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Anaerobic microbiota facilitate *P. aeruginosa* access to the airway epithelium in a novel co-culture model of colonization

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ABSTRACT

The role(s) of anaerobic microbiota in chronic airway disease are poorly understood due to inherent limitations of existing laboratory models. To address this knowledge gap, we use a dual oxic-anoxic co-culture approach that maintains an oxygen-limited apical epithelial microenvironment while host cells are oxygenated basolaterally. Reduced oxygen culture did not alter the physiology or gene expression of Calu-3 cells but supported anaerobe-epithelial interactions for 24h without affecting bacterial or host cell viability. Anaerobe challenge led to increased expression of inflammatory marker genes and compromised integrity of apical mucins, leading to our hypothesis that anaerobe-host interactions prime the airways for chronic infection. Indeed, anaerobe pre-treatment of Calu-3 cells led to an increase in *Pseudomonas aeruginosa* colonization. This model system offers new insight into anaerobe-host interactions in airway disease pathophysiology and motivates further study of the lung, gut, and oral cavity, where etiological roles of anaerobes have been proposed but specific pathogenic mechanisms remain unclear.

INTRODUCTION

Decades of clinical lab culture have focused on a limited set of pathogens associated with acute and chronic airway disease (e.g. *Pseudomonas aeruginosa, Staphylococcus aureus, Mycobacterium tuberculosis*). More recently, culture-independent studies of airway microbiota have identified more complex bacterial signatures, lending evidence to polymicrobial disease etiologies. Notably, oral and supraglottic-associated facultative and obligate anaerobes – *Prevotella, Veillonella, Streptococcus* spp. – are present at low densities in the healthy respiratory tract (1-4) and are both prevalent and abundant in chronic obstructive pulmonary disease (COPD) (5, 6), cystic fibrosis (CF) (7), non-CF bronchiectasis (8), lung abscess (9), sinusitis (10), idiopathic pulmonary fibrosis (11), and tuberculosis (12). While salivary contamination during sampling remains controversial, consensus is that the development of hypoxic microenvironments within diseased airway mucus provides a niche for anaerobe proliferation, often reaching densities equal to or greater than those of canonical pathogens (7, 13).

The function of anaerobic microbiota in airway disease is poorly understood though several roles have been proposed. In healthy individuals, anaerobe abundance in bronchoalveolar lavage fluid correlates with expression of proinflammatory cytokines, elevated Th17 lymphocytes, and a blunted TLR4 response, implicating a compromised first line of defense against bacterial infection (1). Indeed, epidemiologic and *in vitro* data suggest that anaerobes may facilitate secondary colonization by canonical airway pathogens. In non-CF bronchiectasis, *Prevotella* and *Veillonella* positively correlate with Th17 cytokines and non-tuberculosis mycobacterial infection (14). Similarly, in HIV subjects, anaerobes suppress expression of interferon gamma and IL-17A via production of short-chain fatty acids (SCFAs) and are thought to impair the host response to consequent *M. tuberculosis* colonization (12). In CF, anaerobe-derived SCFAs increase with age and disease progression (15), mediate excessive production of IL-8 by bronchial epithelial cells (in turn promoting neutrophil mobilization) (16) and potentiate the growth and virulence of canonical CF pathogens *in vitro* (17, 18). CF anaerobes have also been reported to increase in abundance during pulmonary exacerbations prior to antibiotic therapy (19), further implicating their role in pathogenesis.

While these data collectively support the causality of anaerobic microbiota in airway disease, direct mechanistic studies of anaerobe-host and anaerobe-host-pathogen interactions have been limited by the paucity of compatible laboratory methods. Animal models poorly reflect chronic infection pathologies and are prohibitively expensive for high throughput analyses. As an alternative, the development of three-dimensional (3D) cell cultures have greatly expanded our knowledge of host-microbe interactions at the respiratory epithelial interface (20-22). However, incorporation of anaerobic microbiota into these models is restricted by the inherent challenge of maintaining host cell viability under hypoxic or anoxic culture conditions. New models are needed for a deeper understanding of the role of anaerobic microbiota in acute and chronic airway disease.

A novel system was recently described that enables co-culture of human intestinal enteroids with obligately anaerobic microbiota under reduced oxygen conditions that recapitulate hypoxic microenvironments found *in vivo* (23). Here, we adopted this approach to overcome limitations of studying anaerobe interactions with the airway epithelium in 3D cell culture. First, polarized monolayers of the mucus-overproducing epithelial cell-line, Calu-3, were maintained at air-liquid interface in an anaerobic chamber while O_2 and CO_2 were delivered through a chamber entry port. This setup allows for maintenance of an anoxic microenvironment in the apical compartment while host cells are oxygenated basolaterally. Using this culture system, we establish its utility for the *in vitro* study of anaerobe-airway interactions. We then use this model to test the hypothesis that anaerobic microbiota enhance colonization of the epithelial surface by *P. aeruginosa.* Data presented here not only demonstrate the power and versatility of the anoxic co-culture approach, but also offer new insight into the mechanisms of pathogen colonization and a potential role of anaerobic microbiota in the development of airway infection.

RESULTS

Optimization and validation of a dual oxic-anoxic airway epithelial culture system. The primary objective was to establish a cell culture system that facilitates study of anaerobe-host interactions (Figure 1). We first cultured polarized monolayers of the adenocarcinoma cell line, Calu-3, at air-liquid interface (ALI) for 21-28 days under standard (normoxic) conditions. As shown previously (24), polarized Calu-3s produce a distinct mucus layer on the apical surface (Figure S1), mimicking aberrant mucin accumulation

associated with chronic airway disease. Once polarized, cell cultures were placed in a gas-permeable multi-well plate manifold (Figure S2), transferred to anaerobic chamber, and mixed gas (21% O_2/ 5% CO_2/ 74% N_2) was delivered through a chamber port to the basolateral compartment of the Transwell-containing plate. Cells were cultured for an additional 24h at anoxic liquid interface (ANLI) prior to analysis.

