Activation of critical, host-induced, metabolic and stress pathways marks neutrophil entry into cystic fibrosis lungs

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Cystic fibrosis (CF) patients undergo progressive airway destruction caused in part by chronic neutrophilic inflammation. While opportunistic pathogens infecting CF airways can cause inflammation, we hypothesized that host-derived metabolic and stress signals would also play a role in this process. We show that neutrophils that have entered CF airways have increased phosphorylation of the eukaryotic initiation factor 4E and its partner the 4E-binding protein 1; 2 key effectors in the growth factor- and amino acid-regulated mammalian target of rapamycin (mTOR) pathway. Furthermore CF airway neutrophils display increased phosphorylation of the cAMP response element binding protein (CREB), a major transcriptional coactivator in stress signaling cascades. These active intracellular pathways are associated with increased surface expression of critical adaptor molecules, including the growth factor receptor CD114 and the receptor for advanced glycation end-products (RAGE), a CREB inducer and sensor for host-derived damage-associated molecular patterns (DAMPs). Most CF airway fluids lack any detectable soluble RAGE, an inhibitory decoy receptor for DAMPs. Concomitantly, CF airway fluids displayed high and consequently unopposed levels of S100A12; a potent mucosa- and neutrophil-derived DAMP. CF airway neutrophils also show increased surface levels of 2 critical CREB targets, the purine-recycling enzyme CD39 and the multifunctional, mTOR-inducing CXCR4 receptor. This coordinated set of events occurs in all patients, even in the context of minimal airway inflammation and well-preserved lung function. Taken together, our data demonstrate an early and sustained activation of host-responsive metabolic and stress pathways upon neutrophil entry into CF airways, suggesting potential targets for therapeutic modulation.

CFTR | EN-RAGE | flow cytometry | S6 ribosomal protein | stromal derived factor-1

Airway disease is the most common cause of morbidity and mortality in cystic fibrosis (CF), a recessive disease affecting more than 70,000 individuals worldwide (1). Early, massive, and sustained inflammation by neutrophils is a hallmark of CF airway disease and neutrophil by-products drive disease progression (2). In particular, human neutrophil elastase (HNE) destroys the elastic fabric of airways and its activity is the best known predictor of CF lung function (3). Also, neutrophil-derived DNA and actin strings spill into mucous secretions and increase their viscosity, a characteristic of CF airway disease that underlies its other common name, mucoviscidosis.

Neutrophils are first-line immune sentinels, endowed with a plethora of receptors and weapons to sense and eliminate pathogens (4). In CF patients, neutrophils function properly throughout the body, except for the airway lumen where their presence seems to favor, rather than antagonize, chronic infections. Hence, CF airways must provide specific conditions that promote early neutrophil dysfunction (5). In a xenograft model of human airway development, we observed that before any infection, neutrophils from the mouse host would migrate into and destroy CF, but not non-CF, xenografts (6); this evidence further supports a role for host-derived cues in the conditioning of airway neutrophils.

The common view of peripheral neutrophils is that of a terminally differentiated population, with little if any ability to become anabolic and escape the default apoptotic program that they embark on as they leave the bone marrow. However, recent studies have outlined the ability of human neutrophils to modify their transcriptional profile upon migration to organs, likely under the influence of local conditioning by host-derived cues (7). Indeed, neutrophils are also endowed with the ability to recognize host-derived metabolites and stress signals (8). Some of these signals, collectively referred to as damage-associated molecular patterns (DAMPs), are emerging as important biomarkers in inflammatory diseases and represent a heterogeneous class of self-proteins that have undergone chemical transition (e.g., oxidation), relocation (e.g., nuclear protein in the extracellular milieu), or other stress-induced modifications (9).

For a long time, neutrophil dysfunction in CF airways has been equated with necrosis and passive release of elastase, DNA, and actin. However, we established recently by direct ex vivo analysis of airway neutrophils from CF patients that a large fraction of these cells are viable and appear to actively release HNE-containing granules (10). We also provided evidence that neutrophils undergo a highly unusual set of surface and intracellular changes suggesting significant functional reprogramming upon their migration to CF airways. Here, we hypothesized that viable CF airway neutrophils would bear the signs of pathophysiological conditioning by endogenous cues such as DAMPs, including significant activation of key metabolic and stress signaling pathways.

To test this hypothesis, we undertook direct ex vivo analyses of CF airway neutrophils focusing on critical intracellular phosphoepitopes and surface molecules associated with metabolic and stress pathways. Our data demonstrate an early and sustained activation of the anabolic mammalian target of rapamycin (mTOR) pathway and the DAMP-/stress-responsive cAMP-response element binding protein (CREB) pathway upon neutrophil entry into CF lungs. These results shed new light onto neutrophil regulatory pathways and emphasize the importance of host-derived signaling cues in CF pathobiology.

Results

Direct Profiling of Intracellular Phosphoepitopes in CF Airway Neutrophils. We demonstrated previously (10) that viable CF airway neutrophils, as compared to their blood counterparts, main-

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The authors declare no conflict of interest.

