Published in final edited form as:

J Leukoc Biol. 2018 October ; 104(4): 665–675. doi:10.1002/JLB.5HI1117-454RR.

PATHOLOGICAL CONDITIONING OF HUMAN NEUTROPHILS RECRUITED TO THE AIRWAY MILIEU IN CYSTIC FIBROSIS

Osric A. Forrest^{1,2}, Sarah A. Ingersoll^{1,2}, Marcela K. Preininger^{1,2}, Julie Laval^{1,2}, Dominique H. Limoli^{1,2}, Milton R. Brown^{1,2}, Frances E. Lee³, Brahmchetna Bedi⁴, Ruxana T. Sadikot^{3,4}, Joanna B. Goldberg^{1,2}, Vin Tangpricha^{3,4}, Amit Gaggar^{5,6}, Rabindra Tirouvanziam^{1,2}

¹Department of Pediatrics, Emory University, Atlanta, GA, USA

²Center for CF & Airways Disease Research, Children's Healthcare of Atlanta, Atlanta, GA, USA

³Department of Medicine, Emory University, Atlanta, GA, USA

⁴Atlanta VA Medical Center, Decatur, GA, USA

⁵Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, USA

⁶Birmingham VA Medical Center, Birmingham, AL, USA

Abstract

Rationale—Recruitment of neutrophils to the airways, and their pathological conditioning therein, drive tissue damage and coincide with the loss of lung function in patients with cystic fibrosis (CF). So far, these key processes have not been adequately recapitulated in models, hampering drug development. Here, we hypothesized that the migration of naïve blood neutrophils into CF airway fluid *in vitro* would induce similar functional adaptation to that observed *in vivo*, and provide a model to identify new therapies.

Methods—We used multiple platforms (flow cytometry, bacteria-killing, and metabolic assays) to characterize functional properties of blood neutrophils recruited in a transepithelial migration model using airway milieu from CF subjects as an apical chemoattractant.

Main findings—Similarly to neutrophils recruited to CF airways *in vivo*, neutrophils migrated into CF airway milieu *in vitro* display depressed phagocytic receptor expression and bacterial killing, but enhanced granule release, immunoregulatory function (arginase-1 activation), and metabolic activities including high Glut1 expression, glycolysis, and oxidant production. We also identify enhanced pinocytic activity as a novel feature of these cells. *In vitro* treatment with the leukotriene pathway inhibitor acebilustat reduces the number of transmigrating neutrophils, while the metabolic modulator metformin decreases metabolism and oxidant production, but fails to restore bacterial killing. Interestingly, we describe similar pathological conditioning of neutrophils in other inflammatory airway diseases.

Corresponding Author: Rabindra Tirouvanziam, PhD, Emory + Children's Center, 2015 Uppergate Dr NE, Rm 344, Atlanta, GA 30322-1014, USA, Tel: +1 (404) 712-7684, tirouvanziam@emory.edu.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors do not have any conflict of interest to declare relevant to this study.

Conclusions—We successfully tested the hypothesis that recruitment of neutrophils into airway milieu from patients with CF *in vitro* induces similar pathological conditioning to that observed *in vivo*, opening new avenues for targeted therapeutic intervention.

Summary sentence

Active degranulation and metabolic licensing of neutrophils recruited to cystic fibrosis airways can be recapitulated and modulated by drugs *in vitro*.

Graphical Abstract

Keywords

AMPK; Energy Metabolism; Exocytosis; Inflammation; Phagocytes

INTRODUCTION

Cystic fibrosis (CF) is the most frequent fatal recessive disease in Caucasians, caused by mutations in the CF transmembrane conductance regulator (cftr) gene [1]. Most of the morbidity and mortality in CF is due to airway disease, with a triad of obstruction, infection, and inflammation driven by polymorphonuclear neutrophils (PMNs) recruited from blood [2]. Inflammation occurs early in CF airway disease and persists through the patients' lifetime [3]. The first symptoms of airway damage are detected in small airways of CF infants [4], with bronchiectasis coinciding with both PMN presence in the lumen, and extracellular neutrophil elastase (NE) activity [5]. Extracellular NE activity is also a predictor of lung function in CF adults [6]. Currently, no therapy exists to counter PMNs and PMN-derived NE in CF disease.

In prior *in vivo* studies, we showed that NE is primarily released via exocytosis by live PMNs [7, 8]. In the CF airway milieu, PMNs also lose the surface phagocytic receptor CD16

[8], and modulate T-cell inhibitory molecules arginase-1 (Arg1) and programmed death-ligand 1 (PD-L1) [9]. These functional changes occur in the context of anabolic signaling [7], with increased surface Glut1 expression and glucose uptake [10], and track with high extracellular lactate levels [11], high oxygen consumption and reactive oxygen species (ROS) production [12], and redox imbalance [13]. Critically, such changes are not discernable in blood PMNs, and PMNs maintain diploid DNA content while undergoing changes in CF airways [7–10].

Together, changes undergone by PMNs in the CF airway milieu constitute a distinct fate, characterized by enhanced primary granule release leading to NE exocytosis, immunoregulatory function, and metabolic activities, henceforth dubbed the “GRIM” fate. Because of the major impact on CF airway disease of NE [14] and other GRIM PMN-dependent factors, these cells constitute a *bona fide* target for therapy [15]. However, a key limitation has been the absence of models recapitulating the GRIM fate [16, 17].

Importantly, PMN-driven inflammation is not only a hallmark of CF, but also of chronic obstructive pulmonary disease (COPD) and other chronic lung diseases (LD) such as severe asthma [18, 19], although the precise functional adaptations that recruited PMNs undergo in these contexts have been described in less details. Here, we describe a simple, yet robust *in vitro* model based on blood PMN transepithelial migration into airway milieu from patients with CF and other chronic inflammatory airway diseases, which recapitulates the multifaceted functional adaptations corresponding to the GRIM fate *in vivo* [17]. Using this model, drug effects on effector and metabolic pathways relevant to the GRIM fate of human airway PMNs can be tested *in vitro*, opening new avenues for drug development.

METHODS

Human subjects

The protocol for collection and handling of human samples was approved by the Institutional Review Board at Emory University. Informed consent was obtained from all subjects for collection and use of their samples. CF was diagnosed by sweat chloride (60 mEq/L), using a quantitative iontophoresis test and/or documentation of two identifiable *cfr* mutations. COPD and LD patients were diagnosed using American Lung Association criteria. HC subjects were non-smokers, with no history of chronic disease. CF patients were assessed at either one or two visits more than 6 months apart. HC, COPD and LD subjects were assessed at one visit. Suppl. Table 1 presents demographic data for all subjects.

