Innate immune reprogramming in circulating neutrophils of COPD patients

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Background: Chronic obstructive pulmonary disease (COPD) involves both local and systemic neutrophilic inflammation, with dysregulation in blood neutrophil numbers, frequencies, and functions.

Objective: We sought to characterize the transcriptional and epigenetic profiles of circulating neutrophils in patients with COPD and explore correlations with neutrophil dysfunction and clinical disease parameters.

Methods: Circulating neutrophils of patients with COPD and control donors were subjected to RNA-sequencing and genome-wide analysis of histone 3 lysine 4 trimethylation (H3K4me3) by chromatin immunoprecipitation coupled with sequencing. Neutrophils' activation was assessed by cytofluorimetric analysis, O_2^- release, and *Candida albicans* phagocytosis assays.

Results: RNA- and chromatin immunoprecipitation–sequencing analysis of H3K4me3 revealed a poised state in genes involved in innate immune activation, resembling the phenotype observed in neutrophils from individuals who are BCG-vaccinated, referred to as "trained," that is marked by weak or no expression under resting conditions but ready to be expressed at higher levels on stimulation. The epigenetic signature identified in neutrophils from subjects who are BCG-vaccinated was enriched in COPD neutrophils. In particular, and consistent with what has been described in "trained" neutrophils, COPD neutrophils exhibited transcriptional reprogramming of metabolically relevant genes. Functionally, COPD neutrophils produced higher CXCL8 and IL1B levels, released more O_2^-, and displayed greater phagocytic activity on *in vitro* stimulation.

Conclusions: These findings suggest that COPD neutrophils undergo epigenetic, transcriptomic, and metabolic reprogramming, which enhances their responsiveness and aligns with the phenotype of neutrophils previously identified as trained, offering mechanistic insight into the functional dysregulation observed in COPD. (J Allergy Clin Immunol 2025;■■■:■■■-■■■.)

Key words: *COPD, neutrophils, epigenetic, trained-immunity*

Abbreviations used

ChIP:	Chromatin immunoprecipitation
COPD:	Chronic obstructive pulmonary disease
GSVA:	Gene set variation analysis
H3K4me3:	Histone 3 lysin 4 trimethylation
R848:	Resiquimod
RNA-seq:	RNA-sequencing
ROS:	Reactive oxygen species

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease, distinguished by local and systemic manifestations. At the systemic level, the inflammatory response, whose intensity is related to the underlying disease severity, is characterized by elevated levels of circulating acute phase proteins, cytokines, chemokines, and other inflammatory mediators or circulating leukocyte abnormalities.[1] Numerous studies performed genome-wide transcriptomic profiling of peripheral blood cells in the attempt to identify specific signatures of COPD phenotypes, progression, exacerbation, and/or response to therapy.[2,3] Among the different circulating leukocyte populations quantitatively and/or qualitatively modified in COPD, neutrophils have been reported to be altered in number, frequency, migratory accuracy, degranulation, reactive oxygen species (ROS) release and antimicrobial function.[4,5] This is particularly relevant because neutrophils, through their critical role in lung parenchymal destruction and perpetuation of the systemic inflammatory response,[6] are recognized as key players in COPD pathogenesis and progression.[7] Recently, transcriptomic analysis of circulating neutrophils of patients with COPD identified 5 neutrophil cellular states collectively characterized by a reduced interferon signaling pathway and a parallel upregulation of pathways including degranulation genes, RHO guanosine triphosphatase signaling, and ephrin signaling, all of which can promote inflammation.[8]

In addition to transcriptional dysregulation, several studies have linked epigenetic changes to COPD. In particular, alteration in DNA methylation in circulating blood cells has been shown to be associated with COPD and lung function.[9,10] As expected, given that DNA methylation is a crucial event controlling gene expression, epigenetic and transcriptional modifications were found to be concomitant, and dysregulated DNA methylation was associated with altered expression of genes and pathways important for COPD pathology.[11]

Other than DNA methylation, histone modifications such as acetylation and methylation are critical events in the control of gene expression and have been shown to be relevant to several aspects of lung diseases, including COPD.[12] However, few studies have identified histone modifications functionally linked to COPD.[12] Precisely, a reduction in the level of histone H3 lysine 4 acetylation, consequent to impaired HDAC activity, has been