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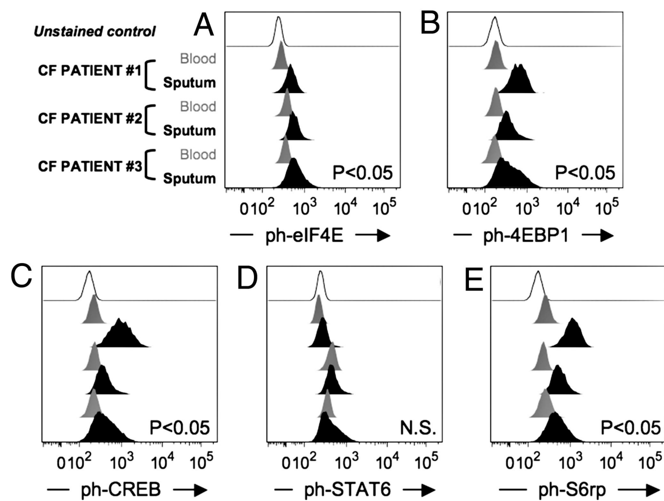

Fig. 1. Activation of mTOR and CREB pathways in CF airway neutrophils. (*A–E*) Viable neutrophils from induced sputum (black histograms) are compared to their blood counterparts (gray histograms) for expression of phospho-eIF4E, 4E-BP1, CREB, Stat6, and S6rp, respectively. A fluorescence control (left unstained in the relevant channel) is also shown (open histogram, first line of each panel). Data are from 3 representative patients with low, medium, and high airway neutrophil count, respectively (from *Top* to *Bottom* in each panel). Differences in median fluorescence intensities between airway and blood neutrophils were similar across all patients in the study (N.S.: not significant; see Table S1 for detailed statistics).

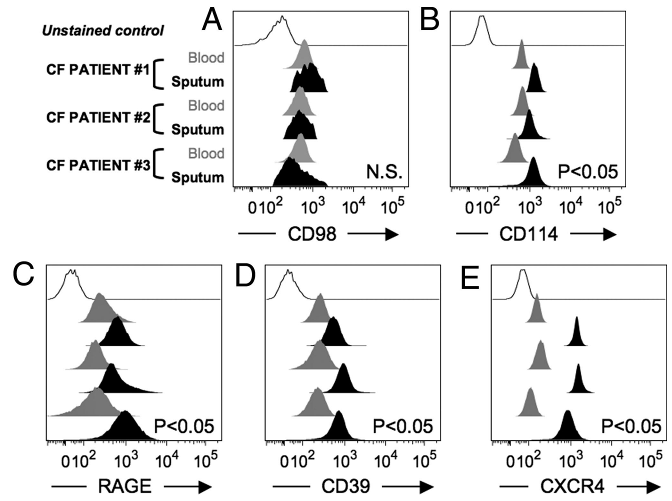

Fig. 2. Modulation of mTOR and CREB-associated surface adaptor molecules in CF airway neutrophils. (*A–E*) Viable neutrophils from induced sputum (black histograms) are compared to their blood counterparts (gray histograms) for expression of CD98, CD114, RAGE, CD39, and CXCR4, respectively. A fluorescence control (left unstained in the relevant channel) is also shown (open histogram, first line of each panel). Data are from 3 representative patients with low, medium, and high airway neutrophil count, respectively (from *Top* to *Bottom* in each panel). Differences in median fluorescence intensities between airway and blood neutrophils were similar across all patients (N.S.: not significant; see Table S1 for detailed statistics).

tained similar levels of phosphorylated intermediate kinases (Akt, p38 mitogen-activated kinase, p44/42 and cJun N-terminal kinase) and significantly increased their levels of phosphorylated S6 ribosomal protein (S6rp). A final effector in the anabolic mTOR pathway, S6rp can also be activated in mTOR-independent fashion (11). Here, we assembled an independent cohort of CF patients with variable ongoing inflammation, lung function, age, gender, and genotype ($n = 20$, see supporting information (SI) Table S1 for demographic details) and screened their airway neutrophils, at baseline, for the above epitopes and 4 new ones relevant to the question of stress signaling (see SI Methods). Analytical gating of viable blood and neutrophils was as described previously (10) and illustrated in Fig. S1.

Activation of mTOR and CREB in CF Airway Neutrophils. We observed increased phosphorylation within CF airway neutrophils of the eukaryotic initiation factor 4E (eIF4E), a translation apparatus-associated effector and anabolic switch (12) in the mTOR pathway (Fig. 1*A*; median change from blood neutrophils, +24%, see Table S2 for detailed statistics). We also observed increased phosphorylation within CF airway neutrophils of the 4E-binding protein 1 (4E-BP1, Fig. 1*B*; median change from blood neutrophils, +68%), an inhibitory binding partner of eIF4E that is itself inhibited by phosphorylation (11). Phosphorylation of the host stress-responsive CREB transcriptional co-activator (13) was also increased in these cells (Fig. 1*C*; median change from blood neutrophils, +67%). Meanwhile, the cytokine-regulated signal transducer and activator of transcription (STAT) 1 was not detectable in CF airway neutrophils (not shown) while STAT6 was detectable but not significantly modulated compared to blood neutrophils (Fig. 1*D*). Data for this new cohort confirmed previous data (10) on Akt, p38 mitogen-activated kinase, p44/42, cJun N-terminal kinase (stable expression, not shown), and S6rp (increased expression, Fig. 1*E*; median change from blood neutrophils, +69%).

Modulation of the mTOR-Associated Surface Molecules CD98 and CD114 in CF Airway Neutrophils. The mTOR pathway is the main orchestrator of cell anabolism; responding to nutrient (notably amino acids) availability and growth factor exposure (12). Extracellular amino acids are in large excess in CF lungs, driving opportunistic bacteria toward auxotrophy (14). Thus, we tested whether high amino acid levels would drive up expression of CD98, the common heavy chain subunit in heterodimeric amino acid transporters (15). CD98 expression (Fig. 2*A*) was neither up- nor down-regulated on CF airway neutrophils compared to blood neutrophils, unlike previous data in activated macrophages (16). Next, we assessed modulation on CF airway neutrophils of CD114, receptor for the granulocyte-colony stimulating factor (G-CSF), and a major neutrophil growth factor (17). CF neutrophils showed strongly up-regulated CD114 expression in airways compared to blood (Fig. 2*B*; median increase from blood to airways, +46%), although G-CSF was detectable in some, but not all, CF airway fluids (Table 1).