Sample processing and airway supernatant preparation

Blood was collected by venipuncture. Sputum was collected from CF patients by spontaneous expectoration, and from HC, COPD and LD subjects by induction. Blood and sputum were processed as previously described [8]. In brief, blood was spun at 400 g to separate cells from platelet-rich plasma, which was further spun at 3,000 g to generate platelet-free plasma. Sputum was gently dissociated using repeated passage through an 18G needle after addition of 6 ml of PBS with 2.5 mM EDTA. Dissociated sputum was then spun at 800 g generating cell and fluid fractions. The fluid fraction was further spun at 3,000 g at

4°C for 10 minutes to generate a purified, cell and bacteria-free, airway supernatant -ASN-, which was stored at -80°C until use. Blood and airway cell fractions were re-suspended in PBS-EDTA and used for flow cytometric analysis. Blood was also used to prepare PMN fractions for in vitro experiments, as previously described [7]. In brief, blood layered onto Polymorphprep (PMP, Nycomed Pharma) was centrifuged at 400 g with minimal brake for 45 minutes at room temperature. The PMN layer was collected and erythrocytes removed by hypotonic lysis by 30-second incubation in water, after which PMNs were washed and resuspended in RPMI until use.

Transepithelial migration (TM) model

PMN transepithelial migration and subsequent inflammation occur first in the small airways of CF patients, i.e., the bronchiolar region, which is lined with a microvilli-covered epithelial monolayer dominated by Club cells [3–5]. Therefore, to mimic PMN transmigration into the small airway lumen, we selected the H441 human Club cell line [20] to grow epithelial monolayers at air-liquid interface (ALI). To enable PMN loading in the lamina propria and transepithelial migration (Fig. 1A), we used Alvetex (Reinervate) 200 µm-thick inert 3D scaffolds with >90% porosity (pore sizes of 36–40 µm, with interconnects of 12–14 µm). In brief, inserts were activated with 70% ethanol, coated overnight at 37°C with rat-tail collagen I (3 mg/mL, Sigma) and seeded with H441 cells at 2.5×10^5 cells per 12-well insert. Cells were first grown in submerged cultures with DMEM/F12 supplemented with 10% heat-inactivated serum, penicillin, and streptomycin. After 2 days, cells were supplemented basally with serum-free DMEM/F12 with 10% Ultrosor G (Pall Life Sciences) to establish ALI. Cultures were grown for 2 weeks at ALI and supplemented basally with fresh medium every 48 hours. For TM experiments, the ALI cultures were placed with the apical compartment exposed to RPMI, leukotriene B4 (LTB4, 100 nM), CXCL8 (100 ng/mL), formyl-methionine-leucine-phenylalanine (fMLF, 100 ng/mL), lipopolysaccharide (LPS, 500 ng/mL), or airway supernatant (ASN) from CF, HC, COPD, and LD subjects. TM experiments with $0.5\text{--}1 \times 10^6$ PMNs loaded onto the 200 µm-thick basal compartment of the Alvetex scaffold (situated upside), and allowed to migrate at 37°C at 5% CO₂ through the collagen and epithelial layers into the apical compartment (situated downside, and bathed with either control medium with chemoattractant, or ASN). In some experiments, drugs were added to apical ASN and/or basal PMN suspensions. In other experiments, LPS-RS (competitive inhibitor of LPS binding to TLR4) was added to apical LPS or CF ASN. LPS and LPS-RS were purchased as ultrapure reagents from InvivoGen.

Flow cytometry

Blood and airway PMNs collected from human subjects and from our transmigration model were assessed by flow cytometry using standard templates for staining, acquisition and analysis, as described, enabling quantitative comparison of in vivo and in vitro samples throughout the whole study [21]. Reagents used for the study included Live/Dead (Invitrogen), and antibodies against Arg1 (6G3, Hycult Biotech), as well as CD11b (M1/70), CD16 (3G8), CD41a (HIP8), CD45 (HI30), CD63 (H5C6), CD66b (G10F5), CD62L (DREG-56), and PD-L1 (29E.2A3) from BioLegend, and the ROS probe CellRox (ThermoFisher), using the gating strategy in Suppl. Fig. 1A. Phosphorylated 5' adenosine monophosphate-activated protein kinase (AMPK) α 1 levels were assessed using an antibody

against phospho-Thr172 (Biorbyt) using a previously published protocol [7]. For Glut1 detection, we used a specific receptor-binding domain probe (Metafora Biosciences), as detailed before [10]. Data were acquired on a LSRII cytometer (BD Biosciences) and compensated, gated and analyzed in Flowjo (Treestar). Live PMNs were counted using CountBright beads (Life Technologies), and expression of all markers is reported as median fluorescence intensities.

Pinocytosis assay

Pinocytosis was assessed via Lucifer Yellow (LY, Biotium) uptake, as described previously [22], with some modifications. For *in vivo* samples, a NH₄Cl solution (StemCell Technologies) was used to lyse erythrocytes from whole blood, yielding blood leukocytes. Airway cells were obtained as described above. Blood leukocytes and airway cells were then stained with probes for viability (Live/Dead) and antibody markers for PMNs (CD66b) and exocytosis (CD63), as described above. Cells were then incubated with LY diluted at 1 mg/mL in RPMI for 30 mins at 37°C. For *in vitro* samples, PMNs were transmigrated to CFASN containing LY (1 mg/mL) for 2, 10, and 18 hours, after which the cells were washed with PBS-EDTA and stained with viability probes and antibodies, as above. Cells were then washed with RPMI and pinocytosis was measured by flow cytometry.

Bacteria killing assay

P. aeruginosa strain PAO1 was grown in LB broth (BD Biosciences) at 37°C with aeration to an optical density (600 nm) of 0.5. Bacteria were pelleted by centrifugation at 14,000 g for 5 min at room temperature, resuspended in 500 µl 10% autologous plasma in RPMI and opsonized for 30 minutes at 37°C. Opsonized bacteria were added to PMNs at a multiplicity of infection of 0.1. After an hour of incubation tumbling at 37°C, the cultures were lysed using 0.1% Triton for 2 minutes, after which the bacteria were serially diluted and plated in triplicate on LB plates and incubated at 37°C in a non-CO₂ incubator overnight. Viable bacterial CFUs were counted the following day and bacterial viability was calculated as a percentage of bacterial counts at time 0.

