Transcriptional firing represses bactericidal activity in cystic fibrosis airway neutrophils

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In brief
Neutrophils are often considered terminally differentiated and poised for bacterial killing. Here, Margaroli et al. show that human neutrophils are transcriptionally and translationally plastic after migration into the cystic fibrosis lung, leading to adaptations that can affect even canonical functions (i.e., bacterial clearance).

Highlights
- Neutrophils recruited to cystic fibrosis airways undergo de novo RNA transcription
- Neutrophil adaptation to CF airways leads to downregulation of their antimicrobial genes
- Transcriptional blockade restores CF airway neutrophil bactericidal capacity
Transcriptional firing represses bactericidal activity in cystic fibrosis airway neutrophils

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SUMMARY

Neutrophils are often considered terminally differentiated and poised for bacterial killing. In chronic diseases such as cystic fibrosis (CF), an unexplained paradox pits massive neutrophil presence against prolonged bacterial infections. Here, we show that neutrophils recruited to CF airways in vivo and in an in vitro transmigration model display rapid and broad transcriptional firing, leading to an upregulation of anabolic genes and a downregulation of antimicrobial genes. Newly transcribed RNAs are mirrored by the appearance of corresponding proteins, confirming active translation in these cells. Treatment by the RNA polymerase II and III inhibitor a-amanitin restores the expression of key antimicrobial genes and increases the bactericidal capacity of CF airway neutrophils in vitro and in short-term sputum cultures ex vivo. Broadly, our findings show that neutrophil plasticity is regulated at the site of inflammation via RNA and protein synthesis, leading to adaptations that affect their canonical functions (i.e., bacterial clearance).

INTRODUCTION

Postnatal neutrophil development occurs in the bone marrow over 10–14 days, with cells of the neutrophilic lineage progressing from progenitor to mitotic and post-mitotic stages. In humans, five stages of neutrophil differentiation are identified that depend on the wave-like expression of specific transcription factors controlling the production of antimicrobial receptors and effector proteins that are sequentially packaged into three granule subsets and secretory vesicles. This differentiation is accompanied by progressive chromatin condensation, which culminates in the formation of mature neutrophils that are released into the bloodstream, featuring a characteristic multilobulated nucleus.

Upon the presence of inflammatory stimuli, neutrophil interaction with soluble factors, such as cytokines and danger- or pathogen-associated molecular patterns, as well as migration into tissues, induces neutrophil priming, a pre-activated state reflected by specific phenotypic and functional changes. These changes are mediated by intracellular signaling pathways and physical adaptation dictated by the passage through the tissue, both of which may lead to the modification of the chromatin state in neutrophils and the transcription of new genes.

Chronic respiratory diseases are one of the main causes of morbidity and mortality worldwide, and are often characterized by unresolved neutrophil inflammation. Among these diseases, cystic fibrosis (CF) represents a prototypical example of sustained inflammation driven by lifelong recruitment of neutrophils from the blood into the airway lumen, progressive connective tissue damage by neutrophil enzymes, and fatal loss of lung function. Previously, we established that neutrophil recruitment to CF airways is followed by profound phenotypic and functional adaptations dictated by the local microenvironment. We dubbed this adaptive fate GRIM, because it is characterized by primary granule release, immunomodulatory activities toward other immune subsets, reduced bacterial clearance, and metabolic licensing. The latter is reflected by an increased expression of nutrient transporters, enhanced glycolytic capacity, increased number of polyribosomes, and sustained activation of the mechanistic target of rapamycin (mTOR) anabolic pathway, a master regulator of cellular metabolism and nucleotide synthesis. Despite this sustained activation, CF airway neutrophils are unable to contain bacteria that progressively...
colonize patients’ lungs and worsen their outcomes. The mechanisms underlying adaptive changes in CF airway neutrophils and their lack of bactericidal activity remain poorly understood. Here, we investigated whether these adaptive changes were dependent upon de novo transcription and translation.

RESULTS

Airway neutrophils display profound transcriptional changes in vivo and in vitro

The recruitment of neutrophils into inflamed CF airways induces profound changes in their phenotypic and functional profile, called the GRIM fate, which includes metabolic adaptation to the airway microenvironment. To determine whether the acquisition of the GRIM fate was concomitant with changes in the RNA profile, blood and airway (sputum) neutrophils from seven adult patients with CF (Table S1) were analyzed by flow cytometry (Figure 1A). CF airway neutrophils displayed the characteristic primary granule hyperexocytosis, reflected by increased surface CD63 expression, and loss of the phagocytic receptor CD16. In addition to these previously described changes, airway neutrophils showed a median 3.5-fold increase in total RNA content compared to blood neutrophils (Figure 1B).

