Airway surface liquid from smokers promotes bacterial growth and biofilm formation via iron-lactoferrin imbalance

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Abstract

Background: Smoking is a leading cause of respiratory infections worldwide. Tobacco particulate matter disrupts iron homeostasis in the lungs and increases the iron content in the airways of smokers. The airway epithelia secrete lactoferrin to quench iron required for bacteria to proliferate and cause lung infections. We hypothesized that smokers would have increased bacterial growth and biofilm formation via iron lactoferrin imbalance.

Methods: We collected bronchoalveolar lavage (BAL) samples from non-smokers and smokers. We challenged these samples using a standard inoculum of *Staphylococcus aureus* and *Pseudomonas aeruginosa* and quantified bacterial growth and biofilm formation. We measured both iron and lactoferrin in the samples. We investigated the effect of supplementing non-smoker BAL with cigarette smoke extract (CSE) or ferric chloride and the effect of supplementing smoker BAL with lactoferrin on bacterial growth and biofilm formation.

Results: BAL from smokers had increased bacterial growth and biofilm formation compared to non-smokers after both *S. aureus* and *P. aeruginosa* challenge. In addition, we found that samples from smokers had a higher iron to lactoferrin ratio. Supplementing the BAL of non-smokers with cigarette smoke extract and ferric chloride increased bacterial growth. Conversely, supplementing the BAL of smokers with lactoferrin had a concentration-dependent decrease in bacterial growth and biofilm formation.

Conclusion: Cigarette smoking produces factors which increase bacterial growth and biofilm formation in the BAL. We propose that smoking disrupts the iron-to-lactoferrin in the airways. This finding offers a new avenue for potential therapeutic interventions to prevent respiratory infections in smokers.

Background

Respiratory infections are one of the leading causes of mortality worldwide and smoking is considered a risk factor for developing upper and lower respiratory infections [1–3]. In addition, exposure to cigarette smoke is associated with increased risk of airway bacterial colonization compared to non-smokers [4, 5].

The pathogenesis of infectious airway disease caused by cigarette smoke is complex. Smoking damages airway epithelia, increases mucus production, decreases mucociliary clearance, impairs cell immunity and the production of antimicrobial peptides and proteins (AMPs) in the airway [6–11]. Besides impairing the host response to infectious challenges, cigarette smoke can also affect bacterial virulence [12].

The airway surface liquid (ASL) is a layer of fluid covering the airways that is a first line of defense responsible for antimicrobial activity against airborne pathogens. One of the most abundant AMPs present in the ASL is lactoferrin, a bacteriostatic protein that chelates iron, which is required for bacteria to grow and form biofilms [13]. The impairment of AMP activity plays a fundamental role in the origin of infectious lung diseases. Several factors can alter the activity of AMPs such as decreased pH, increased
Tobacco contains particulate matter and multiple chemicals that can potentially alter the iron homeostasis in the lungs [19, 20]. Since iron promotes bacterial growth and biofilm formation and inhibits AMP activity against pathogens, we hypothesized that the ASL from smokers would grow more bacteria and develop more biofilm compared to non-smokers [21–23]. Repeated respiratory infections in smokers influences the development of chronic inflammation and lung function decline leading to chronic obstructive pulmonary disease (COPD) [24, 25].

We have chosen *Staphylococcus aureus* and *Pseudomonas aeruginosa* as models to study relevant culturable airway pathogens. They are both representative airway Gram positive and negative pathogens. *S. aureus* colonizes the nostrils of smokers in a higher prevalence than non-smoking population [26]. This carrier state has been associated with an increased risk of lethal infections by endogenous strains [27]. *Pseudomonas aeruginosa* is a pathogen present in the airways of patients with COPD at both baseline and during exacerbations [28]. This organism is more prevalent in severe COPD and is associated with poor clinical outcomes in hospitalized patients [29, 30]. Therefore, it is relevant to determine mechanisms implicated in increased risk of respiratory infections in smokers.

Sampling ASL is extremely challenging as it is present at a very small volume in the lungs [31]. Therefore, we have used bronchoalveolar lavage as a surrogate of ASL. Bartlett et al. examined the protein composition of bronchoalveolar lavage (BAL) and ASL from new born pigs and found they had 514 protein in common, including AMPs such as lactoferrin, lysozyme and cathelicidins [32]. We challenged the BAL from smokers and non-smokers with bacteria and assessed growth and biofilm formation.