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identified in lung parenchyma and circulating immune cells, and found to correlate with COPD severity and responsiveness to corticosteroid therapy or to IL-10.[13,14] Additionally, histone methylation, particularly at the histone 3 level, is an important marker of gene expression, and its alterations have been implicated in several chronic diseases.[15,16] Reduction of histone 3 lysine 9 trimethylation, a mark for heterochromatin and transcriptional silencing, has been detected in lung-infiltrating and in circulating immune cells in COPD.[17] Conversely, alterations of histone 3 lysine 4 trimethylation (H3K4me3), a marker of active transcription, have been shown to be associated with emphysema in a cigarette smoke extract–induced murine model,[18,19] but have not been reported in patients with COPD. Overall, the relationship between cellular dysfunction and histone 3 trimethylation has not been identified, and their clinical relevance remains largely unknown. In addition, the epigenetic alterations that lead to the transcriptional and functional changes of circulating neutrophils from patients with COPD described above are still poorly understood and are often the subject of controversy.

Elucidating how short-lived cells such as neutrophils, which have a half-life of only a few hours, can sustain epigenetic modifications is a critical issue. Nevertheless, addressing this challenge, Kapellos et al[8] provided compelling evidence that transcriptional alterations in circulating neutrophils of patients with COPD arise early in the disease course and originate from epigenetic modifications occurring at the level of bone marrow precursors.

Similarly, another condition has been identified that depends on modifications at the level of bone marrow precursors. Specifically, circulating neutrophils purified from BCG-vaccinated individuals have been shown to undergo epigenetic reprogramming within the bone marrow. As these progenitors differentiate into neutrophils, the acquired epigenetic changes persist, endowing the mature neutrophils with an augmented capacity to respond to subsequent challenges despite their short lifespan.[20]

In this context, this study aims to characterize the role of the epigenetic H3K4me3 signature of circulating neutrophils from patients with COPD in driving neutrophil transcriptional reprogramming and the implication in neutrophil functional dysregulation.

METHODS

Detailed methods are provided in this article's Online Repository (available at www.jacionline.org).

Patients

Patients with COPD (n = 41) and age- and sex-matched controls (n = 38) were recruited from the Respiratory Medicine Unit of the Azienda Ospedaliera Universitaria Integrata of Verona, Verona, Italy. All the subjects enrolled subscribed to an informed consent form approved by the local ethics committee (protocol 42052/2015) and in agreement with the principles of the Declaration of Helsinki. Clinical data of enrolled donors are reported in Table I. All participants met the following criteria: (i) older than 50 years; (ii) not affected by chronic or acute infections, inflammatory, autoimmune, active or previous neoplastic conditions, and lung diseases (other than or associated to COPD); (iii) not under treatment with oral or systemic steroids. Lung function procedures were performed according to international recommendations as detailed in the Supplemental Methods in the Online Repository. All patients (N = 79) underwent RNA-sequencing (RNA-seq) analysis, while a subset of these individuals (11 patients with COPD and 10 controls, identified as chromatin immunoprecipitation sequencing [ChIP-seq] subcohort) simultaneously underwent ChIP-seq analysis (Fig E1, *A* in this article's Online Repository at www.jacionline.org). Subsequently, we recruited additional 14 subjects (7 with COPD and 7 controls) for functional experiments (independent cohort) (Table I). The recruitment of these additional subjects was conducted following the same criteria. Not all donors, particularly control donors, agreed or were able to undergo spirometry for the assessment of lung function at the time of sample collection.

RNA-seq

RNA-seq libraries were prepared using the QuantSeq 3'mRNA-Seq Library Prep Kit (Lexogen Inc, Greenland, NH) starting from 100 ng total RNA per sample. Differentially expressed genes between COPD and control donors were identified according to Wald test implemented in DESeq2.[21]

ChIP-seq

ChIP of H3K4me3 was performed as previously described.[22] ChIP-seq libraries were generated using the NEXTflex Rapid DNA Sequencing Bundle Illumina Compatible (Bioo Scientific, a PerkinElmer Company, Austin, Tex). H3K4me3 enriched regions were identified by *callpeaks* function of MACS2 (https://pypi.org/project/MACS2/).

RESULTS

Epigenetic and transcriptional reprogramming of circulating neutrophils from patients with COPD

The molecular characterization of peripheral blood neutrophils was conducted in 41 patients with COPD and 38 age- and sex-matched controls. The main features of patients with COPD and controls enrolled in this study are summarized in Table I.