To study this phenomenon further, we used a transmigration in vitro model that recapitulates the GRIM fate. In this model, naive blood neutrophils are migrated through a small airway epithelium grown at the air-liquid interface toward CF airway fluid supernatant (CFASN, which corresponds to sputum sequentially centrifuged to remove cells and bacteria) or leukotriene B4 (LTB4, transmigration control), placed apically (Figure 1C). Similar to our in vivo observations in CF sputum samples and as previously shown in our model, healthy donor neutrophils transmigrated in vitro to CFASN showed increased surface CD63 and decreased surface CD16 expression, as well as increased total RNA content (>10-fold) (Figures 1D and 1E). Meanwhile, neutrophils transmigrated to LTB4 did not differ from blood neutrophils, except for a 2-fold increase in total RNA content. Similar modulations of RNA content were observed in CF blood neutrophils transmigrated in vitro, suggesting that the origin of the airway fluid, but not that of the blood neutrophils, is a major influence on their behavior upon recruitment to the airways (Figure S1A).

Next, to establish whether in vitro induction of CF airway neutrophils induces transcriptional differentiation, we compared the fold change between airway and blood neutrophils. Among the 2,010 genes that showed a differential change in CF airway compared to blood neutrophils in vivo, 639 genes were also found upregulated in vitro, while 560 were downregulated both in vivo and in vitro (Figure S1B). The transcriptional profile of airway neutrophils, analyzed using the genes that showed the same regulation in vivo and in vitro, mirrored the functional adaptation previously observed in these cells (Figure 1F). Genes coding for anabolic pathways and production of new proteins were upregulated compared to matched blood neutrophils, whereas canonical antimicrobial and cell death pathways were downregulated, suggesting that de novo transcription and translation are involved in the pathological conditioning of neutrophils by the CF airway microenvironment.

The CF airway microenvironment imprints a unique transcriptional and proteomic profile onto recruited neutrophils

To better define the changes observed in CFASN-transmigrated neutrophils, we performed both targeted and untargeted analyses of the transcriptome obtained from blood neutrophils pre-transmigration, as well as CFASN- and LTB4-transmigrated neutrophils. These three conditions were completely discriminated by principal component analysis (PCA) (Figure 2A), and comparison of the CFASN- and LTB4-transmigrated neutrophils showed considerable overlap in genes that were up- or downregulated by at least by 4-fold (|log2| >2) compared to blood (Figure S1C). Of those, 1,407 genes were upregulated and 1,221 were downregulated in both LTB4 and CFASN conditions. However, a distinctive profile was observed in CFASN-transmigrated neutrophils, with 3,602 and 3,148 uniquely upregulated and downregulated genes, respectively (Figure S1C).

Next, we investigated phenotypic changes defined as hallmarks of CF lung disease and the GRIM fate of CF airway neutrophils. Of those, the active release of primary granules and resulting increased extracellular presence of neutrophil elastase (NE) and myeloperoxidase (MPO) are among the best correlates of lung function and structural damage in pediatric and adult CF patients. In addition to increased extracellular NE, we previously observed an increase in cell-associated NE in CF airway GRIM neutrophils compared to matched blood neutrophils in vivo. Therefore, we investigated whether GRIM neutrophils were producing NE and other effector proteins through de novo transcription by cross-referencing our results with those obtained in a prior comprehensive proteomic study of human neutrophil granules. Most of the upregulated transcripts in CFASN-transmigrated versus blood neutrophils coded for proteins related to the structure, transport, and docking of granules to their target compartment (Table S2), while most of the granule effector proteins were downregulated (Figure 2B).

To confirm these comparative results, we determined whether the transcripts of downregulated effector proteins were present at all in neutrophils transmigrated to CFASN and LTB4 in vitro (Figure S2A). Apart from matrix metalloproteinase 9 (MMP9), other effector proteins such as NE, MPO, and arginase-1 had transcripts that were below the detection limit. To confirm these results with samples collected in vivo, we quantified the levels of the same transcripts by multiplex qPCR (Fluidigm) in blood and airway neutrophils sorted from patients. Similar to our in vitro results, the levels of NE, MPO, and arginase-1 were below the detection limit in CF airway GRIM neutrophils in vivo (Figure S2B).

These findings suggest that the pathological accumulation of primary granule effector proteins in the airway fluid of CF patients and within airway neutrophils is likely not caused by de novo transcription, but rather by the uptake of proteins discharged by waves of GRIM neutrophils and accumulated in the airway lumen over time.

Neutrophils recruited to CF airways revert to an anabolic state with active transcription and translation

The above findings suggest that the transcriptional burst seen in neutrophils transmigrated to CFASN is regionally controlled, rather than dependent upon a global switch from...
heterochromatin to euchromatin. This observation prompted us to investigate the breadth of transcriptional burst observed in CFASN neutrophils, which revealed activity across all chromosomes (Figure S2 C). To distinguish the relative effects on the acquisition of the GRIM fate by neutrophils of (1) transepithelial migration and (2) conditioning by CFASN, we focused our analysis on the comparison between CFASN incubation and transepithelial migration and LTB4 and CFASN transmigration conditions. Incubation in CFASN increased the neutrophil lifespan compared to those incubated in LTB4 (Figure S3 A), suggesting that the microenvironment in the CF airways could delay neutrophil apoptosis. However, incubation in CFASN (Figure S3 B) or transmigration toward chronic obstructive pulmonary disease (COPD) ASN (Figure S3 C) failed to induce the