We also investigated the effect of supplementing iron, cigarette smoke extract, and lactoferrin to explore the role of iron in the bactericidal and anti-biofilm properties of the airway.

**Methods**

**Human BAL collection and processing**

We used biobank-stored BAL samples from non-smokers (*n* = 11) and smokers (*n* = 11) from the study Human Lung Responses to Respiratory Pathogens that aimed to study the relationship of vitamin D levels and the innate defense of the lung against inhaled bacteria. Participants were selected if they were between the age 18–60, if smoker, FEV1 had to be more than 60% of predicted. Participants characteristics were similar between smokers and non-smokers (Table 1). Participants were excluded if they had history of positive tuberculin test or tuberculosis, pneumonia, recent airway infections, antibiotic use or vaccination, were taking vitamins or medications with selected exceptions, were pregnant, breast-feeding, had asthma, diabetes, heart disease or allergy to lidocaine. More detailed inclusion and exclusion criteria for this study were previously published [33]. The collection was approved by the Institutional Review Board at the University of Iowa (IRB# 200607708). BAL collection was performed as previously described [33]. Briefly, after subjects signed an informed consent, and pregnancy was ruled out using a urine test, participants were premedicated with atropine (0.6 mg intramuscularly (IM)), and either morphine (10 mg IM) or meperidine (12.5–25 mg IM). The airways were locally anesthetized using 2–4% lidocaine. A pulmonary physician performed the bronchoscopies by a standard procedure using a flexible bronchoscope (model P160 or P180; Olympus) at the University of Iowa Hospitals and Clinics (Iowa City, Iowa, USA). Under clinical monitoring, five BAL samples (20 mL) were suctioned into a trap container from three segments of the lungs. The liquid in the collection traps was transferred into conical 50 mL centrifuge tubes. Tubes were centrifuged to separate cells from the rest of the BAL. The supernatant of the tubes was thawed at once on ice, aliquoted into working samples, and stored again at −80 °C. The selection of flasks that was retrieved from the biobank were thawed at once on ice, aliquoted into working samples, and stored at −80 °C. Working samples were thawed and used once for every experiment to avoid multiple freeze-thaw cycles.

**Assessment of BAL bacteriostatic effect on bacteria**

To assess bacterial growth, we used bioluminescent *Pseudomonas aeruginosa* Xen 05 and *Staphylococcus aureus* Xen 29 (Caliper Biosciences, USA). It has been reported that Relative Light Units (RLU) correlate closely with Colony Forming Units (CFU) [16]. Briefly, *P. aeruginosa* Xen 05 was cultured overnight in tryptic soy broth (TSB) and then subculture in iron-free media M9 (BD Difco®, USA) overnight at 37 °C, and then washed twice with Phosphate Buffered Saline without calcium and magnesium (PBS−/−). Thereafter, we combined 100 μL of BAL samples with 10 μL of bacteria (~ 5 × 10⁵ CFU) in a 96-well plate (Optiplate-96, Perkin Elmer, USA). We measured RLU (527 nm) 6 h after bacterial challenge as a surrogate of live bacteria.

| Table 1 Comparison of participant characteristics by smoking status |
|---------------------------------------------------------------|
| Characteristics  | Smokers | Non-Smokers | p-value |
| Age mean         | 32.8 (11) | 37.7 (13)  | 0.3881 |
| Male gender (%)  | 54      | 81          | NA     |
| Recovery rate (%)| 77.3 (10.5) | 70.7 (16.4) | 0.2542 |

Data expressed as mean and standard deviation.
S. aureus Xen 29 was cultured overnight in TSB. The next day we washed a subculture of mid-log phase bacteria, twice with PBS/−/. Because S. aureus does not grow in M9 iron-free media, we resuspended in minimal essential media (10 mM Sodium phosphate buffer, pH 7.4, 100 mM NaCl, 1% TSB). Thereafter, we combined 10 μL of BAL samples with 10 μL of bacteria (~5 × 10⁵ CFU) in a Optiplate-96 and measured RLU 30 min after challenge as a surrogate for live bacteria.