Modulation of the Receptor for Advanced Glycation End-Products (RAGE), a Potent CREB Inducer, in CF Airway Neutrophils. The CREB pathway mediates the response to various host-derived stress signals, including DAMPs. A versatile DAMP sensor and CREB inducer (18) is the receptor for advanced glycation end-products (RAGE). We show that RAGE expression is up-regulated on CF airway neutrophils compared to their blood counterparts (Fig. 2*C*; median increase from blood to airways, +291%). We also observed that the soluble form of RAGE (sRAGE), an inhibitory decoy receptor up-regulated in pulmonary injury settings (19) was not detectable in 18 out of 20 CF airway fluids tested, although it was present in high amounts in plasma (Table 1). Furthermore, the major RAGE inducer S100A12 (5, 20), also called EN-RAGE, was induced by about 100-fold in CF airway fluids compared to plasma (Table 1). Another RAGE ligand, S100A4 (8), was detected in some but not all CF fluids (Table 1).

Modulation of Inflammation-Associated CREB Targets, CD39 and CXCR4, in CF Airway Neutrophils. Several neutrophil-associated surface effector molecules have been previously shown to crit-

Table 1. Measures of metabolic and stress signaling in CF patient fluids

Analyte	Detectability	Median [IR]	Correlations	Difference
CXCL12 (pg/ml)	Plasma: 8/20	142 [75.6; 287]	None	None
	Sputum: 11/20	377 [83.8; 617]	None	
G-CSF (pg/ml)	Plasma: 8/20	20.8 [5.1; 27.2]	None	Plasma $<$ Sputum ($P = 0.02$)
	Sputum: 9/20	1197 [75.1; 1415]	None	
S100A4 (ng/ml)	Plasma: 20/20	10.8 [6.8; 27.2]	None	Plasma $>$ Sputum ($P = 0.02$)
	Sputum: 12/20	3.4 [3.1; 6.2]	None	
S100A12 (pg/ml)	Plasma: 20/20	41.3 [27.6; 91.8]	None	Plasma $<<$ Sputum ($P < 0.01$)
	Sputum: 20/20	6762 [3948; 7629]	Neutrophil sputum count ($P = 0003$)	
sRAGE (pg/ml)	Plasma: 20/20	1354 [1119; 1520]	None	Plasma $>>$ Sputum ($P < 0.01$)
	Sputum: 2/20	2 datapoints 1091; 3058	None	

IR, interquartile range defined by encompassing 25th and 75th percentile. Correlations were sought between the fluid measures above and disease predictors. These included age, gender, genotype (homozygous for DF508 mutations, compound heterozygotes harboring one DF508 and one other mutations and patients with other mutations), infection with opportunistic pathogens, ongoing Pulmozyme, TOBI, Zithromax, inhaled steroid treatments, functional expiratory volume in 1 sec (a measure of lung function), neutrophil blood and sputum counts. Differences were calculated between fluid measures in blood and sputum.

ically depend on CREB activation for expression. CD39 is up-regulated in activated myeloid cells via the CREB pathway (21) and is the rate-limiting enzyme in neutrophil autocrine/paracrine feedback regulatory loops involving purines (22). Here, we show a strong up-regulation of surface CD39 on CF airway neutrophils compared to their blood counterparts (Fig. 2*D*; median increase from blood to airways, +162%). CXCR4, a CREB target (23) with high expression in both immature young and mature senescent neutrophils that causes their sequestration in the bone marrow (24, 25), was also highly up-regulated on mature, nonsenescent CF airway neutrophils (Fig. 2*E*). The median increase in CXCR4 expression as neutrophils migrate from blood to airways (+634%) exceeded that measured for conventional granule activation markers such as CD11b or CD66b (+233% and +545%, respectively; see Table S1 for detailed statistics). Interestingly, CXCL12 (also called stromal-derived factor 1), the only known ligand for CXCR4, was detected in some but not all CF airway fluids, with median levels comparable to those in plasma (Table 1).

Metabolic and Stress Signaling in CF Airway Neutrophils Occurs Early and Independently of Conventional Disease Predictors. The coordinated set of events described above, featuring increased eIF4E and CREB phosphorylation in CF airway neutrophils and up-regulated surface CD114, RAGE, CD39, and CXCR4 expressions, occurred in all CF patients tested and did not correlate with age, gender, genotype, airway and blood neutrophil count, infection status, and lung function ($P > 0.1$ for all) (Table 1). In particular, the presence or *P. aeruginosa* bacteria in the airways of patients did not influence this phenomenon. We did not find any indication that chronic therapies, some of which have been claimed to modulate neutrophilic inflammation (e.g., Pulmozyme, TOBI, Zithromax, or inhaled steroids as listed in Table S1), had any influence on the induction of mTOR and CREB in CF airway neutrophils. Additional data are required however to study potential correlations within complex combined classes of patients (e.g., with or without *P. aeruginosa*, combined with specific therapies). Finally, and of significant importance for our understanding of CF airway inflammation, we found that functional CF airway neutrophil subsets defined by high and low CD16 expression (an indication of active HNE release; see Fig. 3 and ref. 10) did not differ in the expression of the above metabolic/stress markers (Fig. S1). Thus, metabolic and stress signaling occurs right upon entry of neutrophils into CF airways, earlier than HNE release, and is sustained throughout their lifespan.