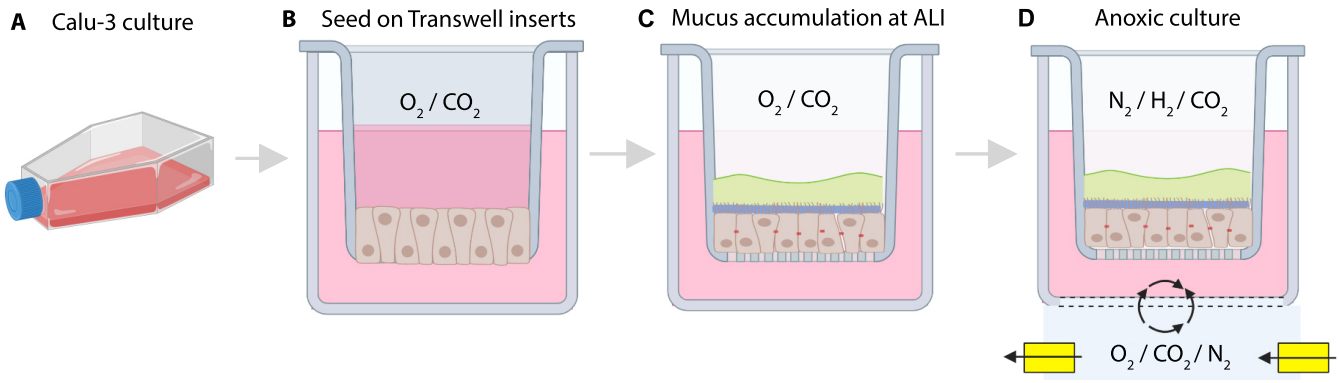

Figure 1. Calu-3 culture at anoxic liquid interface (ANLI). (A) Calu-3 cells are cultured in MEM with 10% FBS. **(B)** Cells are seeded on 6.5mm Transwell inserts and grown to confluency (~5 days). **(C)** Apical medium is removed, and cells are cultured at air-liquid interface (ALI) for 21-28 days prior to **(D)** incubation under anoxic liquid interface (ANLI) conditions, where the apical compartment is oxygen limited and mixed gas is delivered basolaterally. Figure created with BioRender.com.

We then determined the effects of ANLI culture, if any, on Calu-3 cell physiology. Quantification of lactate dehydrogenase release showed a negligible increase between normoxic and ANLI culture conditions (p=0.42), suggesting little to no change in viability after 24h (Figure 2A). Transepithelial electrical resistance (TEER) (Figure 2B) and E-cadherin concentrations (Figure 2C), both proxies of epithelial barrier integrity, were also similar between culture conditions (p=0.38 and 0.55, respectively). These data were further supported by immunofluorescence microscopy which revealed confluent monolayers and well-defined staining of the tight junction zonula occludens protein which appeared as near-continuous rings localized to the periphery of each cell (Figure 2D).

Previous work has shown hypoxia-induced expression of pro-inflammatory cytokines in primary pulmonary fibroblasts (25). Thus, we used enzyme-linked immunosorbent assays to quantify IL-6 and IL-

8 production by Calu-3 cells. Both cytokines showed no significant increases after 24h under ANLI culture conditions relative to normoxic controls (Figure 2E, F, p=0.21 and 0.61, respectively), suggesting that basolateral supply of O_2/CO_2 is sufficient to prevent a pro-inflammatory response.

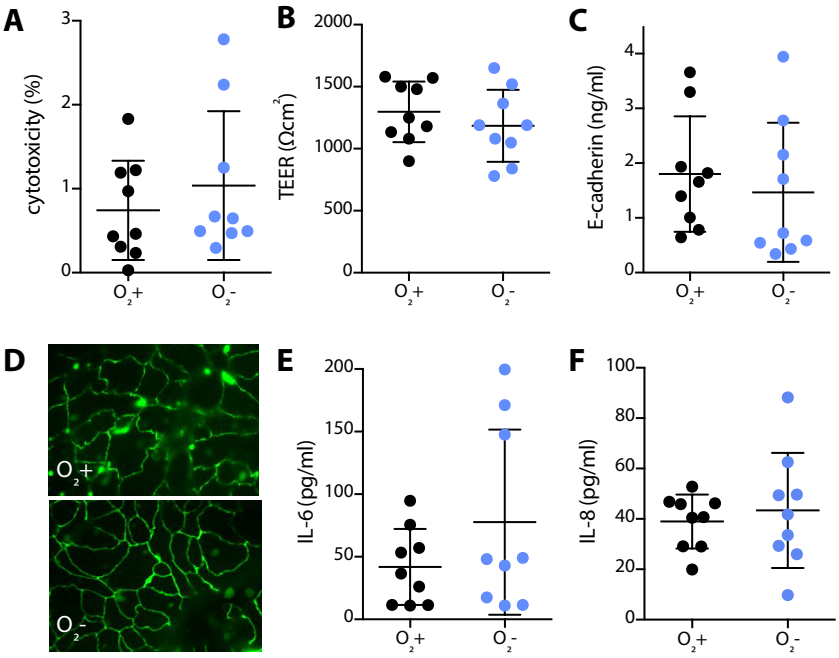

Figure 2. Culture of human bronchial epithelial cells (Calu-3) at ANLI has minimal effect on cell physiology. (A) Cytotoxicity as measured by lactate dehydrogenase (LDH) in the culture medium, **(B)** transepithelial electrical resistance (TEER), **(C)** E-cadherin concentrations, **(D)** immunofluorescence of zonula occludens, and cytokines **(E)** IL-6, and **(F)** IL-8 showed no significant differences between normoxic and ANLI culture conditions. All data shown are for three independent experiments using three biological replicates (n=9) and were compared using an unpaired t-test with Welch's correction.

To gain a broader understanding of the physiological response of Calu-3 cells to ANLI culture, we used RNAseq to compare global Calu-3 gene expression to culture under normoxic conditions. Transcriptome analysis revealed 148 differentially expressed transcripts (117 upregulated, 31 downregulated, l2fc ≥ 1, padj<0.001; out of ~16,000 total genes) (Figure 3A, Table S1). With the exception of *ANGPTL4* (encoding angiopoietin-like 4) and *SERPINA1* (alpha-1 antitrypsin)(Figure 3B) which are induced in response to hypoxia and acute inflammation, respectively, few markers of cell stress were differentially expressed, including genes involved in tight junction formation, oxidative stress, and endoplasmic reticulum stress. Importantly, HIF-1α, which is constitutively expressed at low levels under

normoxia but upregulated under hypoxia, was also consistent between culture conditions after 24h, suggesting Calu-3 cells were sufficiently oxygenated (Figure 3B). Among inflammatory biomarkers, only *ICAM1* (intracellular adhesion molecule 1) and *TGFβ1* (transforming growth factor beta 1) showed significant differences, further demonstrating that ANLI culture did not yield an appreciably pro-inflammatory microenvironment (Figure 3C). Finally, since we use this model to assay bacterial colonization of the mucus layer, we compared mucin-related gene expression between conditions. Among detectable transcripts (*MUC1, MUC3A, MUC5AC, MUC5B,* and *MUC13*), no significant differences were observed (Figure 3D). These data demonstrate that culture of a respiratory epithelial cell line at anoxic liquid interface yields minimal changes in host cell physiology.