To test the effect of lactoferrin supplementation on bacterial growth, we combined 10 μL of increasing concentrations of recombinant lactoferrin from human milk (10, 30, 100, or 300 μg/mL, final concentration) (Sigma-Aldrich) or phosphate buffer control with 90 μL of the BAL for 30 min at 37 °C. Subsequently, we added 10 μL of bacteria (~5 × 10⁵ CFU) and measured RLU at 30 min for S. aureus and 6 h for P. aeruginosa after bacterial challenge.

Assessment of biofilm formation
We investigated the formation of biofilms in the BAL from smokers and non-smokers using two methods. For S. aureus we used a microtiter dish biofilm formation assay as previously described [34]. Briefly, we combined 10 μL of BAL with 190 μL of bacteria Xen 29 (~5 × 10⁵ CFU) suspended in minimal media in a 96 well plate. After 48 h, we extracted the liquid, washed the wells with distilled water, stained the biofilm using concanavalin-A conjugated with Texas-red. The resulting mixture was filtered through a 0.22 μm filter unit (EMD Millipore). CSE was made fresh for each experiment using the OD₃₂₀ to standardize the solution. An OD₃₂₀ equal to 1.00 corresponded to 100% CSE.

Cigarette smoke extract (CSE) production
We produced the CSE as previously described with modifications [35]. We filled a 50 mL syringe with 10 mL of media and inserted the filter end of a research cigarette from the University of Kentucky (Code 3R4F) into the wide end of a 1000 μL pipette tip. We lit the cigarette and the narrow end of the pipette was placed into the tip of the 60 mL syringe. The syringe plunger was pulled smoothly to aspirate smoke from the cigarette, through the pipette tip, and into the syringe and mixed with the media by vigorous shaking. We repeated this process of smoke aspiration and media/smoke mixing until one cigarette was consumed. The resulting mixture was filtered through a 0.22 μm filter unit (EMD Millipore). CSE was made fresh for each experiment using the OD₃₂₀ to standardize the solution. An OD₃₂₀ equal to 1.00 corresponded to 100% CSE.

Bacterial growth in the presence of CSE alone
For S. aureus, we combined 50 μL of increasing concentrations of CSE solutions in PBS −/− (0.1, 0.3, 1, or 3%) with 150 μL of ~500 CFUs of log phase Xen 29 S. aureus bacteria in sodium phosphate buffer (10 mM, 100 mM NaCl, 1% TSB) in a round bottomed 96-well plate for 30 min at 37 °C. After 30 min, each condition was plated onto tryptic soy agar (TSA) plates, incubated overnight at 37 °C, and CFUs were counted.

For P. aeruginosa, Xen 05 bacteria were cultured overnight in TSB media and subculture in M9 media for 12 h at 37 °C. We combined 100 μL of bacterial solution in M9 (2.25 × 10⁶ CFUs) with 100 μL of CSE in M9 (0.01, 0.1, 1, or 3%) in a 96 well-plate and incubated for 18 h at 37 °C. The next day the samples were plated on TSA plates, incubated overnight at 37 °C to count colonies the following day.

Bacterial growth in BAL supplemented with CSE and ferric chloride
To supplement BAL with CSE we coincubated 50 μL of increasing concentrations of CSE (0.1, 0.3, 1, or 3%) with 140 μL of non-smokers BAL for 30 min. Thereafter, we added 10 μL of S. aureus Xen 29 (~1.5 × 10⁵ CFU) and measured RLU at 30 min.

For P. aeruginosa, we coincubated 100 μL of a combination of non-smokers BAL with either CSE (1%), FeCl₃ (1 μg/mL) or control with 100 μL of Xen 05 in M9 (~1.5 × 10⁷ CFU) in a 96 well-plate. The sealed plate was incubated overnight and RLU were measured at 18 h.

Measurement of lactoferrin in the BAL
We diluted BAL 1:5 using water. Thereafter, we measured lactoferrin using a human lactoferrin (HLF2) ELISA kit buffer control in the samples and assessed biofilm as previously described.
Abcam, USA) interpolating the unknowns to a standard curve to calculate the lactoferrin concentration in the BAL samples. The data was corrected for the dilution factor.