Activation and maturation state of circulating neutrophils was assessed by cytofluorimetric analysis for changes in the expression levels of physiologically relevant surface proteins, including L-selectin (CD62L), which is shed from the surface of neutrophils on activation[23]; CD11b and CD35, which are stored in secondary granules of neutrophils and the surface levels of which increase following degranulation[24,25]; and CD11c and CD10, which mark mature neutrophils.[26,27] No difference in the level of the membrane-bound activation/maturation markers in neutrophils from patients with COPD compared to control donors was observed (Fig E2, *A* and *B* in this article's Online Repository at www.jacionline.org).

Neutrophils purified from all the subjects enrolled in the study were subjected to RNA-seq analysis. Differential gene expression analysis was performed using a multifactorial design to control for variation due to variables affecting neutrophil transcriptome, such as smoking and sex. Differential gene expression analysis identified 1045 genes as significantly ($P < .05$, Wald test) modulated in neutrophils from patients with COPD compared to controls (Fig 1, *A*). Specifically, 718 genes were upregulated ($\log_2$ fold change: >0), while 327 genes were downregulated ($\log_2$ fold change: <0) (Fig 1, *B*).

TABLE I. Demographic and clinical data of COPD and control subjects

	Control donors		COPD patients		
	n	Median (Q1-Q3)	n	Median (Q1-Q3)	*P* value
Enrolled donors					
Sex (M/F)	38	22/16	41	24/17	.999*
Age	38	78 (68.5-80)	41	76 (69-79)	.476†
BMI	30	24.9 (23.9-27.70)	34	26.3 (24.1-29.3)	.589†
Smoker (never/former/current)	38	26/9/3	39	4/23/12	<.0001*
CC index‡	27	2 (0-3)	40	2 (1-3)	.566†
FEV_1 (L)	24	2.5 (2.07-2.99)	39	1.07 (0.78-1.79)	<.0001†
FEV_1, % of predicted	24	111 (103-129.8)	39	52 (47.5-62.5)	<.0001§
FVC (L)	24	3.18 (2.53-3.54)	38	2.80 (2.16-3.76)	.103§
FVC, % of predicted	24	108.5 (100.8-121.3)	38	105 (90.5-112.5)	.075§
FEV_1/FVC, %	24	82.5 (79.0-85.1)	38	51.9 (40.1-59.7)	<.0001†
mMRC			39	1 (1-2)	
GOLD 2023 (A/B/E)			37	20/10/7	
Independent cohort					
Sex (M/F)	7	4/3	7	4/3	.999*
Age	7	62 (61-62.5)	7	61 (56-62.5)	.518†
BMI	7	25.14 (23.17-26.25)	5	24 (23-25)	.755†
Smoker (never/former/current)	7	5/2/0	7	0/5/2	.007*
CC index‡	7	2 (2-2)	7	3 (2-3)	.085†
FEV_1 (L)			5	1.53 (0.76-2.42)	
FEV_1, % of predicted			7	49.0 (37.5-65.00)	
FVC (L)			5	3.53 (2.06-4.34)	
FVC, % of predicted			5	86.0 (56.0-114.0)	
FEV_1/FVC (%)			6	42.5 (38.0-52.1)	
mMRC			5	2 (2-3)	
GOLD 2023 (A/B/E)				0/1/6	

BMI, body mass index; *CC* index, Charlson Comorbidity index; *FVC*, forced vital capacity; *GOLD*, The Global Initiative for Obstructive Lung Disease; *mMRC*, Modified British Medical Research Council Questionnaire.
*Fisher exact test; $P < .05$ differences are significant.
†Mann-Whitney test.
‡COPD pathology was not included in the Charlson comorbidity index.
§Student t-test.

To determine whether epigenetic modifications account for the altered transcriptional profile of neutrophils from patients with COPD, genome-wide H3K4me3 ChIP-seq was performed on the same samples subjected to RNA-seq analysis. Because we could not obtain a number of neutrophils sufficient to conduct RNA- and ChIP-seq studies in parallel from all the subjects, ChIP-seq was performed on 11 of the 41 patients with COPD and 10 of the 38 controls enrolled in the study (Fig E1, *A*). It should be emphasized that the very low RNA content of neutrophils (1 $\mu g/10^7$ cells on average), which is one-tenth of the RNA content of other leukocyte populations, together with the volume of blood that could be drawn from patients posed a constraint on the feasibility of molecular studies performed in parallel. In terms of demographic and clinical parameters, as well as transcriptomic data, this subset of subjects (from now on identified as ChIP-seq subcohort) was representative of the cohort as a whole. In fact, the ChIP-seq subcohort was representative of the whole cohort for clinical parameters of lung function (Fig E1, *B*) and the mean gene expression level of each gene in neutrophils from the 11 COPD and 10 control donors that were available for ChIP-seq analysis directly correlated with the mean gene expression level of the entire cohort (control donors: $\rho = 0.989$, $P < 2.2 \times 10^{-16}$; patients with COPD: $\rho = 0.988$, $P < 2.2 \times 10^{-16}$) (Fig E1, *C*).