Figure 1. GRIM neutrophils show an increase in RNA content in the cystic fibrosis lung in vivo and in vitro
(A) Blood (red) and sputum (blue) neutrophils from patients with CF (N = 7) were analyzed by flow cytometry.
(B) Airway neutrophils displayed a characteristic loss of the phagocytic receptor CD16 and release of primary granules, measured by surface CD63. Increased total RNA content compared to their matched counterpart was measured by flow cytometry.
(C) Blood neutrophils (red, N = 5) were transmigrated in vitro to sputum supernatant from patients with CF (CFASN, blue) or the chemoattractant leukotriene B4 (LTB4) as a transmigration control (gray).
(D and E) Flow cytometry analysis revealed an increase in RNA content in CFASN airway neutrophils compared to matched blood neutrophils, which was confirmed by quantification by bioanalyzer (E).
(F) Genes that were commonly upregulated or downregulated both in vivo and in vitro were used to conduct a Gene Ontology (GO) term RNA analysis. Top 20 pathways for the upregulated and downregulated genes are shown; significance of enriched pathways was established using the false discovery rate (FDR) q < 5%.

Results are shown as median and interquartile range. Statistical analysis was performed by Wilcoxon matched-pairs signed rank test; **p < 0.01, n.s., not significant.
full transcriptional burst observed in neutrophils transmigrated toward CFASN, pointing toward a “two-hit” mechanism requiring both transmigration and subsequent exposure to the CF airway milieu to fully alter the behavior of lung-recruited neutrophils.
transcriptional signature of neutrophils transmigrated to CFASN, but not those transmigrated to LTB4. Among those, genes of the glycolytic and mTOR pathways and genes previously identified in blood myeloid-derived suppressor cells in cancer patients\[28\] were highly enriched in the former (Figure 2C). Moreover, of the gene subsets that were significantly different between CFASN and blood neutrophils (Figure S1C), 769 genes were significantly downregulated and 1,735 were significantly upregulated in CFASN compared to the LTB4 condition (Figure 2D).

Interestingly, untargeted pathway enrichment analysis using the hallmarks (H), reactome (R), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (K) datasets showed a clear downregulation of transcripts implicated in neutrophil and innate immune responses (Figure 2D) and a concomitant upregulation of metabolic pathways in CFASN-transmigrated neutrophils. This highlights the unique properties of that pathological milieu, which is necessary for the acquisition of the GRIM fate.

To validate these transcriptional findings, we sought to determine whether they were mirrored by proteomic changes. Proteomic results of blood neutrophils (pre-transmigration), and both CFASN- and LTB4-transmigrated neutrophils showed a core of 1,798 common proteins, some of which were shared only between 2 conditions, and a pool of proteins unique to each condition (Figures S3D and S3E). Pathway enrichment analysis of the unique proteins revealed overlap with transcriptional signatures, with protein enriched for antimicrobial and cell death pathways in blood neutrophils, while in CFASN-transmigrated neutrophils, proteins related to the transcriptional activation of immunomodulatory and anabolic pathways were enriched. These findings support the hypothesis that neutrophil conditioning after recruitment into the CF airway microenvironment and subsequent acquisition of the GRIM fate are driven by de novo transcription and translation.

**Acquisition of the GRIM fate by CF airway neutrophils occurs in stepwise fashion**

Since most transcriptional and functional changes seen in CF airway neutrophils occur over the course of several hours, we performed a kinetic assay to better understand the dynamics of the acquisition of the GRIM fate. Using the in vitro model, airway neutrophils were collected after 1, 2, 4, and 6 h of transmigration toward CFASN. We observed that the GRIM fate developed along a rapid time course, with stepwise increases in the release of primary granules (Figure 3A), loss of the phagocytic receptor CD16 (data not shown), and increase in the total RNA content compared to blood (Figure 3B) and LTB4 (Figure S4A), suggesting a co-regulation of degranulation and transcription. Analysis of the transcriptome of CFASN-transmigrated neutrophils compared to blood neutrophils showed profound changes as early as 1 and 2 h post-transmigration (Figure 3C), with the upregulation of specific genes occurring before the significant downregulation of other gene modules. Further analysis revealed an upregulation over time of genes related to transcription, translation, and metabolism (Figure S4B). Interestingly,
among the pathways that were significantly downregulated compared to blood (Figure S4C; Table S3), genes belonging to the neutrophil degranulation reactome gene set were prominent (Figure 3D), mirroring the difference previously observed between CFASN and LTB4 neutrophils (Figure 2D). Of those genes, the majority of downregulated genes coded for effector proteins required for host defense against foreign microorganisms (Figure 3D), suggesting a possible relationship between transcription and neutrophil ability to clear pathogens.