**Measurement of metals in the BAL**

Trace metals in BAL were analyzed using a Thermo X series II inductively coupled plasma mass spectrometer ICP-MS with a collision cell (ThermoFisher Scientific). Samples were spiked with 0.5 mL of a 1 part per million indium solution (Inorganic ventures CGIN1–1) to serve as an internal standard. A 1:10 dilution was performed with 2% nitric acid (Fisher Chemical, Trace metal grade) and final sample volume was 10 mL. Calibration curves were prepared for each analyte of interest from a multi-element standard (Inorganic Ventures QCP-QCS-3 source). Standards were prepared in concentrations from 1 to 200 parts per billion with the same internal standard. Standards were diluted with 2% nitric acid. Calibration curves were plotted using the response ratio of analyte/internal standard on the Y axis and concentration of analyte on the X axis. The slope was then used to obtain concentration of analyte in samples after a blank subtraction (2% nitric acid). The data was corrected for the dilution factor and samples that were below blank level concentration were considered as below the limit of detection and plotted as 0.

**Data analysis**

Data are expressed as mean ± SEM. For BAL growth, we used raw RLU and for the CSE experiments we normalized data as percent of control using this formula:

\[
\text{Percent of live bacteria} = \frac{\text{RLU from sample}}{\text{RLU from control vehicle}} \times 100
\]

All experiments had \( n = 11 \), were done in replicates in at least two independent experiments. The exception was the metal measurements in the BAL that was done once with all samples using the same standard to ensure that they were comparable. We determined the statistical significance between two related groups using paired \( t \)-tests.

We used multiple comparison ANOVA and Kruskal-Wallis to compare three or more concentrations to their respective control. We also used the Pearson test to calculate correlation coefficients. Data analysis was performed using Graph Pad Prism 6.00 (GraphPad Software, California, USA).

**Results**

**Bacteria grows more in the BAL from smokers compared to non-smokers**

We hypothesized that BAL from smokers would have increase bacterial growth compared to non-smokers. To investigate this hypothesis we challenged BAL samples, collected via bronchoscopy, with bioluminescent \( S. \ \text{aureus} \) and found significantly more live bacteria in RLU from the smokers BAL compared to non-smokers. (Fig. 1a). We also challenged the BAL with \( P. \ \text{aeruginosa} \) and found an average of about 64% more RLU at 6 h in the BAL from smokers compared to non-smokers (Fig. 1b). These results suggest that there is increased bacterial growth in the BAL of smokers compared to non-smokers.

**Bacteria develops more biofilm mass in the BAL from smokers compared to non-smokers**

Since BAL from smokers had more \( S. \ \text{aureus} \) and \( P. \ \text{aeruginosa} \) growth compared to non-smokers, we investigated the ability of these bacteria to form biofilms. We challenged BAL samples with \( S. \ \text{aureus} \) and assessed biofilm formation at 48 h by measuring the optical density of crystal violet staining of the biofilm. We found that \( S. \ \text{aureus} \) in the BAL from smokers had about 52% more biofilm mass compared to non-smokers (Fig. 2a). We also challenged BAL samples with \( P. \ \text{aeruginosa} \) and quantified the biofilm formation at 48 h by measuring the RFUs of concanavalin-A conjugated with Texas red staining of the bacterial biofilm matrix. We found that \( P. \ \text{aeruginosa} \) in the BAL of smokers had three times more biofilm matrix compared to non-smokers (Fig. 2b and c). These results

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**Fig. 1** \( S. \ \text{aureus} \) and \( P. \ \text{aeruginosa} \) grow more in the BAL from smokers compared to non-smokers. **a** \( S. \ \text{aureus} \) bacterial growth assessed by RLU at 30 min (**\( p < 0.0001 \) by unpaired \( t \)-test). **b** \( P. \ \text{aeruginosa} \) bacterial growth by RLU at 6 h (\( p = 0.0493 \) by unpaired \( t \)-test)
suggest that bacteria grow more as a biofilm in the BAL from smokers compared to non-smokers.

**Metals concentration in the BAL**

Since tobacco contains iron containing particulate matter, we hypothesized that smokers would have higher concentration of metals in the BAL. To investigate this hypothesis, we measured the concentration of selected metals in the BAL of smokers and non-smokers using ICP-MS. We found that some metals, such as aluminum, lead, and vanadium were only detected in the BAL of smokers. Despite that the mean concentration of metals was higher in smokers (e.g. iron was almost four times higher) they were not statistically different (Table 2 and Fig. 3a).