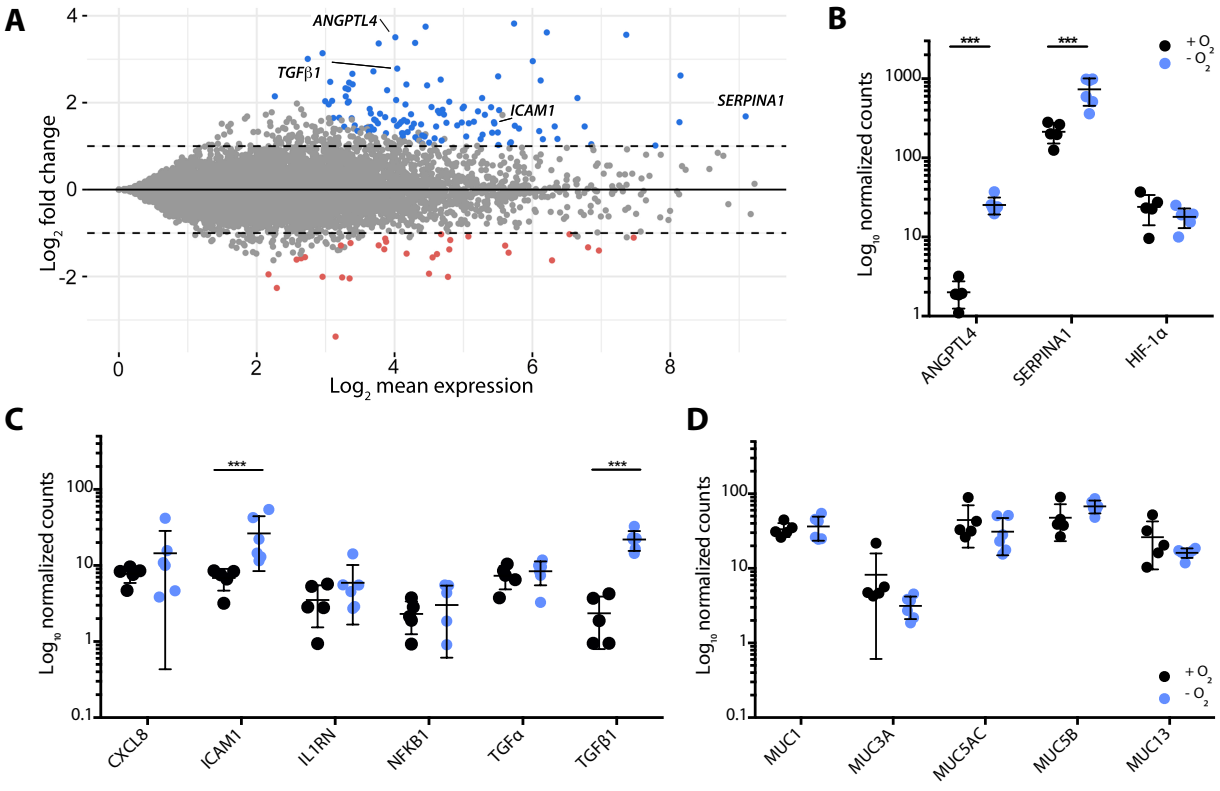

Figure 3. **Culture of Calu-3 cells at ANLI yields a similar transcriptomic profile to normoxic culture conditions (ALI). (A)** MA plot representation of Calu-3 gene expression at ANLI relative to normoxic culture (ALI). **(B)** *ANGPTL4* and *SERPINA1* were differentially expressed, though HIF-1α was consistent between cultures. Few changes in **(C)** inflammatory biomarkers and **(D)** mucin gene expression were observed. Data shown in panels B-D are log_{10}-normalized gene counts from five or six independent biological replicates and were compared using the Wald test, Benjamini-Hochberg adjusted (***, p<0.001).

Anaerobic airway microbiota induce an inflammatory host response. To date, the lack of tractable cell culture systems compatible with hypoxic or anoxic growth has limited our understanding of host-anaerobe interactions. Prior work has shown that culture supernatants of anaerobic bacteria elicit pro-inflammatory cytokine expression *in vitro* through mixed-acid fermentation and production of SCFAs (*1,* 15, 16). However, it is not yet known how the host responds to the physical presence of anaerobes at the airway epithelial interface. To address this knowledge gap, we used our ANLI culture approach to assess the response of Calu-3 cells to co-culture with anaerobic microbiota.

As a starting point, we used a defined anaerobic bacterial consortium (ABC) enriched from airway mucus derived from an individual with chronic sinusitis. This representative community was chosen for its dominant bacterial taxa (*Veillonella, Prevotella, Streptococcus*) associated with both healthy and diseased airways (Figure 4A). These genera are also known for their mucin-degradation capacity and the ability to support pathogen growth through nutrient cross-feeding (17). After 3h of equilibration at ANLI, Calu-3 cells were apically challenged with the anaerobic consortium (~8 x 10^6 CFUs) and incubated for an additional 24h. Colonization was confirmed using scanning electron microscopy which revealed bacterial cells at the epithelial interface (Figure 4B). Importantly, anaerobes (4 x 10^6 CFUs) were recovered after 24h by washing with PBS and plating on Brain Heart Infusion agar (BHI), while washing with Triton X-100 resulted in a 0.7-log increase in recovery (1.6 x 10^7 CFUs), suggesting both anaerobe growth at the epithelial surface and either robust attachment or bacterial invasion of host cells (i.e., cells were not removed by PBS washing alone). Recovery of ~7 x 10^5 CFUs on a *Prevotella*-selective medium (Brucella Blood Agar, BBA) confirms that apical oxygen concentrations were sufficiently reduced to facilitate strict anaerobe growth. Despite this growth, cytotoxicity was not induced by anaerobe challenge after 24h (Figure 4D).

We then used RNAseq to profile the Calu-3 transcriptional response to anaerobe (ABC) challenge (Figure 4E, Table S2). Contrary to our expectation, only five genes were differentially expressed relative to untreated ANLI cell cultures (all upregulated, l2fc≥1, padj <0.001), though all were markers of inflammation. These included *ICAM1*, *TNFAIP2* (mediated by TNFα in response to bacterial challenge) (26), chemokines *CXCL1* and *CXCL5* (neutrophil chemoattractants primarily expressed as an acute