Metabolic assays

Real-time analysis of the extracellular acidification rate (ECAR) as a measure of glycolysis, and Oxygen Consumption Rate (OCR), were performed using a Seahorse XFp Extracellular Flux Analyzer (Agilent). In brief, PMNs were collected after transmigration, resuspended in Seahorse assay medium (DMEM-based, without serum, glucose or bicarbonate) and plated at 7.5×10^5 cells/well on CellTak (BD Biosciences)-coated Seahorse assay plates. PMNs were attached to the bottom of the wells by centrifugation at 350 g (without brake), and incubated for 45 minutes before the assay in a non-CO₂ incubator at 37°C. The glycolysis stress test kit (Agilent) was used to obtain real-time measurements of ECAR and OCR upon sequential injections of glucose (10 mM) to induce glycolytic activity, oligomycin (complex V inhibitor, used at 3 µM) to shut down mitochondrial contribution to glucose consumption, 2-deoxyglucose (2-DG, 0.1 M) to competitively inhibit glucose use by hexokinase, and when indicated diphenyleneiodonium (DPI, 10 µM), an NADPH oxidase inhibitor. Extracellular lactate was measured in culture supernatants after Seahorse runs using the lactate assay kit (Biovision), according to manufacturer's instructions.

Murine PMN isolation and transmigration

Bone marrow PMNs were collected from the tibias and femurs of wild-type mice by flushing with RPMI with 10% FBS using a 25-gauge needle. Cells were then washed with PBS and allowed to migrate in our model to either CFASN or LTB₄ for 5 or 10 hours. Recruited cells were stained with antibodies and analyzed by flow cytometry as detailed above for CD11b (M1/70) and Ly6G (1A8) expression to identify mature PMNs, and for CD63 (NVG-2) expression to assess primary granule release. Antibodies were from BioLegend.

Data analysis

Statistical analyses were performed using JMP12 (SAS Institute). Between-group and matched-pair statistical analyses used the Wilcoxon rank sum and signed rank tests, respectively. Correlations were tested using the non-parametric Spearman test. A threshold of $p < .05$ was used to determine significance.

RESULTS

Core features of CF airway PMNs are recapitulated upon blood PMN transmigration *in vitro*

We designed a model (Fig. 1A) in which purified airway supernatant (ASN) from CF patients leads to the transepithelial migration of blood PMNs, yielding large numbers of “airway-like” PMNs that can be identified by flow cytometry (Suppl. Fig. 1A). PMNs transmigrated into CF ASN shed the CD62L receptor, as expected after transmigration (Fig. 1B). Remarkably, these cells also recapitulated core phenotypes of PMNs recruited to the CF airway lumen *in vivo*, namely downmodulation of the phagocytic receptor CD16 (Fig. 1C), and high CD63 expression (Fig. 1D), reflecting active release of NE-rich granules [18]. While *in vivo* samples contain a mixed population of PMNs recruited to CF airways over hours/days, our model enables collection of transmigrated PMNs at precise intervals, so that the dynamic course of pathological conditioning can be resolved (Suppl. Fig. 1B). Next, we assessed the status of the epithelial layer after PMN transmigration induced by LTB₄ and CF ASN. First, using confocal imaging (Suppl. Fig 2A), we observed that both conditions grossly preserved the integrity of the epithelial layer based on nuclear staining, and ZO-1 expression (reflecting junctionality). Second, using an extracellular lactate dehydrogenase assay (Suppl Fig 2B), we observed that epithelial cell viability was only slightly decreased after PMN transmigration compared to baseline conditions, and did not differ between LTB₄ and CF ASN conditions. Third, we checked viability of EpCAM⁺ epithelial cells after detachment from the filters using flow cytometry (Suppl Fig 2C), and confirmed that they were highly viable, with no difference between LTB₄ and CF ASN conditions. Finally, we observed that epithelial permeability to 4 kDa (significant) and 70 kDa (non significant trend) dextrans after LTB₄- and CF ASN-induced PMN transmigration compared to baseline (Suppl. Fig 2D). However, it remained lower in LTB₄ and CF ASN conditions than in the Triton-X maximum permeability control.

Healthy control airway milieu promotes transmigration, but not pathological conditioning, of PMNs

PMNs transmigrated to HC and CF ASN showed similar loss of CD62L, while CD16 downregulation and CD63 upregulation were less pronounced at all time points for PMNs transmigrated to HC ASN (Fig. 2A–C). In addition, HC ASN recruited PMNs in lesser numbers than CF ASN at 10 and 18 hours PTM (Fig. 2D). These patterns recapitulate differences between HC and CF airway PMNs observed *in vivo* [8] (independently reproduced in Suppl Fig. 3A–C). We also showed previously [9] that the immunomodulatory receptor PD-L1 is uniformly increased *in vivo* on HC airway PMNs compared to HC blood PMNs, as also seen in our model, while CF airway PMNs cluster into PD-L1^{Lo} and PD-L1^{Hi} subsets, a bimodal pattern also recapitulated in our model (Suppl. Fig. 4A). In both HC and CF subjects, airway PMNs increase surface expression of Arg1 compared to blood PMNs [9], a pattern again recapitulated *in vitro* (Suppl. Fig. 4B).

Pathological conditioning induced by CF ASN on transmigrated PMNs is independent of their origin

Next, we investigated whether the origin of blood PMNs affected transmigration and pathological conditioning. We found that blood PMNs from HC and CF subjects do not markedly differ in their recruitment and exocytosis profile after transmigration to CF ASN (Suppl. Fig. 3D). Murine PMNs (from wild-type animals) also undergo transmigration and primary granule hyperexocytosis in the context of CF ASN (Suppl. Fig. 3E). Thus, our data demonstrate a dominant role for CF ASN in causing pathological conditioning of transmigrated PMNs, irrespective of their origin.

CF airway milieu and transmigration are both critical to pathological conditioning of PMNs

A low dilution of CF ASN (1:3) leads to levels of primary granule exocytosis in transmigrated PMNs matching those seen in CF patients *in vivo*, while intermediate (1:30) and high (1:300) dilutions lead to two- and four-fold lower levels, respectively (Fig. 2E). Diluting CF ASN also decreases transmigrated PMN count (Fig. 2F). CF ASN contains multiple factors able to recruit and activate PMNs, including host chemoattractants [23] like LTB₄ and CXCL8, and bacterial products [14] like fMLF and LPS. In our model, LTB₄, CXCL8, and fMLF alone failed to induce primary granule exocytosis, despite promoting transmigration (Fig. 2G–H). Importantly, direct incubation of blood PMNs in CF ASN without transmigration failed to induce primary granule exocytosis, and delayed the downregulation of CD16 (Fig. 2I–J). Thus, pathological conditioning of blood PMNs by CF ASN requires transmigration, and can be modulated by experimental dilution of the ASN.