**Transcriptional blockade antagonizes the GRIM fate and restores bactericidal activity in CF airway neutrophils**

To investigate a potential causal relationship between de novo transcription and GRIM fate acquisition, we transmigrated neutrophils in the model for 2 h into CFASN and then treated them with the RNA polymerase II and III-specific transcriptional blocker α-amanitin (Figure S5A). As expected, the total RNA content was reduced upon α-amanitin treatment (Figure S5B), showing the effectiveness of the blockade. Analysis of the transcriptome of neutrophils treated with α-amanitin revealed the downregulation of transcriptional and translational pathways, consistent with the effectiveness of the treatment. Furthermore, blockade of transcription by α-amanitin led to the upregulation of neutrophil degranulation and antibacterial genes compared to non-treated neutrophils (Figure S5C). Among the former, treatment with α-amanitin led to the upregulation of 20% of those genes that were significantly downregulated at 6 h post-transmigration. Of the genes recovered upon α-amanitin treatment, the majority belonged to proteins contained in the secretory vesicles.

Finally, since CFASN-transmigrated, α-amanitin-treated neutrophils showed decreased exocytosis and a concomitant upregulation of effector granule proteins, we questioned whether this would affect their bactericidal activity. To this end, we incubated α-amanitin-treated and -untreated neutrophils with Pseudomonas aeruginosa, a common CF pathogen, and assessed total bacterial count in an in vitro co-culture assay after 30 min. Remarkably, the bactericidal activity of CFASN-transmigrated neutrophils increased from 40% to 95% with α-amanitin treatment. Meanwhile, LTB4-transmigrated neutrophils remained stable at 65% bactericidal activity whether treated or not with α-amanitin, suggesting that the effects of α-amanitin are confined to CFASN-transmigrated neutrophils and do not perturb bacterial growth per se (Figure 4D).

To provide in vivo validation for the reduced exocytosis and gain of bactericidal activity observed with α-amanitin treatment in vitro, sputum neutrophils collected from patients with stable CF disease were treated with α-amanitin in a short-term culture...
(2 h), followed by flow cytometry analysis or incubation with *P. aeruginosa* (30 min). Consistent with our *in vitro* findings, the blockade of *de novo* transcription by α-amanitin reduced neutrophil exocytosis (Figure 4E) and increased bactericidal activity by primary CF airway neutrophils from 32% to 53% (Figure 4F). These results suggest that *de novo* transcription and translation are key to driving the neutrophil adaptation (GRIM fate) observed in the CF airway milieu, in part via active repression of their bactericidal activity.

**DISCUSSION**

Neutrophil adaptation to the CF airway microenvironment features profound changes at the metabolic and functional levels, including increased numbers of polyribosomes, increased glycolytic activity, activation of the mTOR pathway, active release of primary granules, immune modulation, and decreased bacterial killing. 17-20 Our findings here demonstrate that this adaptation is driven by active transcription and translation and highlight the plasticity of neutrophils at the target organ.

Our findings provide an explanation for paradoxical bacterial infections occurring in the face of neutrophil recruitment and sustained metabolism of these cells in inflammatory sites such as the CF lung. This conundrum has traditionally been approached from the ability of the microorganisms to manipulate and escape the immune system. Here, we show that the local airway microenvironment is able to condition recruited neutrophils by inducing quick and extensive *de novo* transcription and translation.

Moreover, we show that blocking *de novo* transcription in airway neutrophils produced in an organotypic *in vitro* model and in short-term *ex vivo* culture of patients’ airway neutrophils, restores their bactericidal activity, unveiling a surprising mechanism by which this canonical function of neutrophils is modulated. Combined with our prior discovery that neutrophilic inflammation occurs before chronic infection in CF infants, 16 these findings suggest a cascade of events in which infection may be facilitated by preexisting adaptations of tissue neutrophils. As evidenced by the decrease in expression of genes related to innate immune and cytokine signaling, we speculate that the cells may be actively conditioned to respond to types of stressors other than bacteria. This behavior, persisting into later stages, would contribute to the permissivity of CF airways to bacteria. 30

Neutrophil plasticity at the site of inflammation is becoming increasingly apparent. In addition to known transcriptional changes in the bone marrow, there is a growing body of literature describing *de novo* transcription of inflammatory genes upon neutrophil priming, 10,31 transcriptional firing during NETosis, 32,33 and in a variety of chronic diseases, 34-37 in which phenotypic and functional changes have been related to disease outcomes and therapeutic success. In line with our results, a prior study described the dynamic regulation of the neutrophil transcription during the transition from bone marrow to blood and upon migration to the site of inflammation in a mouse model, 38 further supporting the plastic nature of neutrophils and the impact of the local microenvironment on their transcriptional profile. However, while there is increasing evidence of neutrophil plasticity through the transcription of new genes, its impact on neutrophil phenotype and function remains poorly understood, particularly in human diseases.