Discussion

Research on CF airway disease has often focused on mechanisms of epithelial dysfunction and opportunistic infections, relegating the

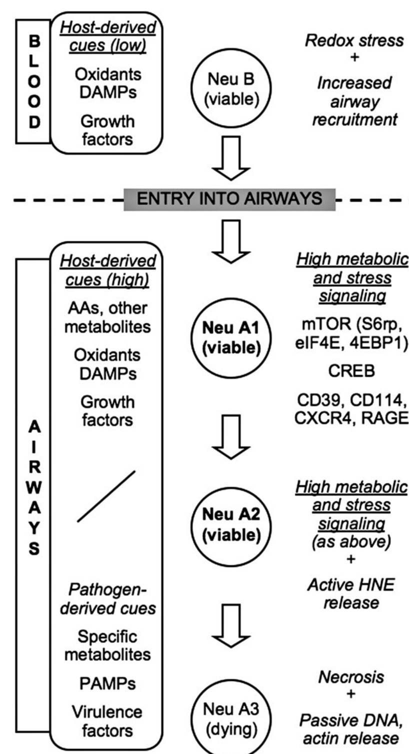

Fig. 3. Putative model of CF airway inflammation highlighting the role of host-derived metabolic and stress signals. Results presented here and previously (refs. 6, 10, 28), support the existence of 4 discrete states of neutrophils, 1 in CF blood (Neu B) and 3 in the airways (Neu A1–3). We propose that host-derived cues such as oxidants, DAMPs, growth factors, and, in the airways, amino acids (AAs) and other metabolites, drive the transition between these discrete states. Pathogen-derived cues mirroring the categories defined for host-derived cues (i.e., pathogen-associated molecular patterns or PAMPs, pathogen-derived metabolites and virulence factors) also impact the biology of Neu A1, A2, and A3 subsets. Early and sustained activation of mTOR and CREB pathways and associated surface molecules is readily detectable in the Neu A1 subset, while active HNE release defines the Neu A2 subset. Both Neu A1 and A2 subsets are viable and thus may be therapeutically targeted to prevent their transition into the dying Neu A3 subset, which passively releases undigested DNA and actin fibers in the lumen.

third hallmark of the disease, neutrophilic inflammation, to a secondary role. Here, we demonstrate that the mTOR and CREB pathways, 2 intracellular cascades responsive to host-derived metabolic and stress signals, are activated immediately upon migration of neutrophils to CF airways. Importantly, these events were readily detectable in patients with low airway neutrophil count and normal lung function. Taken together, our results support a pathological model (Fig. 3) that emphasizes the role of host-derived cues in a stepwise, abnormal conditioning of neutrophils entering into CF airways.

The extreme sensitivity of human neutrophils to experimental artifacts make it essential for signaling studies focusing on inflammatory pathologies to rely on cells collected from affected organs and on methods for their direct analysis, as used here. In vivo, neutrophils are exposed to various signals and integrate them into concerted responses, unless signals induce their rapid necrosis. This was believed to occur in CF, until we showed that neutrophils homing to CF airways do, in fact, remain viable, albeit with a highly unusual set of phenotypic changes (10). These included an active, rather than passive, release of HNE and an increased phosphorylation of the S6rp. S6rp is a conventional effector in the anabolic cascade downstream of mTOR activation, yet it can also be phosphorylated in mTOR-independent fashion (11). Our evidence that CF airway neutrophils activate eIF4E and 4E-BP1, in addition to S6rp, demonstrates an activation of the canonical mTOR pathway in these cells.

Our results are the first demonstration of selective mTOR activation in neutrophils in human disease. The mTOR pathway is an evolutionary conserved anabolic cascade generally associated with long-lived cells and triggered by a range of signals such as amino acids and selected growth factors (12). In CF airway fluid, free amino acid levels are high due to: (*i*) high extracellular amino acid production caused by neutrophil- and bacteria-derived proteases, the activity of which is potentiated by redox stress (26); and (*ii*) defective amino acid reuptake, caused by dysfunction of the CF ion channel and downstream transepithelial charge gradient anomalies (27). Neutrophils are equipped with several heterodimeric amino acid transporters involving the heavy CD98 subunit. Our results demonstrate that CD98 expression, while not up-regulated in CF airway compared to blood neutrophils, still remained detectable, consistent with a role in amino acid uptake (15). Comprehensive metabolite profiling experiments will be necessary to assess whether extracellular amino acids are indeed the main/sole inducers of mTOR pathway activation in CF airway neutrophils. Alternatively, mTOR pathway activation in CF airway neutrophils may proceed via receptors to anabolic growth factors such as CD114, which indeed was up-regulated in these cells.

The mTOR pathway may not operate in isolation, but rather in concert with other pathways, to deliver prosurvival and anabolic cues to CF airway neutrophils. Since CF features chronic metabolic and redox stresses (28, 29), we hypothesized that airway neutrophils would display significant activation of the RAGE pathway, a sensor for host-derived DAMPs generated under such stresses. Indeed, CF airway neutrophils demonstrated highly increased RAGE expression, concomitant with decreased levels of the inhibitory decoy receptor sRAGE, as well as high and consequently unopposed levels in the airway fluid of the RAGE ligand S100A12 ($>$100-fold up-regulation compared to plasma). S100A12 is believed to derive chiefly from neutrophils (perhaps actively, like HNE; see ref. 10) and we found a strong correlation between sputum S100A12 levels and neutrophil count. Airway epithelial cells themselves may release this DAMP into the lumen, as demonstrated in other inflamed mucosae (30). Other RAGE ligands, such as nuclear high mobility group box protein 1 (31) from dead epithelial cells and neutrophils, may provide additional host-dependent activation of RAGE in CF airway neutrophils.