A total of 14,628 H3K4me3 peaks was identified in patients and controls, 1,669 of which were significantly ($P < .05$, Wald test) modulated in COPD neutrophils (Fig 1, *C* and Fig E3, *A* in this article's Online Repository at www.jacionline.org). Specifically, 1,033 peaks had increased H3K4me3, whereas 636 peaks showed reduced H3K4me3 (Fig 1, *D*). As expected, given that H3K4me3 usually marks transcriptionally active genes, 84% of H3K4me3 identified was annotated in the promoter region of the associated gene (Fig E3, *B*).

To identify potential functional effects of the increased H3K4me3, a marker of active promoters, we analyzed whether genes characterized by increased promoter-associated H3K4me3 were also expressed at increased levels. Correlation analysis showed that only 49 genes marked by increased H3K4me3 were upregulated (Fig 1, *E* and *F*). Accordingly, 984 upregulated H3K4me3 peaks were localized at promoters of genes expressed at comparable levels in neutrophils from patients with COPD and controls. Because H3K4me3 marks not only actively transcribed but also poised genes, data suggest that in neutrophils from patients with COPD 984 genes might be in a poised state (ie, weakly or not expressed under resting conditions but ready to be highly expressed on cell activation).[28]

Circulating neutrophils from COPD patients display an epigenetic profile resembling that of "trained" neutrophils

To gain insights into the cellular processes and pathways that are in a poised state in COPD neutrophils, we retrieved the 984