Inhibition of LPS and LTB₄ signaling does not impact the pathological conditioning of PMNs by the CF airway milieu

LPS alone failed to induce primary granule exocytosis or CD16 downregulation when used as an apical stimulus in the model (Fig. 3A–B). LPS signals through Toll-like receptor 4, and blockade of this signaling cascade in PMNs using LPS-RS [24] did not affect primary granule exocytosis and CD16 modulation, whether in the context of CF ASN or LPS (Fig. 3A–B). LPS-RS down-modulated surface CD11b expression and intracellular ROS

accumulation in PMNs in the context of both LPS and CF ASN at 2 hours PTM, confirming the activating potential of LPS on airway PMNs, and the inhibitory mode of action of LPS-RS on LPS-induced activation. LTB₄ is a critical host-derived chemoattractant orchestrating PMN migration through the lamina propria [25]. Acebilustat blocks LTB₄ production by inhibiting LTA₄ hydrolase, the rate-limiting enzyme for LTB₄ synthesis [26]. In our model, acebilustat decreased the apical PMN count at 2, 10 and 18 hours PTM (Fig. 3C), but it did not affect primary granule exocytosis or CD16 downregulation in PMNs (Fig. 3D).

ASN from various airway diseases induce the GRIM fate upon transmigration

Severe asthma and bronchiolitis are examples of other diseases in which PMN dysfunction has been implicated as a potential pathogenic process [18]. In our model, airway milieu from patients with these conditions leads to PMN transmigration and primary granule exocytosis, again recapitulating *in vivo* data (Suppl. Fig. 5A–C). In patients with COPD, disease symptoms, airway PMN dysfunction, and release of toxic by-products such as NE are similar to those observed in CF [19]. In our model, COPD ASN leads to PMN recruitment and exocytosis of primary granules, mimicking *in vivo* data (Suppl. Fig. 5D–E). Similarly to what we observed for CF ASN, CD16 is also downregulated upon PMN transmigration to COPD ASN (Suppl. Fig. 5E).

Increased pinocytosis is a newly identified feature of PMNs transmigrated to the CF airway milieu, *in vivo* and *in vitro*

A prior study linked enhanced primary granule release by PMNs to pinocytosis [27]. In CF patients, airway PMNs showed enhanced pinocytosis compared to blood PMNs, and pinocytic activity in CF airway PMNs correlated with primary granule release (Fig. 4A). In our model, PMNs transmigrated to CF ASN increased their pinocytic activity over time, with a similar correlation with primary granule release to that observed *in vivo* (Fig. 4B).

PMN transmigration to CF airway milieu leads to increased metabolism, but decreased bacterial killing

In vivo, CF airway PMNs undergo metabolic activation [7, 10]. *In vitro*, PMNs transmigrated to CF ASN show increased glycolysis, as reflected by a higher extracellular acidification rate, and increased surface Glut1 expression, compared to those transmigrated to LTB₄ (Fig. 5A–D). PMNs transmigrated to CF ASN also showed a strong upregulation of their oxygen consumption rate, which was not sensitive to the inhibitor of mitochondrial oxidative phosphorylation oligomycin (Fig. 5E–F), but was associated with an increased ROS production (Fig. 5G). This is consistent with the notion that activated PMNs use oxygen for ROS production via either NADPH oxidase or mitochondrial complex I, with support from glycolysis [28, 29]. Despite this metabolically activated state, PMNs transmigrated to CF ASN showed lower ability to kill *P. aeruginosa* than those transmigrated to LTB₄ (Fig. 5H). Thus, our model recapitulates a key paradox in CF, namely the inability to kill bacteria [14], despite PMN recruitment and their metabolic activation.

Metformin inhibits key pathological phenotypes of PMNs recruited to the CF airway milieu

Metformin is a metabolic modulator able to curb down glucose-dependent cell activation, ROS production, and inflammation [29, 30]. Metformin had a mild effect on glycolysis, but strongly inhibited oxygen consumption in PMNs transmigrated to CF ASN (Fig. 6A–B). Metformin acts, in part, via activation of the metabolic checkpoint enzyme 5' adenosine monophosphate-activated protein kinase $\alpha 1$ (AMPK $\alpha 1$), by facilitating its phosphorylation [31]. We observed that phosphorylated AMPK $\alpha 1$ levels were indeed reduced in CF airway compared to blood PMNs *in vivo*, and in PMNs transmigrated to CF ASN compared to LTB4 *in vitro*, and that treatment with metformin increased phosphorylated AMPK $\alpha 1$ levels in PMNs transmigrated to CF ASN (Fig. 6C). This effect was associated with a reduction of ROS production and primary and secondary granule exocytosis (Fig. 6D). Interestingly, metformin treatment did not rescue the ability of PMNs transmigrated to CF ASN to kill bacteria (Fig. 6E).

DISCUSSION

Massive airway recruitment of PMNs, and their pathological conditioning resulting in heightened granule release, immunoregulatory, and metabolic activities ("GRIM" fate), and depressed bacterial killing, drive early and chronic lung damage in CF and other intractable diseases. These processes are incompletely understood and remain untapped as potential therapeutic targets, in large part due to the lack of adequate models. The model described here enables transepithelial recruitment and pathological conditioning yielding GRIM PMNs that feature characteristic hyperexocytosis of NE-rich granules, but also decreased surface CD16, increased Arg1 expression, bimodal PD-L1 expression, increased metabolic activity and decreased bacterial killing, all hallmarks of CF airway GRIM PMNs *in vivo*. In addition, we describe a new metabolic and functional feature of these cells *in vivo* and *in vitro*, which relates to their enhanced pinocytosis.

A dominant conditioning effect of CF airway secretions was noted in studies where these were acutely added to healthy or CF airway epithelium [32], and macrophages [33], although their effect on PMNs was not explored. In our model, apical CF ASN was necessary and sufficient to recruit and condition blood PMNs to adopt the GRIM fate, regardless of whether these PMNs originate from CF or HC subjects. Thus, our data demonstrate a dominant environmental effect of factors present in CF airway milieu onto recruited PMNs. However, they do not exclude a contribution of endogenous CFTR in PMNs [34, 35], although such an effect appears to be secondary relative to the induction of the pathogenic GRIM fate. Further studies are needed to fully delineate the still debated issue of whether endogenous CFTR is key to the function of PMNs recruited to CF airways *in vivo*, and in this model.

Our data show that PMN transmigration toward ASN from COPD and LD patients also lead to substantial primary granule exocytosis, similar to the results obtained with CF ASN, while HC ASN induced transmigration but not primary granule exocytosis. Thus, PMN pathological conditioning into the GRIM fate is not unique to CF. Murine PMNs also undergo transmigration and hyperexocytosis of NE-rich granules, suggesting that factors in CF ASN leading to PMN pathological conditioning are cross-specific. This result is

reminiscent of the cross-specific recruitment of murine PMNs in human CF small airway xenografts [36]. It also opens potential avenues for future studies using relevant mouse strains in our model to assess mechanisms of PMN plasticity and conditioning [14], and further delineate the role of endogenous CFTR using PMNs from wild-type and CFTR knockout or mutant mice.