RAGE is a potent inducer of the stress-responsive CREB pathway (18) and our data demonstrate a specific up-regulation of phosphorylated CREB in these cells. CREB induces several adaptor molecules involved in neutrophil regulatory loops, and we identified 2 such CREB targets, CD39 and CXCR4, as being significantly up-regulated in CF airway neutrophils. CD39 is a critical enzyme that metabolizes ATP into AMP and primes purine-dependent regulatory cascades during various neutrophil effector responses (32). CD39 up-regulation in CF airway neutrophils is further evidence for an active, rather than passive, role for these cells. This result is also consistent with the recent evidence that purine levels track neutrophil count and CF airway disease (33).

With regards to CXCR4, the level of up-regulation observed on CF airway neutrophils compared to their blood counterparts is the highest of any of the molecules we have identified so far, including markers of granule mobilization such as CD11b and CD66b (ref. 10 and Table S2). CXCR4 is responsible for neutrophil retention in the bone marrow during maturation and upon senescence, and in that context, signaling via the G-CSF receptor CD114 provides the main driving force for CXCR4 down-regulation as needed for mature neutrophils to avoid bone marrow retention (24, 25). The concomitant up-regulations of CXCR4 and CD114 surface receptors in CF airway neutrophils thus constitute a rather intriguing coexpression pattern. Another potentially important functional interplay is that of CXCR4 with mTOR, the former having been shown to induce activation of the latter in cancer cells (34).

Interestingly, the only known ligand for CXCR4, CXCL12, was detectable in some but not all CF airway fluids tested. CXCL12 was previously measured in other lung diseases (35), and its absence in some CF fluids may be linked to high activity of HNE, which can cleave CXCL12 (36). Whether cleavage products of CXCL12 may retain some bioactivity toward CXCR4 is currently unclear. The absence of full length G-CSF in a large portion of CF airway fluids tested in this study, despite increased CD114 expression on CF airway neutrophils, may follow the same line of explanation since G-CSF can be cleaved by HNE (37) and yet may not be construed as a failure to activate the receptor since cleavage products may retain such bioactivity. Overall, an emerging set of regulatory mechanisms at play in CF airways rely on selective, HNE-dependent cleavage of conventional receptor/ligand pairs endowed with neutrophil-tropic effects (10, 38). Functional mismatches and the emergence of novel functional/signaling modules may ensue, regulating the balance of chronic/acute neutrophilic inflammation and hampering the normal resolution of infections (Fig. 3).

In this context, it is particularly important to consider that active HNE release by CF airway neutrophils occurs at all stages of the disease and that, as shown here, activation of mTOR and CREB pathways occur in these cells even before HNE release. While only associative at this point, the relation between these active metabolic and stress pathways and HNE release are worth exploring, since extracellular HNE is a key contributor to CF airway disease. Due to the sensitivity of neutrophils to exogenous manipulation, accurate mechanistic studies in vitro are difficult to conduct, but are necessary to confirm the relation between these 2 sets of events.

In summary, we show that the mTOR and CREB pathways, 2 key pathways involved in cellular responses to host-derived metabolic and stress signals, are activated upon migration of neutrophils to CF airways, early on in the disease. Within the context of CF, our results suggest that airway neutrophils are active disease contributors and bona fide therapeutic targets. CF airway neutrophils also emerge as a potential source of dynamic cellular biomarkers that could be interrogated to assess the disease course and, possibly, the effect of new targeted therapies. Such biomarkers, identified in baseline conditions on a limited set of patients, require additional data on large cross-sectional (infants to transplantees) and longitudinal (yearly baseline vs. disease exacerbations) cohorts to ascertain their usefulness as potential tools in clinical settings. More broadly, it is interesting to consider that the reliance on host-derived cues to drive innate immunity has been observed and thoroughly described

before in the plant kingdom (39). In humans, such cues may represent a primary evolutionary layer responsible for the robustness of neutrophilic immunity, notably in contexts where pathogen-sensing pathways are genetically or functionally debilitated (40).

Materials and Methods

Human Subjects. This study received the approval of the Stanford Administrative Panel on Human Subjects in Medical Research. All subjects signed informed consent forms before undergoing study procedures. Diagnosis of CF was by documented sweat chloride >60 mEq/L by quantitative iontophoresis test and/or one or more clinical features consistent with CF and/or preexisting documentation of 2 identifiable mutations. The presence in the patients' lungs of common opportunistic pathogens (as indicated in Table S1) was tested by routine sputum culture. Lung function was tested by spirometry using American Thoracic Society criteria.

Collection and Processing of Samples. Blood and airway fluid were simultaneously collected from subjects by venipuncture and sputum induction, respectively. Sputum induction is a minimally invasive, standardized, procedure that was shown by several independent groups to faithfully reflect CF inflammatory lung disease. Since our study focused on characterizing baseline functional and signaling profiles of CF airway neutrophils, we used a protocol for sputum processing that avoids DTT treatment and associated artifacts, as described earlier (10).

Multiparametric Flow Cytometry. Screening of viable CF airway neutrophils for intracellular and surface adaptor molecules of interest was performed as indicated in details in ref. 10 and in the *SI Methods*. In brief, cells were stained with the Live/Dead near infrared probe from Invitrogen to assess viability, as well as with fluorescent antibodies from Invitrogen, BD Biosciences, eBioscience and Cell Signaling Technologies, as listed in the *SI Methods*, online. Cells were acquired on a 4-laser LSRII digital FACS (BD Biosciences).