Here, we showed that the bactericidal activity of neutrophils can be repressed through the expression of new genes, suggesting a more profound role of *de novo* transcription that reaches beyond the expression of defined inflammatory mediators. This observation introduces another concept in neutrophil biology, which to date has been widely underappreciated, in which neutrophil antimicrobial activity is not only dictated by extrinsic factors derived from local damage or pathogen-associated molecular patterns but also modulated intrinsically through active transcriptional regulation, thereby instructing an exclusive fate of increased metabolic activity and adaptation with lower bactericidal performance (GRIM fate). The identification of the factors leading to transcriptional firing and downstream GRIM adaptation is a major focus of ongoing investigations in our laboratory, which due to the richness and variety of factors present in CFASN are outside of the range of the present study. However, preliminary fractionation of CFASN with molecular weight cutoff filters suggest that components larger than 300 kDa are responsible for inducing the GRIM fate (data not shown). In all cases, transmigration is required for the induction of the GRIM fate, as direct incubation does not suffice to induce a major transcriptional burst in neutrophils. In a related study, we found that transmigration (but not incubation) of blood monocytes in CF airway fluid inhibits their bactericidal activity, 39 suggesting that this effect is not restricted to neutrophils. Another line of investigation in our laboratory relates to the potential effects of oxygen tension on the acquisition of GRIM fate by CF airway neutrophils. CF airways can develop pockets of profound hypoxia in which neutrophils are found to be enriched. 40,41 While GRIM neutrophils may consume oxygen to generate oxidants, they may not depend on it for metabolism, as they are competent for glycolysis, 8 for which oxygen is not required.

GRIM neutrophils displayed a transcriptional burst across distinct regions on all chromosomes, emphasizing the extensive nature to which these cells can remodel their gene expression profile within just a few hours. This happened, however, with the notable exception of NE and other granule effector proteins, which are produced in pre-mitotic bone marrow neutrophil precursors, 42 but not in GRIM neutrophils. This observed lack of *de novo* transcription of granule proteins is in concordance with previously published studies showing the absence of transcription factors key to granule biogenesis and of corresponding mRNAs in mature neutrophils. 43-45 The absence of specific transcripts encoding for granule effector proteins normally expressed at early stages of bone marrow development supports the notion that GRIM adaptation of CF airway neutrophils is not linked to overall chromatin derepression, but rather to tightly orchestrated reopening of certain chromatin domains. Focused follow-up studies of neutrophil epigenetics upon recruitment to target organs are needed to shed light on this question. Of particular importance is the identification of factors enabling such extensive and yet fast transcriptional derepression of the hyper-condensed chromatin characteristic of blood neutrophils as that observed in our study.
In conclusion, our findings demonstrate the ability of human neutrophils to actively adapt their canonical functions upon cues from the local microenvironment through the expression of new genes, challenging the age-old paradigm that holds these cells as terminally differentiated, and with little opportunity for adaptation at the site of inflammation. Importantly, these transcriptional changes have a notable impact on neutrophil function in the inflamed airway, including their bactericidal activity, which is traditionally thought as a canonical role of neutrophils. Future studies investigating the time-dependent switch between heterochromatin and euchromatin states will provide new insights in basic neutrophil biology and may open opportunities for host-directed immunotherapies modulating neutrophil behavior, rather than seeking to completely abrogate the benefits of neutrophil presence at a site of infection in CF and similar conditions.

Limitations of study
There are several limitations to this study. First, due to coronavirus disease 2019 (COVID-19) restrictions related to in-person visits for patients with CF, the sample size for validation experiments was suboptimal. Second, while our study mounts further evidence for an anabolic activation of neutrophils upon recruitment to the CF airway microenvironment, it does not formally establish whether the survival of these cells is increased in vivo. Isotope pulse/chase combined with mathematical modeling is needed to directly measure CF blood and airway neutrophil turnover/lifespan in vivo, and this question remains unresolved until such an effort is initiated. Third, our study did not test the potential impact of blood neutrophil heterogeneity on their behavior post-transmigration. Our prior work\(^1\) and the present study established that the origin of blood neutrophils (from healthy or CF donors) does not affect the acquisition of the GRIM fate in vitro. Instead, CF ASN is the dominant factor in the acquisition of the GRIM fate, mirroring the dominant effect on epithelial interleukin-8 (IL-8) secretion exerted by CF mucopurulent medium on healthy control epithelial cultures in prior work by Ribeiro and colleagues.\(^46\) Nevertheless, it remains to be determined whether discrete neutrophil subsets that may arise in the blood of patients with CF (e.g., band cells present during acute exacerbations) would respond differently in our model. Fourth, our prior work\(^1,18\) and the findings of the present study suggest that GRIM adaptation of lung-recruited neutrophils is prominent in CF but may also occur in other chronic airway diseases. However, in-depth studies are required to fully discern shared and unique features conferred to lung-recruited neutrophils in distinct pathological microenvironments. This is a key question, since GRIM neutrophils are likely playing important protective functions, such as the inhibition of potential T cell-driven autoimmunity, notably via the induction of myeloid-derived suppressor cell (MDSC)-like responses, as evidenced here, including the release of arginase-I.\(^17\) Therefore, potential therapeutic interventions directed at these cells in CF and other diseases would have to be carefully calibrated to recover defective bacterial killing and limit the effects of extracellular elastase and MPO, while preserving the beneficial functions of these tissue-recruited neutrophils.