CF ASN is characterized by an abnormal molecular composition (including altered lactate [11], small metabolite [37], and protein [38] contents), itself caused by altered epithelial function, and the sustained presence of bacteria and PMNs in the CF airway lumen [14]. It is interesting to note that at concentrations similar to those used to promote PMN transmigration and conditioning in our model, CF ASN induces T-cell apoptosis [9]. At lower concentrations, T-cells survive but their ability to activate and proliferate is dramatically reduced, due in part to Arg1 activity originating from GRIM PMNs. Both Arg1 and PD-L1, another prominent T-regulatory molecule, are modulated in the model in similar fashion to *in vivo* [9]. Thus, CF ASN exerts paradoxical effects on PMNs and T-cells, promoting the former while demoting the latter, which further contributes to the build-up of a PMN-dominated inflammatory environment in the CF airway lumen.

The ability of CF ASN to license recruited PMNs metabolically toward increased Glut1 expression and glycolytic activity is of particular significance, since PMNs are generally thought of as short-lived, yet thrive and undergo transcriptional changes [10] in this pathological milieu. In addition, GRIM PMNs obtained by transmigration into CF ASN show decreased killing of *P. aeruginosa* as compared to those transmigrated to LTB₄, despite their metabolic licensing, which is consistent with CF pathology *in vivo* [14, 15]. Further studies will focus on this paradox and screen for drugs capable of rewiring GRIM PMNs to enact proper bacterial clearance. The correlation between pinocytic activity and primary granule exocytosis in PMNs migrated to the CF airway lumen *in vivo* and *in vitro* also suggests a link between metabolic activity and abnormal granule mobilization [27], which warrants further study.

In typical Transwell models, PMNs are forced to squeeze one-by-one through pre-drilled 3 µm-wide cylindrical pores before reaching the epithelial basement membrane. By contrast, our model includes a highly porous lamina propria-like scaffold with 36–40 µm-wide pores that can each accommodate 20+ cells, thus enabling PMNs to congregate and migrate in physiologically relevant manner [25]. Confocal imaging confirmed that LTB₄- and CF ASN-induced PMN transmigration largely preserved the integrity of the epithelial layer, including typical peripheral ZO-1 staining pattern. In addition, we ascertained that epithelial viability was preserved, based on extracellular lactate dehydrogenase assay and flow cytometry. Dextran permeability assays showed that LTB₄- and CF ASN-induced PMN transmigration led to an expected increase in transepithelial permeability, which remained well below that observed in Triton X-treated monolayers (maximum permeability control). These results emphasize the physiological relevance and robustness of this experimental model.

While we plan to test the use of primary epithelial cells from CF patients in our model in future studies, the present set-up including a commercial 3D substrate, a small airway epithelial cell line, standard culture media, and human blood PMNs, provides a highly robust

and reproducible platform to mass-produce airway-like PMNs for research. The CF ASN used to induce PMN migration and pathological conditioning is a very abundant material easily collected from expectorations. Our model is advantageous not only because it integrates patient airway milieu as the apical stimulus; but also because blood PMNs from patients can be integrated as target cells; and because drugs can be readily added to blood and/or airway sides, mimicking systemic and airway routes of administration. These combined features provide the ability to de-risk drugs at an early stage, in conditions close to those seen *in vivo*.

Because of the long-held belief that PMNs die rapidly after migration into CF airways, PMN-targeted therapies have focused on reducing their recruitment via corticosteroids or non-steroidal anti-inflammatories [39]. Because GRIM PMNs found in CF airways are alive when they release their toxic contents [7, 8], the pathological conditioning of PMNs, rather than their recruitment, may be a more effective target for new therapeutic agents to benefit patients with CF and other similar diseases [40]. Our data obtained with the metabolic regulator metformin illustrate that point. Indeed, combined effects of metformin on GRIM PMN metabolism (i.e., oxygen consumption), ROS production, and degranulation, suggest that pathogenic functions in these cells are fueled by dysregulated metabolism, and that targeting metabolism in GRIM PMNs may be a relevant strategy for therapeutic intervention. Whether metformin is a relevant drug in the context of CF is questionable, however, since it did not rescue but rather seemed to further depress the ability of PMNs transmigrated to CF ASN to kill bacteria. Further studies are required to determine whether this adverse effect of metformin treatment may be linked to its ability to counter ROS production and degranulation, an otherwise beneficial effect of metformin considering the self-damaging impact of ROS and granule effectors unleashed by pathologically conditioned PMNs in CF airways.

Together, this study emphasizes the complex roles of GRIM PMNs, which represent a promising target for immunotherapy in CF and other human airway diseases. We also introduce a simple, robust and flexible *in vitro* model for basic studies and drug development efforts focused on this key pathogenic subset.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors would like to thank Dr. J. Alvarez, as well as Ms. W. Si Hassen for assistance; the Emory + Children's Pediatric Flow Core (A. Rae and Dr. D. Archer), and Integrated Confocal Imaging Core for access to flow cytometry and confocal imaging systems; Children's Healthcare of Atlanta and Emory University Pediatric CF Discovery Core, Mouse Models Core, and Experimental Models Core (M. Maliniak, J. Flores, J. Wu, B. Imhoff, and Drs. S. Molina, N. McCarty and A. Stecenko) for access to CF patient samples, mouse tissues and confocal microscopy expertise; Drs. A. Fitzpatrick and S. Stevenson for HC sputum collection; and Dr. E. Springman (Celtaxsys, Inc.) for his generous donation of the acebilustat drug.

FUNDING SOURCES

This study was supported by the Emory Pediatrics Startup, EECR Seed, and URC Programs (R.T.), Cystic Fibrosis Foundation (TANGPR12A0 to V.T.; MCCART15RC, TIROUV13P0, and TIROUV15A0 to R.T.), and National

Institutes of Health (T32 AA013528 to S.I.; R01HL102371 to A.G.; UL1TR000454 to V.T., and R.T.; and R01HL126603 to R.T.).

ABBREVIATIONS

ALI	air-liquid interface
AMPKα1	5' adenosine monophosphate-activated protein kinase α 1
Arg1	arginase-1
ASN	airway supernatant
CF	cystic fibrosis
CFTR	CF transmembrane conductance regulator
COPD	chronic obstructive pulmonary disease
ECAR	extracellular acidification rate
fMLF	formyl-methionine-leucine-phenylalanine
GRIM	granule-releasing, immunomodulatory, and metabolically active
HC	healthy control
LD	lung disease
LPS	lipopolysaccharide
LTB4	leukotriene B4
LY	Lucifer Yellow
NE	neutrophil elastase
OCR	oxygen consumption rate
PD-L1	programmed death-ligand 1
PMN	polymorphonuclear neutrophil
PTM	post-transmigration
ROS	reactive oxygen species