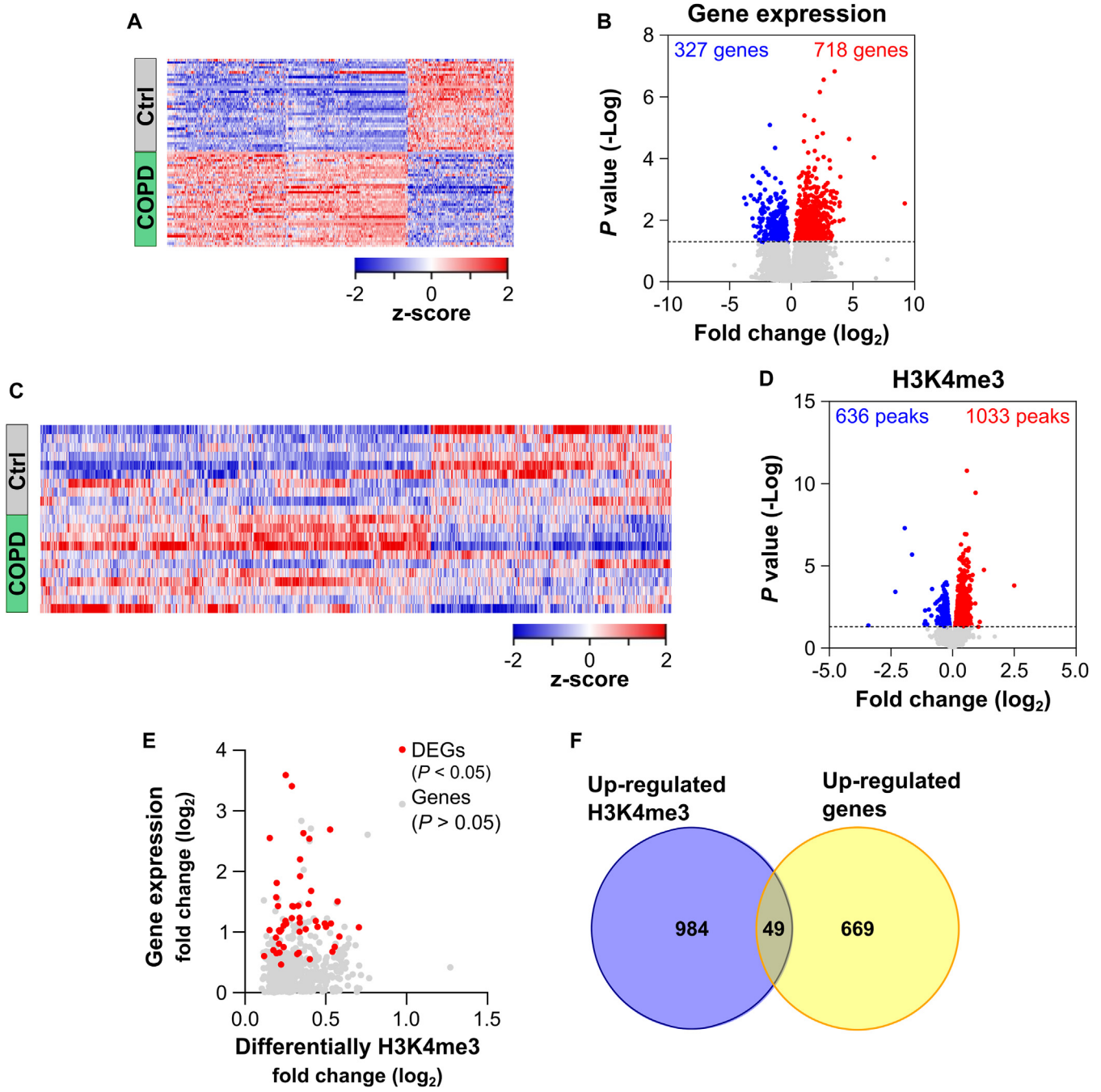

FIG 1. Characterization of the epigenetic and transcriptomic profile of COPD and control neutrophils. Heatmaps and volcano plots of differentially expressed genes (*DEGs*) **(A, B)** and genome-wide H3K4me3 changes **(C, D)** in neutrophils from patients with COPD (n = 41 in **A** and **B**, and n = 11 in **C** and **D**) and controls (*Ctrl*; n = 38 in **A** and **B**, and n = 10 in **C** and **D**) ($P < .05$). Data are shown as *z*-score of the fragment per million (*FPM*) mapped reads **(A)** or as *z*-score of the normalized reads per peak **(C)**. Number of upregulated (*red dots*) and downregulated (*blue dots*) genes **(B)** and peaks **(D)** is shown. **(E)** Correlation analysis between regions showing increased H3K4me3 in patients with COPD (x-axis) and the expression of their nearby localized genes (y-axis). *Red dots* identify increased H3K4me3 ($P < .05$, $\log_2$[fold change] > 0) associated with significantly upregulated genes ($P < 0.05$, $\log_2$(fold change) > 0). **(F)** Venn diagram showing the number of upregulated H3K4me3 (*blue*) associated with significantly upregulated genes (*yellow*).

potentially poised genes (ie, showing increased levels of promoter-associated H3K4me3 but not increased transcription) and performed a gene ontology biological process and Reactome pathway enrichment analysis. Among the top categories, we identified biological pathways associated with response to infection, phagocytosis, cytokine signaling and production, cell death, and innate immune cell activation (Fig 2, *A*). Neutrophils with similar characteristics, specifically displaying significant