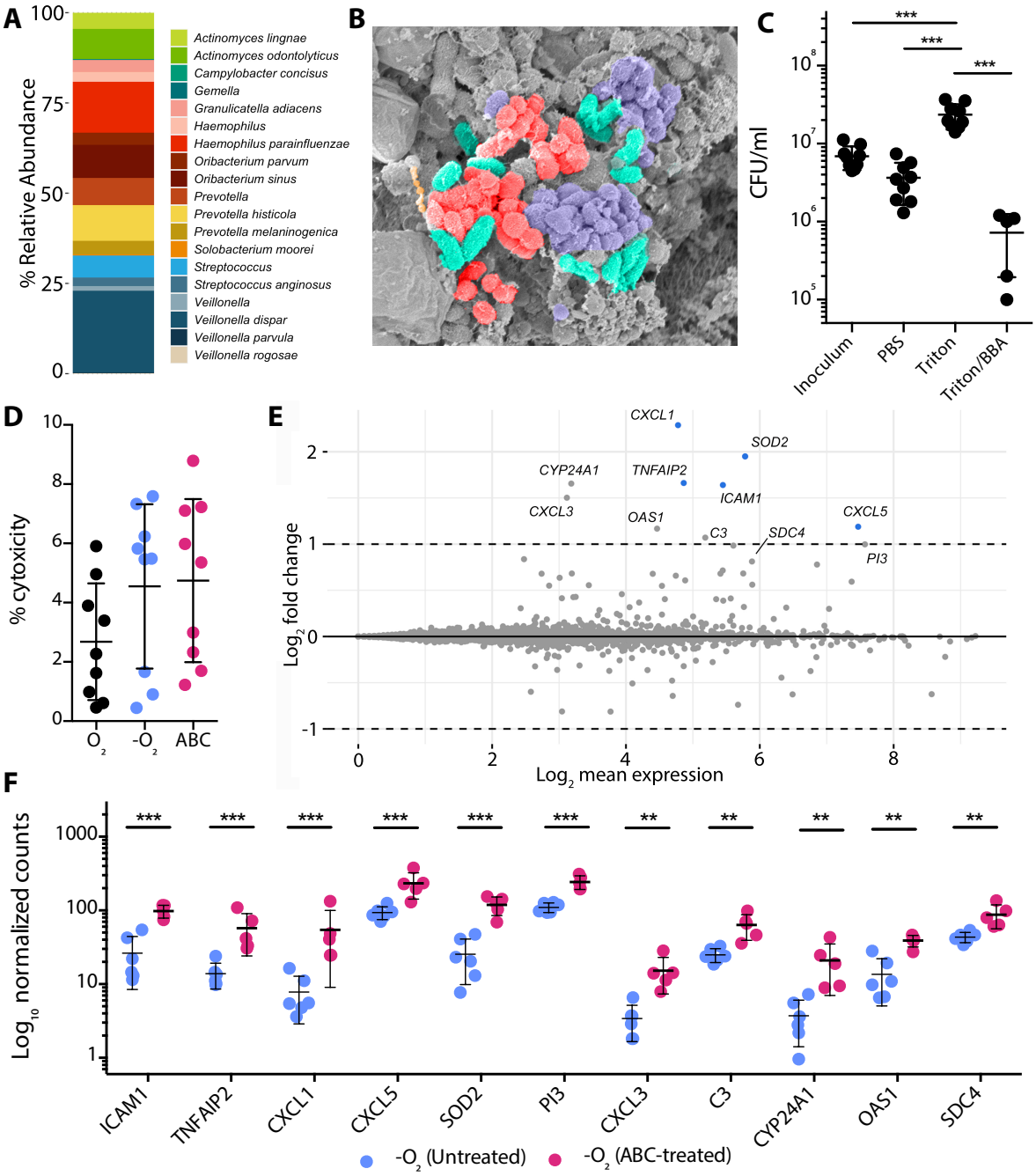

Figure 4. **Anaerobic microbiota colonize the apical surface of Calu-3 cells and induce a pro-inflammatory response. (A)** Taxonomic composition of an anaerobic bacterial consortium (ABC) derived from the upper airways. **(B)** SEM micrograph of Calu-3 cells after CRS challenge. **(C)** Bacterial recovery from Calu-3 cells after 24h by washing with PBS, TritonX-100, and plating on *Prevotella* selective agar (BBA). **(D)** Anaerobe (ABC) challenge did not result in Calu-3 cytotoxicity relative to unchallenged cells. **(E)** MA plot representation of Calu-3 gene expression under ANLI after ABC challenge relative to an untreated ANLI control. **(F)** Log_{10}-normalized gene counts from five or six independent biological replicates. Data in panels C and D were compared using a one-way ANOVA ($p<0.0001$) with multiple comparisons test. Data in panels E and F were compared using a Wald test, Benjamini-Hochberg adjusted (***$p<0.001$, **<0.01).

inflammatory response to infection) (27, 28), and *SOD2* (superoxide dismutase 2, expressed in response to bacterial LPS and has an antiapoptotic role against inflammatory cytokines) (29). Other inflammatory markers including *PI3* (elafin, an elastase inhibitor that can prime innate immune responses in the lung) (30), *CXCL3*, *C3* (complement), *CYP24A1* (cytochrome p450 family 24 subfamily A member 1), *OAS1* (oligoadenylate synthetase), and *SDC4* (syndecan 4) were also differentially expressed, though did not reach our threshold padj of <0.001 (Figure 4F).

Anaerobic microbiota alter the mucosal interface through mucin degradation. Our previous work demonstrated the functional capacity of anaerobic microbiota to degrade airway mucins and support the growth of canonical pathogens via nutrient cross-feeding (17). Thus, in support of downstream pathogen colonization experiments, we used fast protein liquid chromatography (FPLC) to determine whether anaerobe challenge altered Calu-3 mucin integrity relative to unchallenged cells. To do so, we collected and purified mucin from the apical side of the Transwells as previously described (24) and used CL-4B size-exclusion chromatography to assay their integrity. As expected, chromatograms revealed two characteristic peaks; (i) high molecular weight mucins which ran in the void volume of the column, and (ii) a broader inclusion volume peak representative of lower molecular weight mucins (31, 32) (Figure 5A). While differences in the chromatographic profile of peak 1 (high molecular weight mucins) were negligible between culture conditions (p=0.38), peak 2 area was significantly reduced (p=.007) following anaerobe challenge (Figure 5B), reflecting degradation of lower-molecular weight mucin glycoproteins. Together, these data suggest that in addition to eliciting a pro-inflammatory host response, anaerobic colonization alters the physicochemical properties of mucosal interface.

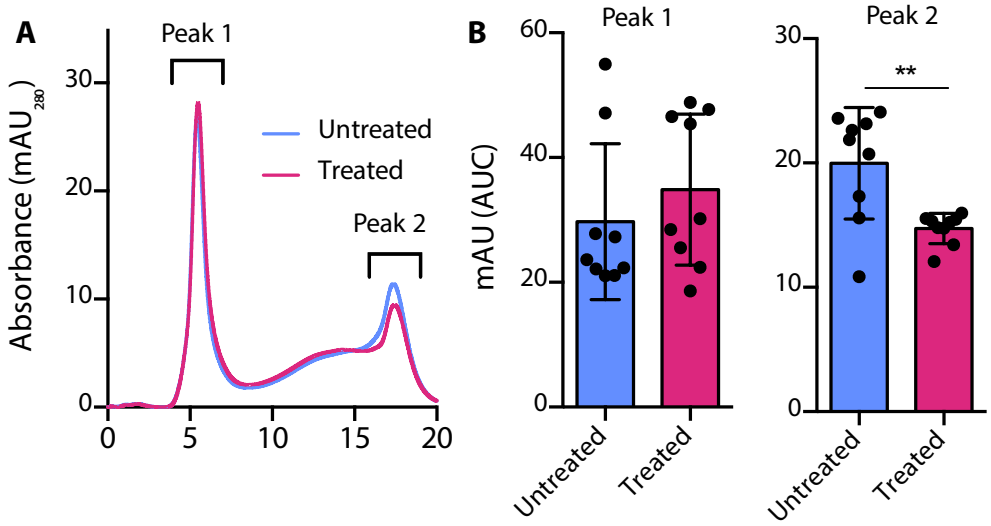

Figure 5. Anaerobic microbiota alter epithelial mucin integrity. (A) Representative FPLC traces of MUC5AC mucins purified from Calu-3 cells grown at ANLI (untreated) and after treatment with an anaerobic bacterial community (ABC, treated). **(B)** Area under curve (AUC) for both peak 1 (high molecular weight mucins) and peak 2 (low molecular weight mucin). Data shown were derived from three independent experiments using three biological replicates (n=9). Data were compared using a unpaired t-test with Welch's correction (**, p<.01).