**Lactoferrin concentration in the BAL**

Exposure to cigarette smoke has been associated with increased lactoferrin concentration in human secretions [36, 37]. Therefore, we decided to measure this AMP in our samples. We found that there was not a significant difference in the concentration of lactoferrin in the BAL of smokers compared to non-smokers (Fig. 3b). Because the

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**Table 2** Concentration of metals present in the BAL from smokers and non-smokers

| Element   | Metal concentration (ng/mL) | P-value* |
|-----------|-----------------------------|----------|
|           | Non-Smokers BAL | Smokers BAL |           |
| Aluminum  | BBL | 0.73 (1.89) | NA |          |
| Arsenic   | 3.46 (0.86) | 4.24 (1.19) | 0.0933 |
| Calcium   | 811.7 (181.61) | 856.8 (183.34) | 0.5691 |
| Chromium  | 0.003 (0.01) | 0.03 (0.06) | 0.1142 |
| Copper    | 4.77 (4.9) | 4 (2.26) | 0.6411 |
| Iron      | 6.38 (9.12) | 23.37 (28.47) | 0.0741 |
| Lead      | BBL | 0.07 (0.16) | NA |          |
| Magnesium | 389.06 (66.93) | 428.76 (110.42) | 0.3200 |
| Manganese | 0.12 (0.16) | 0.21 (0.24) | 0.2785 |
| Nickel    | 0.22 (0.12) | 0.5 (0.55) | 0.1205 |
| Vanadium  | BBL | 0.06 (0.02) | NA |          |
| Zinc      | 40.45 (20.99) | 48.16 (35.06) | 0.5385 |

Data are expressed in mean (SD). BBL below blank level
*Compared by unpaired t-test. NA not apply
BALs can have different dilutions, we calculated the ratio of iron to lactoferrin in the BAL samples. We found that the ratio iron/lactoferrin was five times higher in the smoking group compared to non-smokers (Fig. 3c). These data suggest that smokers have an excess of iron in relation to lactoferrin compared to non-smokers.

**Bacteria grow more in non-smokers BAL supplemented with CSE compared to non-supplemented**

Since we found that smokers had an increased iron to lactoferrin ratio in their BAL, we investigated if adding CSE to non-smokers BAL would have a similar effect as smoking on bacterial growth. First, we investigated if CSE alone had an effect on bacterial growth. We combined increasing concentrations of CSE (0.1–3%), with *S. aureus* and *P. aeruginosa* and measured bacterial growth by standard CFU counting. We found that there was no significant increase in bacterial growth in the presence of CSE alone at these concentrations (Additional file 1: Figure S1). These results suggest that CSE by itself is not sufficient to cause increased bacterial growth. We then supplemented BAL samples from non-smokers with CSE at several concentrations and observed a concentration-dependent increase in *S. aureus* growth at 30 min compared to samples exposed to buffer control (Fig. 4a). Thereafter, we exposed BAL from non-smokers to either CSE (0.1%), ferric chloride (1 μg/mL), or control, challenged with *P. aeruginosa*, and read RLU overnight. We found that BAL from non-smokers supplemented with CSE similar to smoking increased bacterial growth of *P. aeruginosa* (Fig. 4b). We found a similar response to CSE when we added ferric chloride to the BAL of non-smokers (Fig. 4c). These results suggest that CSE, similar to iron, enhances bacterial growth.