Fluid Assays. Fluid mediators were measured by specific commercial ELISA kits, including kits against CXCL12, G-CSF, and sRAGE (from R&D Systems) as well as S100A4 and S100A12 (from MBL International), as per the manufacturer's guidelines. Detection limits were 18 pg/ml, 20 pg/ml, 4 pg/ml, 0.25 ng/ml and 50 pg/ml, respectively. Absorbance data were acquired at 450 nm on a Modulus microplate reader (Turner Biosystems).

Analysis and Presentation of Data. Flow cytometry data were exported to the FlowJo software (Treestar) and compensated using single-stained beads or cells, as detailed elsewhere. Median fluorescence values were calculated and compared to the appropriate background controls. Statistical analysis of flow cytometry and ELISA datasets was performed using the JMP6 software (SAS Institute). Distributions were compared between blood and airway neutrophils using the paired Wilcoxon signed-rank test. Correlations between continuous variables were tested using the Spearman Rho test. Differences in outcome measures following nominal categories (e.g., gender, genotype) were assessed using the unpaired Wilcoxon rank-sum test. Differences or correlations were considered significant with $P < 0.05$.

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Supporting Information

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SI Methods

Foreword. Microbiological assessment of patients as well as methods for blood and sputum sample processing, surface and intracellular staining, data acquisition on the flow cytometer and multistep analytical gating strategy of viable neutrophils were exactly as described in extensive details in the supporting information *(SI) Text* [Tirouvanziam R, et al. (2008) Profound functional and signaling changes in viable inflammatory neutrophils homing to cystic fibrosis airways. *Proc Natl Acad Sci USA* 105:4335–4339.]. Core technical details are given below, as well as additional information regarding antibodies against intracellular phosphoepitopes and surface markers that are specific to this study.

Processing of Samples. To limit artifactual activation of blood and lung neutrophils as much as possible, samples were kept on melting ice immediately upon collection and throughout all experimental procedures. Blood samples were centrifuged at 400G for 10 min to remove platelet-rich plasma and resuspended to their original volume in sterile PBS with EDTA at 5 mM (PBS-EDTA). Sputum samples were weighed, their volume measured and 1–2 volumes of ice-cold PBS-EDTA were added to sputum samples to loosen adherences and inhibit extracellular proteases. Cells were collected after gentle mechanical dissociation by repeated slow passage through a sterile 18"G needle, followed by filtration through a sterile nylon 70 μm sieve (BD Biosciences) and low-speed centrifugation at 400G for 10 min to remove the sputum supernatant. Both platelet-rich plasma and sputum fluid were spun at 3000G for 10 min to pellet down platelet and debris, respectively, and then aliquoted for use in further fluid assays, as described in the *Materials and Methods* section.

Surface Marker Profiling. Whole blood (50 μl) and sputum cells (50 μl, after resuspension at 5×10^6 leukocytes per ml, similar to whole blood) were stained in PBS-EDTA for 20 min on ice, in the dark, with the Live/Dead near InfraRed viability probe (Invitrogen) and several antibodies against surface determinants. Previously tested antibodies (1) that were maintained for the analysis of the present cohort of patients were directed against CD11b, CD16, CD45, CD63, and CD66b (all from BD Biosciences) and CD14 (from Invitrogen). We also included novel antibodies against CD39, from eBioScience, CXCR4 (CD184) from BD Biosciences, and RAGE, from Santa Cruz Biotechnologies. After staining, cells were washed with excess PBS-EDTA, centrifuged, and the supernatant was removed. Upon resuspension in 100 μl of PBS-EDTA, cells were fixed with 2 ml of 1X Lyze/Fix PhosFlow (BD Biosciences). To ascertain the efficiency of individual stains, we used fluorescence-minus-one controls, consisting of all stains but the stain of interest. For quantitative assessment of antibody binding to key markers (CD39, CD98, CD114, CXCR4, RAGE), we used fluorescence channels with minimal overlap with other channels, as previously discussed (2). For standardization of cell staining, antibodies were purchased in large batches and titrated for epitope saturation. Saturating concentrations were used throughout the study.

Intracellular Phosphoepitope Profiling. Whole blood (50 μl) and sputum cells (50 μl, after resuspension at 5×10^6 leukocytes per ml, similar to whole blood) were stained in PBS-EDTA for 20 min on ice, in the dark, with the Live/Dead near InfraRed viability probe (Invitrogen). Cells were then washed with excess PBS-EDTA, centrifuged, and the supernatant was removed. Upon resuspension in 100 μl of PBS-EDTA, cells were fixed with 2 ml of 1X Lyze/Fix PhosFlow (BD Biosciences). After one wash cycle with excess PBS-EDTA, the supernatant was removed. Cells were resuspended in 100 μl of PBS-EDTA and permeabilized by drop-by-drop addition of 400 μl ice-cold Perm III buffer (BD Biosciences) while vortexing. Upon 30 min incubation on ice, the Perm III solution was washed twice with excess PBS-EDTA and the supernatant was removed. Cells were then resuspended in 50 μl PBS-EDTA and stained with antibodies against phosphoepitopes of choice, for 20 min, in the dark, followed by a wash in excess PBS-EDTA. Previously tested anti-phosphoepitope antibodies (2) that were maintained for the analysis of the present cohort of patients were directed against: Akt (phospho-S473), JNK (phospho-T183/phospho-Y185), NFkB p65 (phospho-S536), p38 MAP kinase (phospho-T180/Y182), p44/42 (phospho-T202/phospho-Y204), S6rp (phospho-S235/236), all from Cell Signaling Technologies and STAT5 (phospho-Y694) from BD Biosciences. Novel antibodies introduced in this study were against: CREB (phospho-S133) and 4E-BP1 (phospho-T37/46) from Cell Signaling Technologies; eIF4E (phospho-Y285), Stat1 (phospho-Y701) and Stat6 (phospho-Y694) from BD Biosciences.

