA extraction method [57], analyzed variable region for 16 S rRNA amplicon sequencing [58, 59], or the databases used for assigning taxonomy [60]. Also geographic differences, resulting in possible distinct lung microbiome profiles between Australian (Aus-asthma and Aus-bx) and British (Scot-bx) cohorts [61, 62], could potentially contribute to the difference in relative abundance [61, 62].

The extent to which *Aggregatibacter* spp. are present in the lower airways of people with CAD remains difficult to determine since contamination with the oropharynx during expectoration is possible [25]. However, studies that have used methods to minimize oropharyngeal sampling contamination, for example bronchoalveolar lavage fluid (BAL), have still isolated *Aggregatibacter* spp [63], highlighting the likely presence of these organisms in the lower airways. In this study, efforts were made to minimize contamination during sample collection for each cohort and our results were also consistent when removing samples with high squamous cell counts (i.e. Aus-asthma cohort), which is indicative for salivary contamination [38, 39].

Most lung microbiome studies where *Aggregatibacter* relative abundance is reported are for patients with lung cancer, tuberculosis or pneumonia, and a mean relative abundance of <1 −2% was found [46–50] – which is in

line with the Scot-bx cohort in the present study. One study reported the relative abundance of *Aggregatibacter* spp. in COPD patients as being approximately 5%, which is in the same order of magnitude as the two other cohorts analyzed in the present study (Aus-Bx, Aus-asthma) [64]. As mentioned above, different processing, sequencing and analysis protocols might influence the relative abundance between study cohorts [57–60]. Besides *Aggregatibacter*, various other genera were linked to a lower inflammatory profile in CAD patients in the present study. From these genera, *Rothia*, *Gemella*, *Prevotella* and *Streptococcus* were previously reported to exert immunomodulatory properties in vitro and/or in vivo [17, 20, 65]. In our study, *Neisseria* was associated with a lower inflammatory profile, a finding that contrasts with a previous study where *Neisseria* was proposed as a pathobiont in patients with bronchiectasis [37]. This previous study analyzed both Asian and European patients, with the lung microbiome being most often dominated by *Neisseria* spp. in the Asian population. Demographic differences in *Neisseria* spp. abundance could potentially account for the observed discrepancy between studies. Moreover, in the study by Li et al.., *N. subflava* was further investigated in vitro and in a mouse model of acute infection, and a pro-inflammatory response was observed for this particular species. Hence, it remains to be investigated whether other *Neisseria* species may modulate the immune response differently, which could be another contributing factor to the observed differences between the study of Li et al.. and the present study.

Anti-inflammatory activity of *Aggregatibacter* was confirmed in an in vitro 3-D cell culture model. For this study, we used an *A. actinomycetemcomitans* strain that originated from a patient with periodontitis since no lung isolates were available. The *A. aphrophilus* strain was derived from the lung of a 66 year old woman. In vitro analysis was done under microaerobic conditions (3% O_2, 5% CO_2 and 92% N_2), which closely mimics the environment of the diseased lung since low oxygen is present in viscous mucus [66], and most of the CF pathogens such as *P. aeruginosa* (used in this study) can rely on aerobic or anaerobic respiration [67]. We observed that *Aggregatibacter* spp. could inhibit NF-κB pathway activation and pro-inflammatory cytokine production after stimulation with LPS or *P. aeruginosa* without cytotoxicity towards host cells. It should however be noted that, while cytokine production or NF-κB pathway activation were not induced upon exposure of 3-D lung epithelial cells to *A. actinomycetemcomitans*, *A. aphrophilus* significantly induced GM-CSF production (but not NF-κB pathway activation or other cytokines) in the absence of a pro-inflammatory trigger. This finding, together with the ability of *Aggregatibacter* to cause infections in and outside the respiratory context, should carefully be considered in understanding and defining its contribution to the net inflammatory profile of patients with CAD. In this regard, identifying the bioactive compound(s) in the supernatant of *Aggregatibacter* spp. will provide key insights on how exactly they interact with the host. While the present study indicated that *Aggregatibacter* spp. secrete anti-inflammatory compounds, a previous study that was performed in the context of periodontal infections found that the outer membrane lipoprotein (BilRI) of *A. actinomycetemcomitans* LPS binds IL-8, thereby modulating the host immune response by removing the chemotactic IL-8 gradient in the junctional tooth epithelium [68]. As a result, neutrophil migration is impacted which facilitates periodontal infection. In this particular disease context, cytokine sequestering is used for pathogenicity of *A. actinomycetemcomitans*, yet attenuation of excessive neutrophil infiltration might be beneficial in the context of chronic airway inflammation [69]. Furthermore, cytokine sequestering can also be achieved by secreted proteins, as described for uropathogenic *Escherichia coli* [70]. This demonstrates that the attenuation of NF-κB pathway activation and cytokine production can take place through various mechanisms, and either by membrane-bound or secreted compounds.