STAR+METHODS
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SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
C.M. and R.T. conceived the study; C.M., D.A.G., B.D., V.D.G., O.A.F., and A.G. analyzed the data; A.G., R.W., and G.G. contributed the methods; C.M. and R.T. drafted the manuscript; and all of the authors reviewed and approved the final submission.

DECLARATION OF INTERESTS
The authors declare no competing interests.

INCLUSION AND DIVERSITY
We worked to ensure gender balance in the recruitment of human subjects. One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. The author list of this paper includes contributors...
from the location where the research was conducted who participated in the data collection, design, analysis, and/or interpretation of the work.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| CD45                | Biolegend | Cat#368523, RRID: AB_2810854- |
| CD66b               | Biolegend | Cat#305107, RRID: AB_314496- |
| CD16                | Biolegend | Cat#302043, RRID: AB_11219184- |
| CD63                | Biolegend | Cat# 353003, RRID: AB_10896786- |
| Zombie Aqua         | Biolegend | Cat#423101 |
| **Bacterial and virus strains** |        |            |
| P. aeruginosa, PAO1 (Xen41) | Perkin Elmer | Cat#119229 |
| **Biological samples** |        |            |
| Patient blood and sputum | CF Biospecimen Repository - Children’s Healthcare of Atlanta and Emory University | https://www.pedsresearch.org/research/cores/cf-discovery-core/overview/ |
| Patient blood and sputum | NAC Phase II Trial -Stanford University | https://clinicaltrials.gov/ct2/show/NCT00809094 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| DMEM/F-12           | Corning | Cat#15-090-CV |
| Glutamine           | Sigma   | Cat#G7513  |
| FBS                 | Corning | Cat#35-011-CV |
| Penicillin/Streptomycin | Sigma   | Cat#P4333  |
| Trypsin             | Hyclone | Cat#SH30042.01 |
| Rat tail collagen type I | Sigma   | Cat#C3867-1VL |
| Ultroser-G          | Crescent Chemical Co | Cat#67042 |
| RPMI 1640           | Corning | Cat#10-040-CV |
| CXCL-8              | Biolegend | Cat#574202 |
| LPS                 | Sigma   | Cat#L9143  |
| C5a                 | R&D systems | Cat#2037-CS-025/CF |
| LTB4                | Sigma   | Cat#L0517  |
| NEmo-1 probe        | Sirius Fine Chemicals SiChem GmbH | Cat#SC-0200 |
| SYTO RNASelect      | ThermoFisher | Cat#S32703 |
| a-amanitin          | Sigma   | Cat#A2263  |
| **Critical commercial assays** |        |            |
| Clontech Nucleospin RNA | Takara Bio | Cat#740955 |
| PolymorphPrep       | Cosmo Bio USA | Cat#AXS-1114683 |
| WT-Ovation Pico RNA Amplification System | NuGen | Cat#3302 |
| TruSeq RNA Single Indexes Set B kit | Illumina | Cat#20020493 |
| AmPure beads        | Beckman | Cat#A63881 |
| **Deposited data**  |        |            |
| Proteomics data     | This paper | https://doi.org/10.17632/vkz52nv6nv.2 |
| Microarrays         | This paper | https://doi.org/10.17632/vkz52nv6nv.2 |
| 10hr RNASEq         | This paper | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE167069 |
| 10hr RNASEq with transcriptional blockade | This paper | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE167066 |
| RNASEq kinetic experiment | This paper | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE165265 |