Anaerobes promote *P. aeruginosa* colonization of the airway epithelium. Recent work has shown that viral challenge of the respiratory epithelium potentiates colonization by *P. aeruginosa* via interferon mediated effects (20). Other work has shown that the protective role of the mucus barrier is compromised by *Streptococcus mitis* through hydrolysis of mucin glycans (33). Given that the anaerobes used in our model both elicited an inflammatory response and altered mucin integrity, we hypothesized that in addition to providing nutrients for pathogen growth through cross-feeding (17), anaerobic microbiota enhance *P. aeruginosa* colonization of the airway epithelium (depicted in Figure 6A).

To test this hypothesis, Calu-3 cells were first treated with the anaerobic bacterial consortium (ABC) for 24h, washed to remove spent medium and unbound cells, and subsequently infected with $1x10^6$ CFUs of *P. aeruginosa* PA14 for 2h. Removal of spent medium and a short incubation time ensures that any difference in colonization between anaerobe-treated cells and an unconditioned (i.e., no anaerobe) control was a result of cell attachment and not enhanced growth. *P. aeruginosa* colonization was then determined

by washing and permeabilization of Calu-3 cells followed by plate enumeration. As predicted, anaerobe pre-treatment resulted in an increase in 1.1 x 10^7 *P. aeruginosa* CFUs relative to untreated (i.e., no ABC) controls (p=0.0003, Figure 6B).

To further demonstrate that enhanced pathogen colonization was due to mucin degradation and not some other unidentified process, mucins isolated from Calu-3 cells treated with ABC (and untreated controls) were used to coat the surface of a microtiter plate (34), followed by addition of *P. aeruginosa* PA14. As expected (35), mucin coating led to a 1.5 log-reduction in bacterial attachment relative to uncoated plates (PBS alone). Consistent with our ANLI co-culture assay, ABC-treated mucins resulted in a significant increase (p=0.0007) in *P. aeruginosa* binding compared to untreated mucins (Figure 6C). To confirm that our mucin purification process (e.g., use of guanidine hydrochloride) did not affect *P. aeruginosa* viability, plates were also coated with mucins degraded with human neutrophil elastase (NE) and isolated using the same process. NE-treated mucins resulted in similar PA14 attachment to PBS controls, confirming bacterial viability and that anaerobe-microbiota can enhance pathogen colonization of a mucin-coated interface.

Finally, to assess the contributions of individual anaerobes to *P. aeruginosa* colonization we challenged Calu-3 cells with representative isolates of the three most abundant genera in the anaerobic consortium (*Streptococcus, Veillonella, Prevotella)* prior to *P. aeruginosa* colonization (Figure 6D). Contrary to a recent study (33), we found that *Streptococcus* species (*S. gordonii* and *S. parasanguinis*) had little effect on PA14 colonization, despite their known mucin degradation capacity. Similarly, *V. parvula* resulted in no significant differences between treatment conditions. By contrast, challenge with both *P. melaninogenica* and *P. oris,* two species commonly associated with inflammatory airway disease, resulted in significantly increased PA14 recovery from Calu-3 cells compared to unconditioned controls (p=0.0003 and p=0.0004, respectively). These data demonstrate that while anaerobic microbiota of the respiratory tract likely facilitate enhanced colonization of the airway epithelium via mucin degradation, it is clear they do so in a species-specific manner.