**BAL from smokers supplemented with lactoferrin reverts bacterial growth and biofilm formation**

We hypothesized that chelating iron using lactoferrin would prevent the increase in bacterial growth and biofilm formation found in the smokers BAL. To investigate this hypothesis, we coincubated the BAL from smokers with increasing concentration of lactoferrin (10–300 μg/mL), challenged with *S. aureus* (~ 5 × 10⁵ CFU), and measured live bacteria in RLU after 30 min. We found a concentration dependent decrease in live bacteria in the samples from smokers treated with lactoferrin (Fig. 5a). BAL
samples from smokers treated with lactoferrin (300 μg/mL) were not statistically different from the non-smokers control (7389 ± 139.5, n = 8, vs 6963 ± 202.4, n = 8, respectively p = 0.1055 by unpaired t-test). We also challenged the BAL samples from smokers treated with lactoferrin (300 μg/mL) with P. aeruginosa, and measured RLU at 6 h. We found decreased RLU in the smokers BAL samples treated with lactoferrin compared to control (Fig. 5b). We also supplemented the BAL of smokers with lactoferrin and found a concentration-dependent decrease in the biomass produced by S. aureus (Fig. 5c). BAL samples from smokers treated with lactoferrin (1000 μg/mL) were not statistically different from the non-smokers control (0.06763 ± 0.002432, n = 11 vs. 0.06835 ± 0.001963, n = 11, p = 0.8184 by unpaired t-test). We also found that supplementing BAL from smokers with lactoferrin (300 μg/mL) significantly decreased the biofilm formation by P. aeruginosa at 48 h (Fig. 5d). These results suggest that adding lactoferrin to the smokers BAL decreased bacterial growth and biofilm formation compared to the untreated BAL.

**Discussion**

We investigated the bacteriostatic properties of human BAL collected in vivo from smokers and non-smokers using a standard bacterial inoculum. We found that S. aureus and P. aeruginosa grow more and develop more biofilm in the BAL samples taken from the lungs of smokers compared to non-smokers.

It has been proposed that the surface of the airways is iron-depleted to limit bacterial growth and virulence [38, 39]. Although the study was underpowered to detect significant differences in iron concentrations, we found that smokers had four times higher mean iron concentration in the BAL than non-smokers. This finding is consistent with several reports that have found that smokers have increased iron concentration in their lungs [20, 40–43]. This result and the report of other
investigators is in part explained by the disruption of iron homeostasis mechanisms in the lungs of smokers.

Cigarette smoke particles are rich in iron and can directly increase iron concentration in the airways [20] where particulate matter is deposited. Some of the particles are endocytosed and metal oxides on their surface can adsorb intracellular free iron to form ferruginous bodies decreasing its concentration available for the cell. In turn, low iron can be sensed by iron-regulatory proteins that activate iron-responsive elements to post-transcriptionally increase transferrin receptors in the cell basolateral membranes, upregulating iron uptake and further increase the iron content in the lungs [44–46]. In addition, cigarette smoke contains polyhydroxybenzenes that can react with ferritin to release iron [47]. Furthermore, iron in the airways might come from damage in the airway epithelial cells, which results in serum leakage [40].

Increased iron concentration in the airways correlates with the severity of lung disease in cystic fibrosis and chronic bronchitis [38, 48]. It has been proposed that changes in iron homeostasis can affect the susceptibility of the airway to develop infections [46]. Most bacteria rely on a continuous supply of host iron to proliferate [49] and high levels of serum iron can increase the risk of developing active infections such as tuberculosis [50, 51]. In addition, iron nanoparticles can directly impair airway innate mechanism such as AMP activity [21].

We found that other metals such as aluminum, lead, and vanadium that were not present in the airways of non-smokers. Accumulation of metals other than iron has also been observed in the lung and serum of patients with COPD [52, 53]. Some these metal have been showed to impair mechanism involved in airway immunity associated with the pathogenesis of COPD such as decreased release of the AMP and decreased cystic fibrosis transmembrane conductance regulator (CFTR) function [52, 54–56].

When we exposed *S. aureus* and *P. aeruginosa* to only CSE, there was no increased growth compared to a solution control. It has been reported that CSE has variable effects on bacterial growth. In general, it has an inhibitory effect that is greater in Gram positive than Gram negative bacteria [12, 57]. However, these experiments with only CSE were done using doses that would also cause cell death in airway epithelial cells and do not necessarily recapitulate what occurs in the airways [35].

When we supplemented BAL from non-smoking subjects with CSE, as a way to recreate in part the airway microenvironment, we found that CSE impaired BAL bacteriostatic properties. We found a similar impairment of the BAL when we supplemented with iron chloride. These results might suggest that iron bioavailability could be a mechanism for regulating airway antimicrobial activity. One key AMP in the airways is lactoferrin. One of its major function is iron chelation, which reduces the amount of bioavailable iron in biological fluids, including ASL [13]. The function of lactoferrin is affected when saturated with iron [58]. Therefore, we speculate that cigarette smoke contains iron nanoparticles that might not only be a source of iron for bacterial growth but could also inhibit the bacteriostatic properties of the ASL. Conversely, longer times were needed to notice a difference between control BAL and CSE or iron supplemented BAL in *P. aeruginosa* (6 vs 18 h). These results suggest that other factors present in the BAL from smokers such as heme-iron, inflammatory mediators, different composition of AMP might also contribute to the increased bacterial growth.