Acquisition of Flow Cytometry Data. Data were acquired on a FACS LSRII digital flow cytometer (BD Biosciences) equipped with 4 lasers (407, 488, and 633 nm), 2 light scatter detectors (yielding forward and side scatter data) and 18 fluorescent detectors. Acquisition was controlled using the DiVa software (BD Biosciences). Threshold for data acquisition was set in the forward scatter channel, excluding dead cells and debris with very low size. To standardize data acquisition, several steps were taken. First, to limit day-to-day variability, all antibodies were purchased in large batches and used at saturating concentrations throughout the study. Second, to provide fluorescence background quantification in measurement channels, one aliquot of each sample was stained with the fixable viability dye, but left unstained in other channels (background control aliquots). Third, to standardize signal output by the flow cytometer, we ran before each session a thorough calibration procedure using a standard set of multicolor fluorescence beads.

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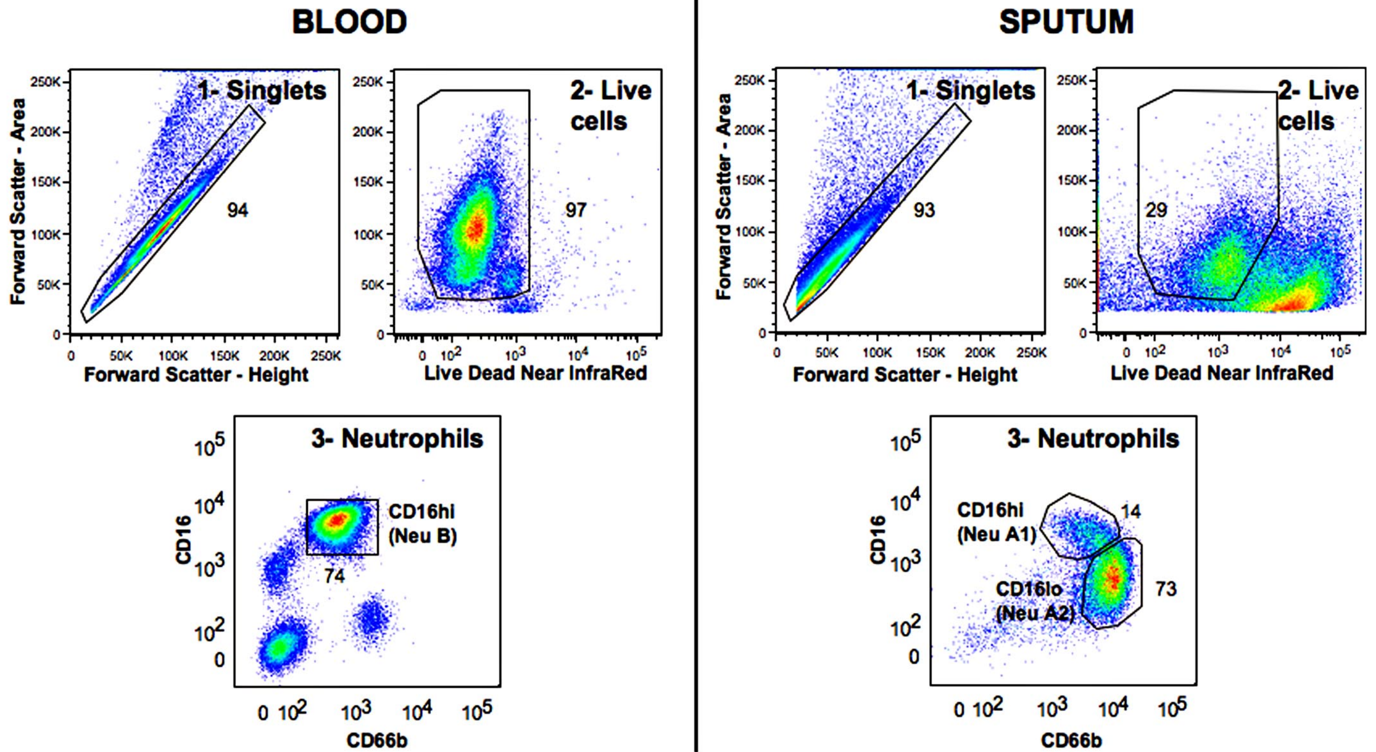