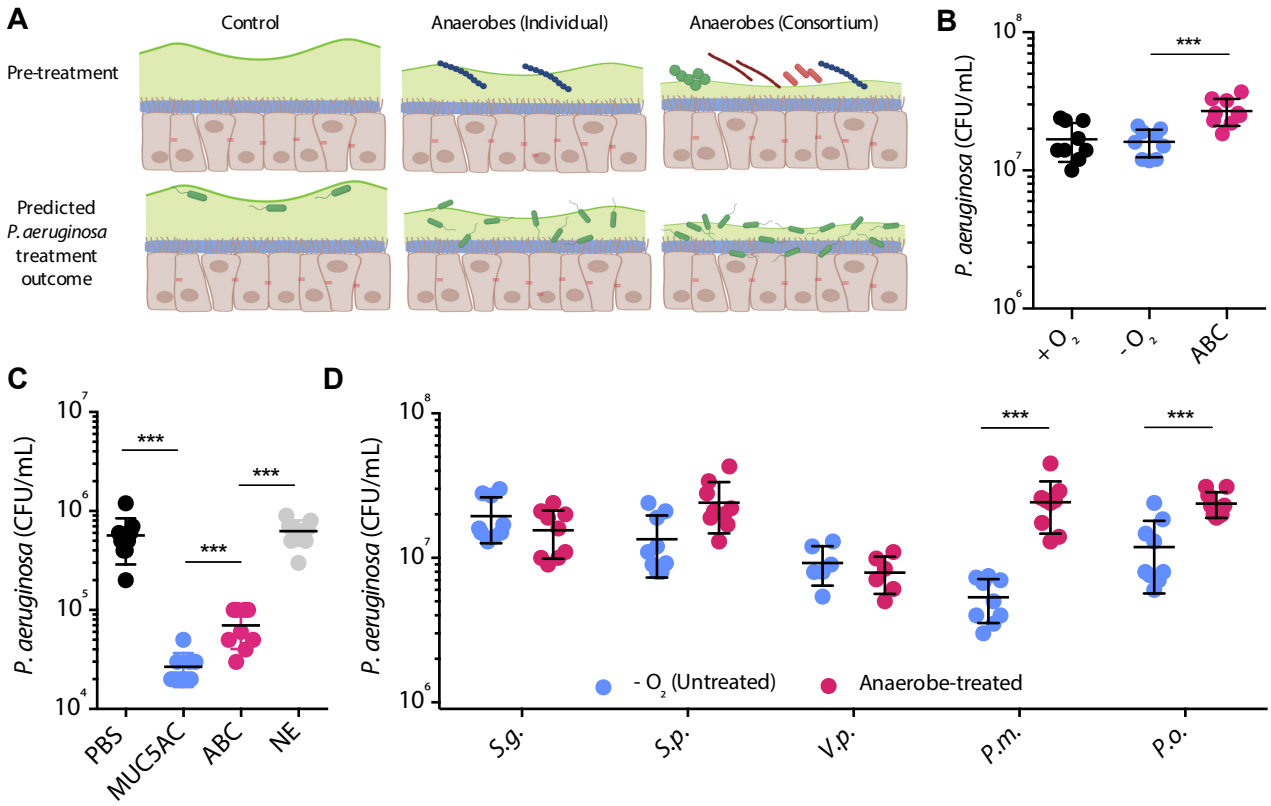

Fig. 6. Anaerobic microbiota condition the epithelial interface for pathogen colonization. **(A)** Schematic of experimental design. **(B)** Calu-3 pre-treatment with an anaerobic bacterial consortium (ABC) potentiates *P. aeruginosa* colonization. **(C)** *P. aeruginosa* adhesion to microtiter plates coated with mucin (MUC5AC), ABC-treated mucin, and neutrophil elastase (NE)-treated mucin relative to an uncoated control (PBS). **(D)** *P. aeruginosa* adhesion to Calu-3 cells after pre-treatment with individual anaerobes (*S.g., Streptococcus gordonii; S.p., S. parasanguinis; V.p., Veillonella parvula; P.o., Prevotella oris; P.m., P. melaninogenica)*. Data shown in panels B-D were derived from three independent experiments with three biological replicates (n=9). Data in C were compared using a one-way ANOVA ($p<0.0001$) with multiple comparisons. Data in C were compared using a non-parametric Kruskal-Wallace test ($p<0.0001$) with multiple comparisons. Pairwise comparisons in panel D were performed using were compared using an unpaired t-test with Welch's correction (*p <0.05, ***p<0001).

DISCUSSION

The recent surge in culture-independent sequencing of human microbiota has spawned renewed interest in the importance of anaerobic bacteria in disease etiologies. However, despite anaerobes comprising a significant component of airway bacterial communities, studies on their contributions to disease pathophysiology have reported seemingly contradictory results (7, 19, 36-41), calling into question their role in patient morbidity. Proposed pathogenic mechanisms are supported by compelling *in vitro* data (15-17, 42, 43), but their relevance is unresolved due to a lack of compatible models with which to test

their interactions with the respiratory epithelium. To address this knowledge gap, here we optimized and characterized a model system that facilitates co-culture of anaerobes and polarized airway epithelial cells. Importantly, provision of oxygen exclusively to the basolateral side of host (Calu-3) cells prevented anoxia-associated cytotoxicity, inflammation, and significant changes in global gene expression over 24h, validating its utility for the extended study of anaerobe-host interactions. Using this model, we demonstrate that anaerobic microbiota stimulate an immune response and promote enhanced epithelial colonization by airway pathogens.

Disparate oxygen demands of epithelial cells and anaerobic bacteria pose significant challenges for their co-culture *in vitro*. Several bacterial-epithelial culture systems have been developed in an attempt to overcome these challenges and recapitulate an oxygen-restricted mucosal interface (44-47), though each has notable drawbacks. Transwell cultures of Caco-2, HaCaT, and primary human gingival cells have been used to demonstrate that anaerobic taxa can adhere to, invade, and alter oral and intestinal epithelia, yet these assays are either limited to short incubation times or poorly mimic atmospheric conditions observed *in vivo* (48-51). Newer microfluidic-based and 'organ-on-a-chip' models have also seen widespread interest due to their ability to establish dual oxic-anoxic interfaces and facilitate study of anaerobe-host interactions (52-56). However, these models either preclude direct host-bacterial contact or are technically challenging to maintain both oxic and strict anoxic microcompartments. More recently, new methods have been expanded to include respiratory epithelial cells and used to study alveolar cell infection by *M. tuberculosis*, but development is still in its infancy (57). Our adapted system offers the distinct advantages of ease-of-use, direct interactions between host and microbiota, reproducibility given the multi-well plate format, and methodological flexibility lending itself to genetic, biochemical, and microscopy studies as demonstrated here. Most notably, this co-culture model elicits few changes in host cell physiology after 24h of anoxia, paving the way for a greater understanding of the mechanistic contributions of anaerobic bacteria to airway pathophysiology.

To demonstrate the utility of our model we used a polymicrobial consortium representative of anaerobic bacterial community signatures observed in acute and chronic airway disease (8, 11, 39, 58). Dominated by *Streptococcus, Prevotella,* and *Veillonella* spp., this consortium (among other bacterial taxa)

306 is thought to seed the airways through microaspiration from the oral cavity and is recognized as a key risk
307 factor in the development of COPD, CF, pneumonia, sinusitis and other diseases. For example, *in vitro*
308 studies have shown that anaerobe-derived supernatants containing proteases and pro-inflammatory short-
309 chain fatty acids modulate the immune tone of bronchial epithelial cell lines and primary cell cultures (15,
310 16). Indeed, anaerobe abundance in both diseased airways and healthy controls is associated with
311 enhanced expression of inflammatory cytokines (1, 12). Our data also support a immunomodulatory role
312 and suggest that colonization of the airway mucosa by aspirated anaerobes may establish a local
313 inflammatory environment known to promote colonization by *P. aeruginosa* and other canonical pathogens
314 (20, 21).

315 We previously reported that anaerobes can also stimulate pathogen growth through mucin-based
316 cross-feeding (17). Specifically, *P. aeruginosa,* which cannot efficiently catabolize mucins in isolation, can
317 gain access to bioavailable substrates via anaerobe-mediated degradation of the mucin polypeptide and
318 O-linked glycans. Though not directly tested here, it is plausible that pre-colonization with an anaerobic
319 bacterial community liberates additional mucin-derived metabolites on which pathogens can thrive at the
320 epithelial interface. While it remains unclear why only low molecular weight mucins were altered, our FPLC
321 data confirm that the mucosal surface is structurally modified as a result of anaerobic colonization. Given
322 that mucin degradation has been shown to compromise its barrier function and enhance pathogen-
323 epithelial interactions (33), we propose that in addition to the host's impaired mucociliary clearance,
324 pathogenic contributions of anaerobic microbiota to airway infection are likely imparted through a
325 multifactorial process (inflammation, cross-feeding, and surface alteration).

326 By focusing on an early time point after *P. aeruginosa* challenge (2h), we targeted anaerobic mucin
327 degradation and its role in re-shaping the epithelial interface while enhancing pathogen attachment, as
328 was previously shown for *S. mitis* and *Neisseria meningitidis (*33*)*. As predicted, anaerobe degradation of
329 apical mucus resulted in a significant increase in *P. aeruginosa* attachment, which has important clinical
330 implications. Most notably, epithelial colonization is known to stimulate rapid *P. aeruginosa* biofilm
331 maturation and associated increases in extracellular polysaccharide production, induction of quorum-
332 sensing and other transcriptional changes (22). In addition, *P. aeruginosa* grown on bronchial epithelial

cells is far more resistant to antibiotic treatment than when grown on abiotic surfaces (22), consistent with their increased tolerance *in vivo*. We propose that anaerobe-mediated colonization further potentiates these phenotypes. Moving forward, it will be important to consider *P. aeruginosa* interactions with the host over longer time periods to further understand differences in pathogen physiology and the inflammatory host response in the presence and absence of anaerobic microbiota.