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**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Rabindra Tirouvanziam (tirouvanziam@emory.edu).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
The datasets generated during this study are available on Mendeley for microarrays and proteomics. https://doi.org/10.17632/vkz52nv6nv.2.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| NCI-H441 | ATCC | Cat# HTB-174 |
| Experimental models: Cell lines |
| | |
| Software and algorithms |
| JMP v13 | SAS Institute | https://www.jmp.com/en_us/software.html |
| Prism v7 | GraphPad | https://www.graphpad.com/scientific-software/prism/ |
| FlowJo v9.9.5 | BD biosciences | https://www.flowjo.com/ |
| R v3.5.2 | R project | https://www.r-project.org/ |
| Microsoft Excel | Microsoft | https://www.microsoft.com/en-us/microsoft-365/p/excel/cfq7tcc0k7dx?activetab=pivot:overviewtab |
| Adobe Illustrator 2020 | Adobe | https://www.adobe.com/products/illustrator.html |
| Robust Multichip Average express software v1.1.0 | Bioconductor | https://rdr.io/bioc/xps/man/rma.html |
| GE2-v5 | Agilent | https://www.agilent.com |
| hisat2 | GitHub | https://github.com/DaehwanKimLab/hisat2 |
| Bowtie2 | Johns Hopkins University | http://bowtie-bio.sourceforge.net/bowtie2/index.shtml |
| Samtools featureCounts | Genome Research Limited | http://samtools.sourceforge.net/ |
| DESeq2 | Bioconductor | https://bioconductor.org/packages/release/bioc/html/DESeq2.html |
| Quilcor Omix Explorer v3.3 | Qilcor | https://quilcore.com/ |
| Short Time-series Expression Miner (STEM, v1.3.12) | Carnegie Mellon University, School of Computer Science | https://www.cs.cmu.edu/~jernst/stem/ |
| GSEA | Broad Institute | https://www.gsea-msigdb.org/gsea/index.jsp |
| SEQUEST algorithm | ThermoFisher | https://www.thermofisher.com/order/catalog/product/OPTON-30945?SID=srch-srp-OPTON-30945#OPTON-30945| OPTON-30945?SID=srch-srp-OPTON-30945 |
| Ideogram | GitHub | https://github.com/eweitz/ideogram |
| Other |
| AlvEtex scaffold for in vitro model | Reprocell | Cat#AVP005 |
Original data for RNASeq analyses are available on GEO Accession viewer at GSE167069 (10 hours transmigration), GSE165265 (kinetics assay), GSE167066 (transcriptional blockade).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

**Human subjects and sample collection**
Blood was obtained from healthy donors and age-matched patients with CF by venipuncture in K$_2$-EDTA tubes (demographics in Table S1). The study was approved by our Institutional Review Board. Airway neutrophils and sputum supernatant were obtained as previously published. Briefly, expectorated sputum from CF patients was solubilized with 2.5 mM final ice-cold PBS-EDTA and gently passed through an 18.5 G needle. Airway neutrophils were recovered after an 800 g, 10 minutes, 4°C centrifugation, while the CFASN (clear supernatant) was collected following an additional 3,000 g, 10 minutes, 4°C centrifugation.

**H441 cell line**
H441 cells were purchased from ATCC (Cat# HTB-174) and cultured in DMEM/F-12 media supplemented with 10% FBS (Corning), 2 mM glutamine (Sigma) and 100 U/mL-0.1 mg/mL penicillin/streptomycin (Sigma). H441 cells from passages 8-10 were harvested and used for the in vitro transmigration model as previously detailed. Briefly, 2.5 x 10$^5$ H441 were cultured at air-liquid interphase on the Alvetex scaffold (Reprocell) coated with rat tail collagen (Sigma) for 14 days. The DMEM/F-12 media supplemented with 2% Ultroser G (Crescent Chemical Co), 2 mM glutamine (Sigma) and 100 U/mL-0.1mg/mL penicillin/streptomycin (Sigma), at the basolateral side was replaced every two days. After two weeks the membranes were inversed to allow the loading of neutrophils on the basolateral side and migration into apical fluid as described below.

**METHOD DETAILS**

**In vitro transmigration**
Blood neutrophils were isolated using the density gradient Polymorphrep (Cosmo Bio USA) following manufacturer protocol. Neutrophil purity and viability was assessed using flow cytometry. Purified neutrophils were loaded in the in vitro transmigration model as previously described. Briefly, 2.5 x 10$^5$ H441 were cultured at air-liquid interphase on the Alvetex scaffold (Reprocell) coated with rat tail collagen (Sigma) for 14 days. The DMEM/F-12 media supplemented with 2% Ultroser G (Crescent Chemical Co), 2 mM glutamine (Sigma) and 100 U/mL-0.1mg/mL penicillin/streptomycin (Sigma), at the basolateral side was replaced every two days. After two weeks the membranes were inversed to allow the loading of neutrophils on the basolateral side and migration into apical fluid as described below.

**Ex vivo transcriptional blockade in CF airway neutrophils**
Neutrophils obtained from sputum as described above were incubated with the transcriptional blocker α-amanitin (Sigma) at 1 μg/mL per 1 million cells. After incubation cells were assessed for their bacterial killing capacity and their phenotype determined by flow cytometry as described previously.

**Neutrophil elastase activity**
Free extracellular NE activity was measured using the FRET-based NEmo-1 probe (Sirius Fine Chemicals SiChem GmbH), as previously described. Samples were measured in duplicate with repeated-measures for values outside the working range.

**Nanoparticle tracking analysis of extracellular vesicles**
Concentration and size of extracellular vesicles (EVs) was determined by Nanosight NS3000 (Malvern Panalytical). Size and median concentration are illustrated in Figure S5.

**Flow cytometry**
Cells were stained with surface markers and dyes as previously described: CD63, CD66b, CD15, CD16, Live/dead zombie dye (Biolegend). Total RNA content was quantified by SytoRNA (ThermoFisher). Cells were acquired on a LSRII cytometer (BD Biosciences) and results were analyzed by FlowJo v.9.9.5 (BD Biosciences).