Furthermore, synergistic anti-inflammatory interactions between different genera of the lung microbiome could potentially contribute to the net inflammatory profile of CAD patients [17]. Although this interaction was not the subject of the present study, *Aggregatibacter* spp. could act in synergy with other anti-inflammatory lung microbiome members, hereby balancing the lung microbiome towards an anti-inflammatory community. In particular, this hypothesis could be relevant for CAD cohorts with very low *Aggregatibacter* abundance where the anti-inflammatory effect of this genus on its own is anticipated to be limited.

This study has several limitations. Firstly, the observed inverse correlation between the abundance of *Aggregatibacter* and inflammatory mediators does not provide insights about the causality of host-microbe interactions in disease onset, progression or severity. To this end, longitudinal studies wherein microbiome composition, microbial anti-inflammatory effectors (that remain to be identified) and patient inflammatory outcomes are monitored with time may provide further insights. Secondly, the adopted in vitro experimental set-up in this study focused on the ability of *Aggregatibacter* spp. to inhibit the host inflammatory response when the pro-inflammatory stimulus and anti-inflammatory species were added simultaneously to the in vitro model system (preventative interaction). Hence, further experimentation is needed to understand whether *Aggregatibacter* spp. can modulate the host inflammatory response once established (curative interaction). Thirdly, this study used a 3-D in

vitro model of an adenocarcinoma cell line (A549) to demonstrate the anti-inflammatory properties of *Aggregatibacter* spp. While the used 3-D model exerts in vivo-like characteristics, it is currently unknown whether this or any other available model faithfully mimics the in vivo response to anti-inflammatory species in patients with chronic airway diseases. Various in vitro and ex vivo model systems are available to study host-microbe interactions in the lung environment (including the air liquid interface model with primary cells, organoids, precision cut lung slices), yet model validation is a key gap that remains to be addressed in the respiratory field to support rational model selection for tackling a specific research question [71].

Taken together, *Aggregatibacter* was identified as an anti-inflammatory genus in the lung microbiome of patients with CAD and could contribute – together with other pro- and anti-inflammatory microbial mediators - to the inflammatory outcome and disease progression in this patient population.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-024-02983-z.

Supplementary Material 1

Supplementary Material 2

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Author contributions

E.G.: formal analysis, investigation, writing (original draft). S.L.T.: methodology, investigation, writing (review and editing). A.D.: investigation, writing (review and editing). L.a.B.: investigation, writing (review). M.W.: investigation, writing (review). M.J.: methodology, writing (review and editing). E.V.B.: funding acquisition, writing (review and editing). J.L.S.: resources, writing (review and editing). L.B.: resources, writing (review and editing). J.D.C.: resources, writing (review and editing). G.B.R.: resources, writing (review and editing). A.C.: conceptualization, writing (review and editing), supervision, funding acquisition.

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Data availability

For Aus-asthma, reads have been deposited in the EBI ENA (PRJEB71587). For Aus-bx, reads have been deposited in the NCBI SRA (SRA128000). For Scot-bx reads have been deposited in the NCBI SRA under the BioProject Numbers PRJNA539959 and PRJNA548310.

Declarations

Ethics approval and consent to participate

The Aus-asthma cohort received ethics approval ACTRN12609000197235 (registration date 20/04/2009) by the Hunter New England human research ethics committee. The Aus-bx cohort received ethics approval ACTRN12608000460303 (registration date 14/07/2009) by the Mater Health Service human research ethics committee. The Scot-bx cohort received ethics approval 12/ES/0059 by the East of Scotland Research Ethics committee. For Aus-asthma, the Human ethics and consent to participate was provided by the Hunter New England human research ethics committee. For Aus-bx, the Human ethics and consent to participate was provided by the Mater Health Service human research ethics committee. For Scot-bx, patients gave written informed consent and the study was approved by the East of Scotland Research Ethics committee (12/ES/0059).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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