We elected to use Calu-3 cells as a representative cell line for several reasons. First, Calu-3 cells reach polarization at ALI within ~21 days, express high levels of occludin and E-cadherin localized at tight junctions, and achieve TEER values (>1000 Ωcm^2) far greater and more stable than those of primary cells (59-61). In addition, Calu-3s express high levels of CFTR (62), demonstrating their suitability and relevance for studies of CF and COPD, for which CFTR-silencing small hairpin RNAs (shRNA) have already been developed (63). Finally, unlike many other respiratory cell lineages, overproduction of mucus on the apical surface of polarized Calu-3 cells mimics a diseased mucosal environment and allowed us to test the hypothesis that mucin degradation enhances pathogen colonization. This was an important consideration as *P. aeruginosa* biofilms, at least in the context of CF, are thought to form within secreted mucus as opposed to the epithelial layer (64, 65). We also acknowledge limitations. Unlike primary cells, which form a pseudostratified epithelium with mucociliary differentiation, Calu-3 cells are derived from human bronchial submucosal glands that comprise a relatively homogenous monolayer. Transcriptional and physiological responses to external stimuli may also be unique to Calu-3s. As an example, *S. aureus* enterotoxin B is known to elicit significant differences in barrier integrity, IL-6 and IL-8 production relative to primary tissue (66). These, among other distinctions, underscore the importance of future work testing additional cell lines and primary tissue.

Despite these limitations, our model represents a tractable co-culture system that facilitates extended interrogation of host-anaerobe interactions. While we use this model here to demonstrate a role for anaerobic microbiota in the initial colonization of the epithelial surface, this work will undoubtedly benefit future studies focused on anaerobe-host and anaerobe-host-pathogen interactions and their dynamics over time. Not only do we anticipate generating a deeper understanding of *P. aeruginosa* pathophysiology under oxygen-limited conditions, we also expect to identify new therapeutic strategies in addition to

360 understanding how existing antimicrobials are impacted by environmental conditions known to exist *in vivo*
361 (64). Finally, while we use an airway-derived bacterial community and *P. aeruginosa* as our model
362 organisms, this work motivates additional studies of the gut, lung, oral cavity, and other sites of infection,
363 where etiological roles of anaerobes have been proposed but specific pathogenic mechanisms remain
364 unclear.
365

366 **METHODS**

367 **Epithelial Cell Culture.** Calu-3 cells were maintained in Minimal Essential Medium (Corning, USA) in 10%
368 fetal bovine serum (FBS, Gene) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin
369 (Gibco) at 37°C in a 5% CO_2 incubator. Upon reaching 80% confluency, 1 x 10^5 cells were passaged onto
370 6.5mm culture inserts (12-well hanging inserts, 0.4 μm pore; Corning). When cells reached confluency (~5
371 days), apical medium was removed to establish an air-liquid interface (ALI). Polarized cells were
372 maintained for an additional 21-28 days to facilitate differentiation and mucus accumulation.

373 Cell cultures were then assembled in a gas permeable multi-well plate manifold based on an
374 enteroid-anaerobe co-culture system recently described (23) (Figure S2). Briefly, once polarized, Calu-3
375 cells were placed into gaskets in a 24-well gas-permeable plate (CoyLabs, Grass Lake, MI) containing
376 800μl of MEM per well. Mineral oil (400μL) was added to unused wells to prevent gas permeation from the
377 basolateral to apical side of the manifold. Once assembled, the apparatus was moved to an anaerobic
378 chamber (90% N_2/5% H2/5% CO_2) while mixed gas (21% O_2/5% CO_2/74% N_2) was delivered to the base
379 of the plate to oxygenate the basolateral side of the polarized monolayer. A schematic of this workflow is
380 summarized in Figure 1.

381 **Cell culture assays.** Calu-3 barrier integrity was determined by trans-epithelial electrical resistance
382 (TEER) measured with a Millicell-ERS2 Volt-Ohm meter (Millipore Sigma). Barrier integrity was further
383 assessed using the Human E-cadherin Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D
384 Systems, Minneapolis, MN). Absorbance was measured at 450 nm using a BioTek Synergy H2 plate
385 reader and concentrations of E-cadherin were determined against a standard curve according to

386 manufacturer's instructions. Tight junction formation was assayed using immunofluorescence with an anti-
387 zona occludens (ZO)-1 monoclonal antibody (Thermo). To do so, Calu-3 cultures on Transwell inserts
388 were chemically fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 1h at room
389 temperature. Cells were blocked in 10% goat serum and 1% bovine serum albumin (Sigma) for 15 min.
390 After blocking, cells were incubated with human anti-mouse ZO-1 (AlexaFluor-488 conjugate;
391 ThermoFisher)(5 μg/mL) for 1h. Transwell membranes were washed, removed from the supporting plastic
392 insert, and mounted on glass slides using Vectashield anti-fade mounting medium. Labeled cells were
393 visualized (ex. 480nm, em. 525nm) on an Olympus IX83 inverted fluorescence microscope using a 20X
394 objective lens (0.75 NA). Finally, cytotoxicity was determined by lactate dehydrogenase measurements on
395 cell-free supernatants using the Cytotoxicity Detection Kit Plus assay (Roche) according to manufacturer's
396 instructions. Data were calculated as percent LDH release compared with a lysed control and reported as
397 %LDH release = [(experimental value low control)/(high control-low control)] x 100.

398 **Immunoassays.** The pro-inflammatory cytokine response of Calu-3 cells was measured after 24h of
399 culture in the anaerobic chamber. To do so, spent medium was collected from the apical side of Transwell
400 cultures, and tumor necrosis factor alpha (TNF-α), interleukin (IL-) 8 and IL-6 were then measured by
401 ELISA per manufacturer instructions (R&D systems). Cells grown under standard incubator conditions (5%
402 CO_2) were used as a control.

403
404 **FPLC.** Secreted mucins were collected from Calu-3 cells as previously described (*24*). Briefly, cells grown
405 on Transwell inserts were solubilized in a reduction buffer consisting of 6M guanidine hydrochloride, 0.1M
406 Tris-HCl buffer, and 5mM EDTA (pH 8). Prior to solubilization, 10mM dithiothreitol (DTT) and a cOmplete
407 Mini protease inhibitor tablet (Roche) were added to 400mL of reduction buffer to minimize mucin
408 degradation. Cell suspensions were gently agitated by pipetting to dislodge biomass, and each of the six
409 Transwell suspensions per plate were pooled into a single aliquot. Cells were rinsed with a reduction buffer
410 to remove residual mucin. This mixture was then incubated for 5h at 37°C, followed by the addition of 25
411 mM iodoacetamide and incubation overnight at room temperature. Mucins were then dialyzed (1000 kDa

MWCO) against 1L of 4M GuHCl buffer containing 2.25 mM NaH_2PO_4-H_2O and 76.8 mM Na_2HPO_4 and proceeded for 36h with buffer exchanges every 12h.