REFERENCES

1. Cutting GR (2015) Cystic fibrosis genetics: from molecular understanding to clinical application. *Nat Rev Genet* 16, 45–56. [PubMed: 25404111]
2. Hartl D, Gaggar A, Bruscia E, Hector A, Marcos V, Jung A, Greene C, McElvaney G, Mall M, Doring G (2012) Innate immunity in cystic fibrosis lung disease. *J Cyst Fibros* 11, 363–82. [PubMed: 22917571]
3. Stoltz DA, Meyerholz DK, Welsh MJ (2015) Origins of cystic fibrosis lung disease. *New Engl J Med* 372, 351–62. [PubMed: 25607428]

4. Tiddens HA, Donaldson SH, Rosenfeld M, Pare PD (2010) Cystic fibrosis lung disease starts in the small airways: can we treat it more effectively? *Ped Pulmonol* 45, 107–17.
5. Sly PD, Gangell CL, Chen L, Ware RS, Ranganathan S, Mott LS, Murray CP, Stick SM (2013) Risk factors for bronchiectasis in children with cystic fibrosis. *New Engl J Med* 368, 1963–70. [PubMed: 23692169]
6. Sagel SD, Sontag MK, Wagener JS, Kapsner RK, Osberg I, Accurso FJ (2002) Induced sputum inflammatory measures correlate with lung function in children with cystic fibrosis. *J Pediatr* 141, 811–7. [PubMed: 12461498]
7. Makam M, Diaz D, Laval J, Gernez Y, Conrad CK, Dunn CE, Davies ZA, Moss RB, Herzenberg LA, Herzenberg LA, Tirouvanziam R (2009) Activation of critical, host-induced, metabolic and stress pathways marks neutrophil entry into cystic fibrosis lungs. *Proc Natl Acad Sci USA* 106, 5779–83. [PubMed: 19293384]
8. Tirouvanziam R, Gernez Y, Conrad CK, Moss RB, Schrijver I, Dunn CE, Davies ZA, Herzenberg LA, Herzenberg LA (2008) Profound functional and signaling changes in viable inflammatory neutrophils homing to cystic fibrosis airways. *Proc Natl Acad Sci USA* 105, 4335–9. [PubMed: 18334635]
9. Ingersoll SA, Laval J, Forrest OA, Preininger M, Brown MR, Arafat D, Gibson G, Tangpricha V, Tirouvanziam R (2015) Mature cystic fibrosis airway neutrophils suppress T cell function: evidence for a role of arginase 1 but not programmed death-ligand 1. *J Immunol* 194, 5520–8. [PubMed: 25926674]
10. Laval J, Touhami J, Herzenberg LA, Conrad C, Taylor N, Battini JL, Sitbon M, Tirouvanziam R (2013) Metabolic adaptation of neutrophils in cystic fibrosis airways involves distinct shifts in nutrient transporter expression. *J Immunol* 190, 6043–50. [PubMed: 23690474]
11. Bense T, Stotz M, Borneff-Lipp M, Wollschläger B, Wienke A, Taccetti G, Campana S, Meyer KC, Jensen PO, Lechner U, Ulrich M, Döring G, Worlitzsch D (2011) Lactate in cystic fibrosis sputum. *J Cyst Fibros* 10, 37–44. [PubMed: 20947455]
12. Kolpen M, Hansen CR, Bjarnsholt T, Moser C, Christensen LD, van Gennip M, Ciofu O, Mandsberg L, Kharazmi A, Döring G, Givskov M, Hoiby N, Jensen PO (2010) Polymorphonuclear leucocytes consume oxygen in sputum from chronic *Pseudomonas aeruginosa* pneumonia in cystic fibrosis. *Thorax* 65, 57–62. [PubMed: 19846469]
13. Tirouvanziam R, Conrad CK, Bottiglieri T, Herzenberg LA, Moss RB, Herzenberg LA (2006) High-dose oral N-acetylcysteine, a glutathione prodrug, modulates inflammation in cystic fibrosis. *Proc Natl Acad Sci USA* 103, 4628–33. [PubMed: 16537378]
14. Cohen TS and Prince A (2012) Cystic fibrosis: a mucosal immunodeficiency syndrome. *Nat Med* 18, 509–19. [PubMed: 22481418]
15. Laval J, Ralhan A, Hartl D (2016) Neutrophils in cystic fibrosis. *Biol Chem* 397, 485–96. [PubMed: 26854289]
16. Lavelle GM, White MM, Browne N, McElvaney NG, Reeves EP (2016) Animal models of cystic fibrosis pathology: phenotypic parallels and divergences. *Biomed Res Int* 2016, 5258727. [PubMed: 27340661]
17. Margaroli C and Tirouvanziam R (2016) Neutrophil plasticity enables the development of pathological microenvironments: implications for cystic fibrosis airway disease. *Mol Cell Pediatr* 3, 38. [PubMed: 27868161]
18. Bruijnzeel PL, Uddin M, Koenderman L (2015) Targeting neutrophilic inflammation in severe neutrophilic asthma: can we target the disease-relevant neutrophil phenotype? *J Leukoc Biol* 98, 549–56. [PubMed: 25977288]
19. Dransfield MT, Wilhelm AM, Flanagan B, Courville C, Tidwell SL, Raju SV, Gaggar A, Steele C, Tang LP, Liu B, Rowe SM (2013) Acquired cystic fibrosis transmembrane conductance regulator dysfunction in the lower airways in COPD. *Chest* 144, 498–506. [PubMed: 23538783]
20. Kulaksiz H, Schmid A, Honscheid M, Ramaswamy A, Cetin Y (2002) Clara cell impact in air-side activation of CFTR in small pulmonary airways. *Proc Natl Acad Sci USA* 99, 6796–801. [PubMed: 12011439]
21. Tirouvanziam R, Diaz D, Gernez Y, Laval J, Crubezy M, Makam M (2011) An integrative approach for immune monitoring of human health and disease by advanced flow cytometry

methods In: Advanced Optical Flow Cytometry. Tuchin V, Ed. Wiley-VCH Verlag GmbH & Co. KGaA 333–362.