**Microarrays**
Blood and sputum neutrophils were sorted from patients with CF (N = 7; Table S1) as previously described. Total RNA was extracted from sorted fractions, mRNA was amplified, and cDNA generated (WT-Ovation Pico RNA Amplification System, NuGen). Next, cDNA from sorted neutrophils from sputum and blood sample pairs was labeled with Cy3 and Cy5, respectively, to generate two-color microarray data by competitive hybridization (GE2-v6, Agilent), per manufacturer’s instructions. RNA profiling data were...
normalized using quantile-normalization with the Robust Multichip Average (RMA) express software v1.1.0, and analyzed using Gene Set Enrichment Analysis (GSEA, Broad Institute) for significant enrichment of GO terms.

**RNA sequenc**

RNA was isolated from purified (blood > 98% purity) and transmigrated (LTB4 or CFASN, > 98% purity) neutrophils using the Nucleospin RNA isolation kit (Clontech, Takara Bio) following manufacturer protocol. RNA quality and concentration were quantified using a Bioanalyzer (Agilent Technologies, Inc.) and only samples with RIN > 8.0 were used for sequencing. Libraries were prepared using the TruSeq RNA Single Indexes Set B kit (Illumina) and AmPure beads (Beckman) and sequenced as paired end (100bp) by high seq rapid run (illumina) with an aim of ~20 million reads per sample. Analysis of transcriptomics data was performed using hisat2 for the alignment to the reference genome GRCh39. Differential gene expression between conditions was determined using DESeq2 and a significance threshold was set at 0.01 for the adjusted p value (Benjamini and Hochberg adjustment for multiple testing), PCA was performed using the Qlucore Omix Explorer v3.3, the Short Time-series Expression Miner (STEM, v1.3.12) for the kinetic assay analysis, and GSEA for pathway enrichment analysis (Broad Institute).

**Fluidigm array**

48 target genes, including NE, MPO, MMP9 and arginase-1, were assessed using a Fluidigm array, and multiplexed qPCR was performed on mRNA from blood and CF sputum neutrophils, as previously described. mRNA levels in Figure S2B are shown as cycle quantification value (Cq), with 30 cycles being the lower detection limit.

**Proteomics**

Proteins were extracted from purified (blood > 98% purity) and transmigrated (LTB4 or CFASN, > 98% purity) neutrophils after cell lysis, followed by reduction and alkylation, and digestion with trypsin. The samples were separated into ten fractions using high-pH reversed phase high-performance liquid chromatography (HPLC, pH = 10). Each fraction was analyzed by an online liquid chromatography / tandem mass spectrometry (LC-MS/MS, LTQ Orbitrap Elite) system. The spectra were searched against a human proteome database using the SEQUEST algorithm. Each protein sequence was listed in both forward and reversed orientations to estimate the false discovery rate (FDR) of peptide and protein identifications. The following parameters were used for the search: 10 ppm precursor mass tolerance; 0.5 Da product ion mass tolerance; fully digested with Lys-C; up to two missed cleavages; variable modifications: oxidation of methionine (+15.9949); fixed modifications: carbamidomethylation of cysteine (+57.0214). Peptides with fewer than seven amino acids were deleted, and peptide spectral matches were filtered to < 1% FDR. In addition, a protein-level filter was further performed in each dataset to reduce the protein-level FDR to be < 1%.

**Bacterial killing**

Overnight cultures of pro-inflammatory bacteria P. aeruginosa (strain PAO1, Perkin Elmer) were sub-aliquoted and grown to reach the exponential growth phase. Bacteria were then incubated in RPMI, supplemented with 10% FBS, on an end-over-end rotating wheel for 30 minutes at 37°C. Transmigrated neutrophils (10^5 cells) were resuspended in RPMI, 10% FBS and incubated at 37°C, 5% CO2 for 15 minutes. Co-incubation of bacteria and leukocytes was performed at a multiplicity of infection of 0.1, in RPMI, 10% FBS, on an end-over-end rotating wheel for 30 minutes at 37°C, 5% CO2. Bacterial killing capacity was calculated using colony forming units (CFU), with the bacteria plus RPMI and 10% FBS condition set as 100% viability.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistical analysis**

Data were compiled in Excel (Microsoft) and transferred to JMP13 (SAS Institute) and Prism v7 (GraphPad) for statistical analysis and graphing, respectively. Differential gene expression between conditions was determined using DESeq2 in R v3.5.2 and a significance threshold was set at 0.01 for the adjusted p value (Benjamini and Hochberg adjustment for multiple testing). Principal component analysis (PCA) was performed using the Qlucore Omix Explorer v3.3, the Short Time-series Expression Miner (STEM, v1.3.12) for the kinetic assay analysis, and GSEA for pathway enrichment analysis (Broad Institute, FDR < 5%). For proteomics analyses, both peptide and protein FDRs thresholds were set at < 1%. Comparison between conditions were performed using non-parametric statistics (median, interquartile range). Statistical details can be found in the legend for each figure.