Fast protein liquid chromatography (FPLC) size-exclusion chromatography was then used to evaluate the integrity of high-molecular weight mucins. Using an Akta Pure FPLC (GE Healthcare BioSciences, Marlborough, MA) housed at 4°C, 500 μL of purified mucin was manually injected and subjected to an isocratic run at a flow rate of 0.4 mL/min for 1.5 column volumes (CV) with 150mM NaCl in 50 mM phosphate buffer (pH 7.2) on a 15mL 10/200 Tricorn column packed with Sepharose 4B-CL beads. Data were collected using Unicorn 7 software (GE Healthcare Biosciences).

Bacterial strains and culture conditions. *P. aeruginosa* PA14 was obtained from D.K. Newman (California Institute of Technology) and was routinely cultured on Luria Bertani (LB) medium. *P. melaninogenica* ATCC 25845, *S. parasanguinis* ATCC15912, and *V. parvula* ATCC10790 were obtained from Microbiologics (St. Cloud, MN). *S. gordonii* was obtained from M.C. Herzberg (University of Minnesota) *and P. oris* 12252T was purchased from the Japan Collection of Microorganisms. All anaerobes were maintained on Brain-Heart Infusion medium supplemented with hemin (0.25 g/L), vitamin K (0.025 g/L) and laked sheep's blood (5% vol/vol) (BHI-HKB) in an anaerobic chamber. A mucin-enriched anaerobic bacterial community (ABC) derived from an individual with chronic rhinosinusitis was also used and was cultured in a minimal mucin medium (MMM) described previously (17).

Bacterial challenge and infection. Forty-eight hours prior to bacterial challenge, Calu-3 cells were incubated in MEM/FBS without antibiotics. On the day of bacterial challenge, Transwells were assembled in the gas permeable culture system and transferred into the anaerobic chamber where they were allowed to equilibrate for 3h. Overnight cultures of each anaerobe and the anaerobic community (ABC) were grown in MMM. Each individual culture was diluted to a concentration of ~1 x 10^6 colony forming units (CFU) in MEM and 10 μL of bacterial suspension was added to the apical side of the Calu-3 cells. Similarly, 10 μL of an adjusted suspension (OD600nm = 0.1) of the anaerobic community was added to separate wells. Co-cultures were then incubated for an additional 24h. Following anaerobe challenge, spent medium was collected and analyzed for cytotoxicity using the LDH colorimetric assay (described above). Mucins were

also collected as described above for integrity analysis via FPLC. In a separate experiment, anaerobe viability was determined using plate enumeration. Briefly, Calu-3 cells were washed with 100μL of PBS (to remove loosely bound cells) or 0.25% Triton X-100 (to recover tightly bound or intracellular bacteria). Resulting washes were serially diluted and plated on BHI or Brucella Laked Blood Agar (BBA) with Kanamycin (100 μg/mL) and Vancomycin (7.5 μg/mL) for enumeration.

For the *P. aeruginosa* colonization assay, Calu-3 cultures were removed from the anaerobic chamber following anaerobe challenge. Cells were gently washed with PBS and subsequently infected with 1 x 10^6 CFU of *P. aeruginosa* for 2h. After incubation, cells were gently washed three times with PBS to remove unbound *P. aeruginosa* and were permeabilized using 0.25% Triton X-100. Bacteria were enumerated by plating serial dilutions of Calu-3 cell lysates on LB agar. All assays were performed using three biological replicates and data are reported as the mean of three experiments.

RNA sequencing. The transcriptomic response of Calu-3 cells to anoxic culture and anaerobe challenge was determined using RNAseq. Calu-3 cells were cultured at ALI as described above and harvested after 24h of anaerobic incubation in the presence/absence of bacterial challenge. Normoxic (unchallenged) cells were maintained under standard incubator conditions. At the conclusion of each experiment, RNAlater (Invitrogen) was added to the apical and basolateral side of each well. For each condition, RNA was isolated from 5 or 6 Transwells using the RNeasy Micro Plus kit (Qiagen) according to manufacturer's instructions. DNase treatment was performed as part of the RNA Clean and Concentrator kit (Zymo). RNA quality (RIN > 9.7) and quantity were assessed using an Agilent Bioanalyzer and RiboGreen, respectively. cDNA libraries were prepared using the SMARTer Universal Low Input RNA Kit (Takara Bio) and submitted for sequencing at the University of Minnesota Genomics Center on the Illumina NovaSeq 6000 platform.

The Ensembl GTF annotation file was filtered to remove annotations for non-protein-coding features. Fastq files were evenly subsampled down to a maximum of 100,000 reads per sample. Data quality in fastq files was assessed with FastQC. Raw reads were mapped to reference Human (Homo_sapiens) genome assembly "GRCh38" using annotation from Ensembl release 98. Gene counts were generated with 'featureCounts' of the RSubread package (67). DESeq2/1.28.1 was used to estimate size factors to

generate normalized count data, estimate gene-wise dispersions, shrink estimates using type='ashr', and perform Wald hypothesis testing (68, 69). Genes with a $\log_2$ fold-change greater than 1 and Benjamini-Hochberg adjusted p-value < 0.001 were considered significant. Code and data files are shared at https://github.com/Hunter-Lab-UMN/Moore_PJ_2020.

Scanning electron microscopy. Untreated, anaerobe-challenged, and *P. aeruginosa*-infected cell cultures were washed three times in 0.2M sodium cacodylate buffer, and submerged in primary fixative (0.15 M sodium cacodylate buffer, pH 7.4, 2% paraformaldehyde, 2% glutaraldehyde, 4% sucrose, 0.15% alcian blue 8GX) for 22h. Transwell membranes were washed three more times prior to a 90 minute treatment with secondary fixative (1% osmium tetroxide, 1.5% potassium ferrocyanide, 0.135M sodium cacodylate, pH 7.4). After three final washes, cells were dehydrated in a graded ethanol series (25%, 50%, 75%, 85%, 2 x 95%, and 2 x 100%) for 10 minutes each before CO_2-based critical point drying. Transwell membranes were attached to SEM specimen mounts using carbon conductive adhesive tape and sputter coated with ~5 nm iridium using the Leica ACE 600 magnetron-based system. Cells were imaged using a Hitachi S-4700 field emission SEM with an operating voltage of 2kV. Images were false colored using Adobe Photoshop CS6.

Microtiter plate binding assay. *P. aeruginosa* adhesion to mucus was tested using an established microtiter plate-based assay (34). 96 well MaxiSorp microtiter plates (Nunc) were coated with 40μg/ml of mucins (MUC5AC) derived from untreated and ABC-treated Calu-3 cells. As a control, MUC5AC treated with neutrophil elastase (1 μg/mL) was also used. After coating, plates were incubated for 24h at 37°C. Mucin-coated wells were then washed three times with sterile PBS to remove any residual unbound mucin. 1 x 10^6 CFUs of *P. aeruginosa* PA14 were added to mucin-coated wells and incubated for an additional 2h at 37°C. Wells were washed 10 times with PBS to remove any unbound bacteria. Bound PA14 was desorbed by treatment with 200μl of 0.25% Triton X-100 for 15 min at room temperature. Bacteria bound to each well were enumerated by plating serial dilutions on LB agar. All assays were performed using three biological replicates and data are reported as the mean of three experiments (n=9).

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