22. Fittschen C and Henson PM (1994) Linkage of azurophil granule secretion in neutrophils to chloride ion transport and endosomal transcytosis. *J Clin Invest* 93, 247–55. [PubMed: 8282794]
23. Mackerness KJ, Jenkins GR, Bush A, Jose PJ (2008) Characterisation of the range of neutrophil stimulating mediators in cystic fibrosis sputum. *Thorax* 63, 614–20. [PubMed: 18245144]
24. Stevens CW, Aravind S, Das S, Davis RL (2013) Pharmacological characterization of LPS and opioid interactions at the toll-like receptor 4. *Br J Pharmacol* 168, 1421–9. [PubMed: 23083095]
25. Afonso PV, Janka-Junttila M, Lee YJ, McCann CP, Oliver CM, Aamer KA, Losert W, Cicerone MT, Parent CA (2012) LTB4 is a signal-relay molecule during neutrophil chemotaxis. *Dev Cell* 22, 1079–91. [PubMed: 22542839]
26. Elborn JS, Horsley A, MacGregor G, Bilton D, Grosswald R, Ahuja S, Springman EB (2017) Phase I studies of acebilustat: biomarker response and safety in patients with cystic fibrosis. *Clin Transl Sci* 10, 28–34. [PubMed: 27806191]
27. Hong CW, Kim TK, Ham HY, Nam JS, Kim YH, Zheng H, Pang B, Min TK, Jung JS, Lee SN, Cho HJ, Kim EJ, Hong IH, Kang TC, Lee J, Oh SB, Jung SJ, Kim SJ, Song DK (2010) Lysophosphatidylcholine increases neutrophil bactericidal activity by enhancement of azurophil granule-phagosome fusion via glycine.GlyR alpha 2/TRPM2/p38 MAPK signaling. *J Immunol* 184, 4401–13. [PubMed: 20237295]
28. Baillet A, Hograindleur MA, El Benna J, Grichine A, Berthier S, Morel F, Paclet MH (2017) Unexpected function of the phagocyte NADPH oxidase in supporting hyperglycolysis in stimulated neutrophils: key role of 6-phosphofructo-2-kinase. *FASEB J* 31, 663–673. [PubMed: 27799347]
29. Zmijewski JW, Lorne E, Zhao X, Tsuruta Y, Sha Y, Liu G, Siegal GP, Abraham E (2008) Mitochondrial respiratory complex I regulates neutrophil activation and severity of lung injury. *Am J Respir Crit Care Med* 178, 168–79. [PubMed: 18436790]
30. Piwkowska A, Rogacka D, Jankowski M, Dominiczak MH, Stepinski JK, Angielski S (2010) Metformin induces suppression of NAD(P)H oxidase activity in podocytes. *Biochem Biophys Res Commun* 393, 268–73. [PubMed: 20123087]
31. Rena G, Hardie DG, Pearson ER (2017) The mechanisms of action of metformin. *Diabetologia* 60, 1577–1585. [PubMed: 28776086]
32. Ribeiro CM, Hurd H, Wu Y, Martino ME, Jones L, Brighton B, Boucher RC, O'Neal WK (2009) Azithromycin treatment alters gene expression in inflammatory, lipid metabolism, and cell cycle pathways in well-differentiated human airway epithelia. *PLoS One* 4, e5806. [PubMed: 19503797]
33. Lubamba BA, Jones LC, O'Neal WK, Boucher RC, Ribeiro CM (2015) X-Box-binding protein 1 and innate immune responses of human cystic fibrosis alveolar macrophages. *Am J Respir Crit Care Med* 192, 1449–61. [PubMed: 26331676]
34. Painter RG, Valentine VG, Lanson NA Jr., Leidal K, Zhang Q, Lombard G, Thompson C, Viswanathan A, Nauseef WM, Wang G, Wang G (2006) CFTR Expression in human neutrophils and the phagolysosomal chlorination defect in cystic fibrosis. *Biochemistry* 45, 10260–9. [PubMed: 16922501]
35. Pohl K, Hayes E, Keenan J, Henry M, Meleady P, Molloy K, Jundi B, Bergin DA, McCarthy C, McElvaney OJ, White MM, Clynes M, Reeves EP, McElvaney NG (2014) A neutrophil intrinsic impairment affecting Rab27a and degranulation in cystic fibrosis is corrected by CFTR potentiator therapy. *Blood* 124, 999–1009. [PubMed: 24934256]
36. Tirouvanziam R, Khazaal I, Peault B (2002) Primary inflammation in human cystic fibrosis small airways. *Am J Physiol Lung Cell Mol Physiol* 283, L445–51. [PubMed: 12114207]
37. Esther CR Jr., Coakley RD, Henderson AG, Zhou YH, Wright FA, Boucher RC (2015) Metabolomic evaluation of neutrophilic airway inflammation in cystic fibrosis. *Chest* 148, 507–15. [PubMed: 25611918]
38. Peters-Hall JR, Brown KJ, Pillai DK, Tomney A, Garvin LM, Wu X, Rose MC (2015) Quantitative proteomics reveals an altered cystic fibrosis in vitro bronchial epithelial secretome. *Am J Respir Cell Mol Biol* 53, 22–32. [PubMed: 25692303]

39. Cantin AM, Hartl D, Konstan MW, Chmiel JF (2015) Inflammation in cystic fibrosis lung disease: Pathogenesis and therapy. *J Cyst Fibros* 14, 419–30. [PubMed: 25814049]
40. Gernez Y, Tirouvanziam R, Chanez P (2010) Neutrophils in chronic inflammatory airway diseases: can we target them and how? *Eur Respir J* 35, 467–9. [PubMed: 20190324]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. *In vitro* model mimicking human PMN transmigration and pathological conditioning in small airways.

(A) We established a model emulating the small airway mucosa by growing the H441 Club-like human small airway cell line at air-liquid interface (upper left insert, arrowheads pointing to apical microvilli typical of Club cells) onto a continuous collagen layer covering a 200 μ m thick, porous scaffold (lower left insert). The apical milieu impacts the conditioning of transmigrated PMNs, depending on its origin, e.g., airway supernatant (ASN) from CF or control subjects, or medium containing chemoattractants. Drugs can be tested in the model, via apical and/or basal addition. Transmigrated PMNs conditioned by the apical milieu of choice, exposed or not to candidate drugs, can be retrieved in large numbers and submitted to downstream assays. (B-D) Analysis of CD62L, CD16, and CD63 expression, respectively, shown in box plots (left panels) and representative histograms (right panels) for blood PMNs at 2 and 18 hours post-transmigration (PTM) into CF ASN (black), compared to PMNs pre-transmigration (grey). * indicates $p < .05$ at 2 or 18 hours PTM vs. pre-transmigration ($n = 8$ experiments).

Figure 2. CF ASN and transmigration are both required to induce pathological conditioning of PMNs *in vitro*.