Both active and passive smoking has been associated with an increase in lactoferrin concentrations in human secretions [36, 37]. In our samples, we found no significant differences in the concentration of lactoferrin between smokers and non-smokers. However, we found an increased ratio of iron to lactoferrin. We speculate that the relative variability in iron/lactoferrin ratio of our samples is responsible to the heterogeneity of some of the results. Especially those experiments using *Pseudomonas* (Figs. 1b, 5b & d). *Pseudomonas* has evolved to efficiently uptake iron by robust and redundant mechanisms [59, 60]. This feature has allowed them to survive in a wide array of environments, such as water currents, plants, nematodes, insects, and in mammals, including humans [61]. Previous studies have suggested that iron in the lungs might be important for *Pseudomonas* airway colonization [62]. As airway disease progresses in COPD, iron deposits in the airways also increases [63]. It is also known that lung diseases such as severe COPD has higher rates of *Pseudomonas* airway colonization. We also speculate that iron/lactoferrin imbalance will also increase the probabilities of being colonized by this pathogen associated with poor clinical outcomes in hospitalized patients [29, 30].

When we supplemented BAL samples with excess lactoferrin we reverted the impaired bacteriostatic activity and biofilm formation observed in the BAL of smokers. One likely mechanism for excess lactoferrin reverting bacterial growth and biofilm formation in smokers is by decreasing bioavailability of iron. Despite that lactoferrin has other antimicrobial mechanisms such as direct binding to LPS, osmotic effect, and bacterial membrane permeabilization, these are also impaired when iron is bound to lactoferrin [64–66]. We acknowledge that the concentration of lactoferrin added to the samples could be considered supraphysiologic. However, commercially available lactoferrin is partially saturated with iron. For this reason, higher doses of AMP were used to observe an effect, particularly in the biofilm experiments.

As a limitation of our study, we acknowledge that *Haemophylus influenzae*, an important organism in COPD...
exacerbations was not considered for this study. However, this organism needs hemin, an iron containing protoporphyrin and nicotinamide adenine dinucleotide to grow in vitro, the addition of these elements could confound the hypothesis of smoking as a source of iron for bacteria.

Several investigators have recently reported that current and former smokers with preserved lung function by spirometry have increased respiratory symptoms and evidence of airway disease by imaging [67, 68]. This study demonstrates that the imbalance between iron content and lactoferrin abundance in the airways can result in conditions that will impair airway innate immune mechanisms, resulting in an increased risk of respiratory infections. Since the development of airway infections has been proposed as an important mechanism for lung function decline and development of chronic bronchitis, our results provide a potential mechanism for some of the recent reports of respiratory symptoms in smokers without spirometry evidence of COPD [24, 25].

The implications of iron/lactoferrin imbalance in the development of COPD might go beyond increasing bacterial growth. A recent discovery demonstrated that a gene that encodes for an iron receptor protein was associated with dysfunctional mitochondrial iron loading affecting mucociliary clearance and contributing to the development of COPD [69, 70]. In the same study, the use of deferiprone, an USDA approved drug that functions as an iron chelator in a mouse model of COPD, improved features of airway disease progression and acute lung injury such as weight loss, pulmonary inflammation, and decreased mucociliary clearance despite continuous cigarette smoke exposure. Further studies will have to determine the feasibility of this intervention but suggest a promising avenue to prevent the progression from smoking to COPD by iron chelation.

Conclusion
We conclude that in vivo BAL collected from smokers grows more bacteria and develops more biofilm compared to non-smokers. We presume that excess iron compared to lactoferrin in the airways of smokers impairs the ability of the lungs to control bacterial airway pathogens.

Additional file

Additional file 1: Figure S1. Cigarette smoke extract alone does not increase bacterial growth. (A) S. aureus growth overnight in the presence of increasing concentrations of CSE assessed by CFU. (B) P. aeruginosa overnight growth in the presence of increasing concentrations of CSE assessed by CFU. (Tiff 601 kb)

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