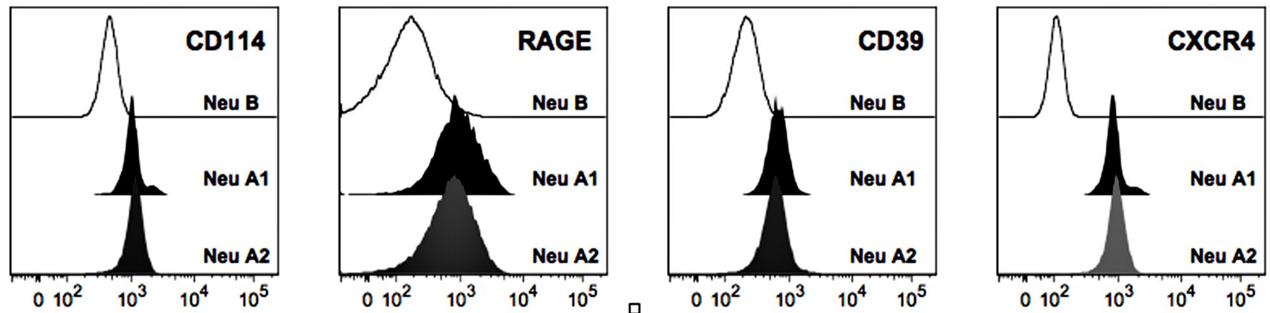

Fig. S1. Modulation of CD114, RAGE, CXCR4, and CD39 in CF airway neutrophils occur before HNE release. (*A*) Example of gating strategy for live blood neutrophils (Neu B, according to Fig. 3) and sputum neutrophils, further subdivided into CD16^{hi} (Neu A1) vs. CD16^{lo} (Neu A2) subsets, which we have previously characterized as having undergone low vs. high HNE release, respectively (2). (*B*) As shown here with samples from a representative CF subject, Neu A1 (black histograms) and Neu A2 (gray histograms) subsets of airway neutrophils show identical up-regulation of CD114, RAGE, CD39, and CXCR4 compared to the blood neutrophil population (Neu B, open histograms). Thus, modulation of these markers in CF airway neutrophils occurs before HNE release.

Table S1. Demographics of patients included in the study (sorted by FEV1, percent predicted)

Gender	Age	Genotype	Neutrophil sputum count (10^6 cells)	Neutrophil blood count (10^6 cells)	FEV1 (%Pred.)	Opportunistic infections	Inhaled steroids	Pulmozyme	TOBI	Zithromax
F	10	HO	10.2	4.1	119	N	N	Y	N	N
M	15	OT	28.3	4.6	106	N	Y	Y	N	N
M	12	OT	7.5	2.3	96	N	Y	Y	N	N
F	18	HO	2.46	6.8	95	Y: PA	Y	Y	Y	Y
F	11	HO	0.48	3.6	91	Y: SA	N	Y	N	N
F	44	OT	15.3	3.5	90	Y: PA, SA, AF, CA	Y	Y	Y	N
M	29	CH	45.7	5.6	89	Y: PA	Y	Y	N	N
F	37	CH	10.2	3.3	85	Y: PA	Y	Y	Y	N
M	11	CH	1.63	7.5	82	Y: PA	Y	Y	N	Y
F	48	HO	0.62	6.2	68	Y: PA	Y	N	N	Y
M	49	HO	60	7.8	67	Y: PA	N	Y	Y	Y
M	22	HO	38.2	5.7	66	Y: PA, CA	N	N	N	N
M	12	HO	0.75	3.4	66	Y: PA, HP, CA	N	Y	Y	Y
M	11	CH	4.5	10.4	65	Y: PA, SA, CA	Y	Y	Y	Y
F	50	CH	31.2	8.7	65	Y: PA, CA	Y	Y	N	Y
M	43	CH	73.5	5.3	65	Y: PA	Y	Y	Y	N
F	44	CH	127	6.4	62	Y: SA	Y	Y	N	N
M	25	HO	96	8.3	59	Y: PA	N	N	N	N
M	33	CH	16.2	4.1	48	Y: PA	Y	Y	Y	Y
F	26	CH	21	3.8	48	Y: PA	N	N	Y	N
M = 11 F = 9	27.5 ± 3.27 (Mean ± SE)	CH = 9 HO = 8 OT = 3	15.8 [2.97; 43.8] (Median [IR])	5.57 ± 0.48 (Mean ± SE)	76.6 ± 4.28 (Mean ± SE)	Y = 17 *N* = 3	Y = 7 *N* = 13	Y = 16 *N* = 4	Y = 11 *N* = 9	Y = 8 *N* = 12

Neutrophil counts are expressed as 10^6 cells for sputum (total count) and as 10^6 cells/ml for blood. Opportunistic infections include *P. aeruginosa* (PA), *S. aureus* (SA), *H. parainfluenzae* (HP), *A. fumigatus* (AF), *C. albicans* (CA). CH = compound heterozygotes, HO = DF508 homozygotes, OT = other mutations, Y = Yes, N = No.

Table S2. Detailed statistics for metabolic and stress markers induced in sputum vs. blood neutrophils (alphabetical order)

Analyte	Type	Percent change: Median [IR]	P
4EBP1	Phosphoepitope	+68.2 [+37.4; +96.8]	0.002
CD114	Surface marker	+46.4 [+41.3; +136]	0.04
CD11b	Surface marker	+233 [+133; +366]	0.03
CD39	Surface marker	+162 [+96.5; +268]	0.03
CD66b	Surface marker	+545 [+230, +1438]	0.03
CREB	Phosphoepitope	+67.5 [+40.0; +117]	$<10^{-3}$
CXCR4	Surface marker	+634 [+335, +722]	0.03
eIF4E	Phosphoepitope	+23.6 [+19.4; +55.4]	0.004
RAGE	Surface marker	+291 [+146; +572]	0.016
S6rp	Phosphoepitope	+69.4 [+53.7; +154]	$<10^{-3}$

All above data relate to new phosphoepitopes and surface markers, as detailed in text, except S6rp, CD11b, and CD66b (initially described in ref. 2), which data was also collected on patients listed in Table S1 and is provided here for comparison. Numerical data presented as percent change [median, with interquartile range (IR) defined by 25th and 75th percentiles]. P values were calculated by the Wilcoxon signed-rank test for paired data.