(A-D) Analysis of CD62L, CD16, and CD63 expression and relative PMN accumulation, respectively, for PMNs transmigrated to HC control (light grey) vs. CF ASN (black) at 2, 10 and 18 hours PTM, compared to PMNs pre-transmigration (grey). The relative PMN accumulation at each time point was calculated as % fold change compared to the number of PMNs transmigrated into CF ASN at 2 hours PTM (set at 100%). *indicates $p < .05$ for HC vs. CF ASN ($n = 8$ experiments). (E-F) Analysis of CD63 expression and relative PMN

accumulation, respectively, for PMNs transmigrated to CF ASN at various dilutions, 2, 10, and 18 hours PTM. The relative PMN accumulation at each time point was calculated as % fold change compared to the number of PMNs transmigrated into CF ASN at the minimal dilution at 2 hours (set at 100%). * indicates $p < .05$ in matched pairs statistics between dilutions ($n = 5$ experiments). **(G-H)** Analysis of CD63 expression and relative PMN accumulation, respectively, for PMNs transmigrated to CF ASN, CXCL8 (100 ng/mL), fMLF (100 nM), or LTB4 (100 nM) at 2, 10, and 18 hours PTM. The relative PMN accumulation at each time point was calculated as % fold change compared to the number of PMNs transmigrated into CF ASN at the minimal dilution at 2 hours (set at 100%). * indicates $p < .05$ in matched pairs statistics between conditions ($n = 5$ experiments). **(I-J)** Analysis of CD63 and CD16 expression, respectively, for PMNs transmigrated to CF ASN (TRM, black), or incubated in it without transmigration (INC, grey) for 2 and 18 hours. * indicates $p < .05$ in matched pairs statistics between conditions ($n = 5$ experiments).

Figure 3. Inhibition of LPS and LTB4 signaling does not impact pathological conditioning of PMNs by CF ASN.

(A-B) Analysis of CD63 and CD16 expression, respectively, for PMNs transmigrated to LPS (500 ng/mL) or CFASN, each tested in the context of vehicle control (black) or LPS blockade by LPS-RS (5 µg/mL, grey), 2 and 10 hours PTM (n = 5 experiments). (C-D) Analysis of CD11b expression and intracellular ROS levels (CellRox probe) in PMNs transmigrated to LPS (500 ng/mL) or CFASN combined with either vehicle (black) or LPS-RS (5 µg/mL, grey) for 2 and 10 hours. Shown here in representative histograms. (E-F)

Analysis of CD63 expression and relative PMN accumulation, respectively, for PMNs transmigrated to CF ASN with vehicle control (black) or the LTA4H inhibitor acebilustat (0.1 μ M, grey) for 2, 10, and 18 hours PTM. The relative PMN accumulation at each time point was calculated as % fold change compared to the number of PMNs transmigrated to CF ASN with vehicle control at 2 hours (set at 100%). * indicates $p < .05$ when comparing PMNs transmigrated to CF ASN with vehicle control vs. acebilustat ($n = 3$ experiments).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. PMN transmigration to CF ASN *in vivo* and *in vitro* induces increased pinocytosis, concomitant with primary granule exocytosis.

(A) Lucifer Yellow (LY) uptake was used to assess pinocytosis in whole blood (WB, grey) and airway (AW, black) PMNs from CF patients as illustrated in box plot ($n = 12$ patients, left panel), and representative histograms ($n = 3$ patients, middle panel). Correlation of primary granule exocytosis (reflected by surface CD63) with pinocytosis (LY uptake) within CF airway PMNs *in vivo* was assessed using the Spearman test ($n = 12$ patients, right panel).

(B) LY uptake was assessed on PMNs transmigrated to CF ASN at 2, 10, and 18 hours PTM as illustrated in box plot ($n = 6$ experiments, left panel), and representative histograms (middle panel). * indicates $p < .05$ comparing pinocytosis at 10 and 18 hours PTM vs. 2 hours PTM (left panel). Correlation of primary granule exocytosis (reflected by surface CD63) with pinocytosis (LY uptake) within CF airway PMNs *in vitro* was assessed using the Spearman test (right panel, all time points, as labelled).

Figure 5. PMN transmigration to CF ASN increases glycolysis, oxygen consumption, and ROS production, but impairs killing of *P. aeruginosa*.

(A) Analysis of extracellular acidification rate (ECAR) by PMNs transmigrated to LTB4 (100 nM, grey) or CF ASN (black) for 10 hours, then washed, and placed in fresh assay medium in a Seahorse XFp system, with subsequent injections of glucose, oligomycin, and 2-DG (representative tracing). (B) Maximal ECAR value was measured after injection of glucose during Seahorse runs. (C) Extracellular L-lactate was measured in PMN supernatants after Seahorse runs. (D) Analysis of surface Glut1 glucose transporter

expression in PMNs transmigrated to LTB₄ (grey) or CF ASN (black) shown in representative histograms at 2, 10, and 18 hours PTM. **(E)** Analysis of oxygen consumption rate (OCR) by PMNs transmigrated to LTB₄ (grey) or CF ASN (black) for 10 hours in a Seahorse XFp system (representative tracing, as above). **(F)** Maximal OCR value was measured after injection of glucose during Seahorse runs. **(G)** Analysis of intracellular ROS levels (CellRox probe) by PMNs transmigrated to LTB₄ (grey) or CF ASN (black) shown in representative histograms at 10, and 18 hours PTM. **(H)** PMNs transmigrated to LTB₄ (grey) or CF ASN (black) for 10 hours were washed and incubated with serum-opsonized *P. aeruginosa* (PAO1 strain), after which bacterial survival was assessed by colony count. Box plots (B, C, F, H) show * for $p < .05$ comparing PMNs transmigrated to CF ASN vs. LTB₄ (n = 5 experiments).

Figure 6. Metformin modulates oxygen consumption, ROS production, granule exocytosis, and bacterial killing capacity by PMNs transmigrated to CF ASN *in vitro*.

(A-B) Analysis of ECAR and OCR, respectively, for PMNs pre-incubated for 4 hours with either metformin or vehicle control, and subsequently transmigrated to CF ASN with vehicle control (black) or CF ASN containing metformin (50 μ M, grey) for 18 hours, followed by washing, and suspension in fresh assay medium in the Seahorse system. Shown are representative tracings (left panels), and box plots for peak ECAR and OCR at baseline and after glucose, DPI, and 2-DG injections (right panels), $n = 4$ experiments, with *indicating

$p < .05$ comparing with vehicle control vs. metformin conditions. **(C)** Representative histograms are shown for the metformin target phosphorylated AMPK α 1 (pAMPK α) in CF blood (grey) and airway (black) PMNs *in vivo* (left panel); in PMNs transmigrated to LTB₄ and CF ASN at 10 and 18 hours PTM (middle panel); and in PMNs transmigrated to CF ASN with either vehicle or metformin treatment at 10 and 18 hours PTM (right panel). Hashed lines in all panels represent median in control conditions (n = 4 experiments). **(D)** Representative histograms are shown for intracellular ROS levels (CellRox probe, left panel), as well as CD66b (middle panel), and CD63 (right panel) expression assessed in PMNs transmigrated into CF ASN with either vehicle (black) or metformin (grey) treatment at 10 and 18 hours PTM, with hashed line in all panels representing median in control conditions (n = 3 experiments). **(E)** PMNs transmigrated for 18 hours into CF ASN combined with either vehicle control (black) or metformin (50 μ M, grey) were washed and incubated with serum-opsonized *P. aeruginosa* (PAO1 strain), after which bacterial survival was assessed by colony count, as shown here in a box plot (n = 4 experiments).