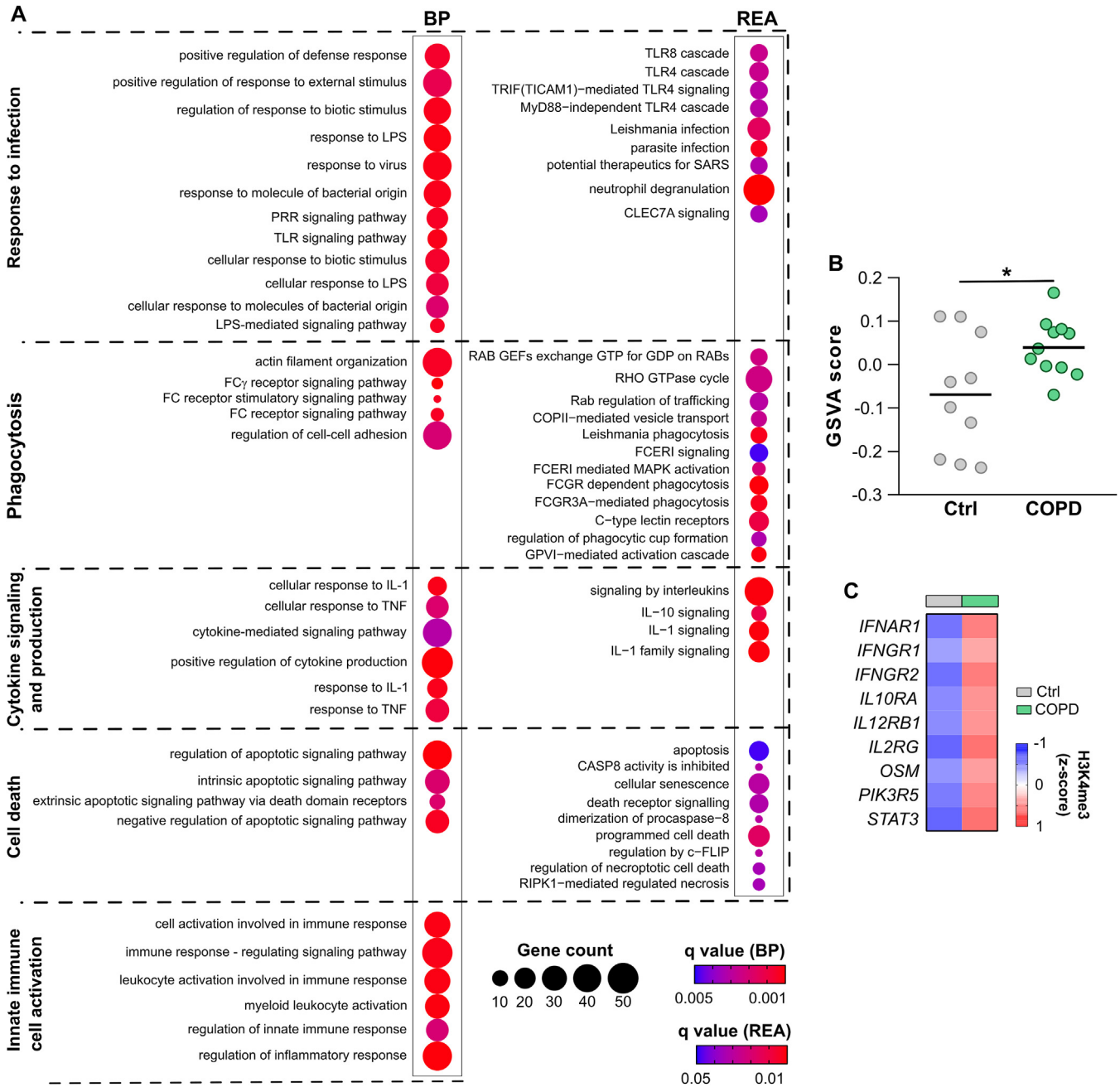

FIG 2. Epigenetic reprogramming in neutrophils from patients with COPD resembles trained neutrophils. **(A)** Gene ontology (*GO*) terms related to biological processes (*BP*) and Reactome (*REA*) pathway enrichment analysis of the 984 genes identified in COPD neutrophils as potentially poised (ie, characterized by increased promoter-associated H3K4me3 but not increased transcription). *Circle size* represents the number of peaks associated with each term; *circle color* shows the significance of the enrichment ($q < 0.05$). GO terms and pathways are grouped according to their semantic similarity. **(B)** GSVA analysis of the H3K4me3 signature retrieved from BCG-treated neutrophils ChIP-seq data[29] in neutrophils from patients with COPD (n = 11, *green dots*) and control donors (n = 10, *gray dots*) (*$P < .05$ according to Mann-Whitney test). **(C)** Heatmap showing H3K4me3 levels at the promoters of *IFNAR1*, *IFNGR1*, *IFNGR2*, *IL10RA*, *IL12RB1*, *IL2RG*, *OSM*, *PIK3R5*, and *STAT3* in neutrophils from patients with COPD and control donors. The mean of H3K4me3 normalized reads per peak after z-score transformation is shown.

alterations in the H3K4me3 epigenetic profile without corresponding transcriptional changes, were identified in BCG-vaccinated individuals. These epigenetic modifications were shown to enhance neutrophil antimicrobial function on secondary stimulation, a phenomenon referred to as "trained immunity."[29] To verify whether the epigenetic signature of trained neutrophils was also present in neutrophils from patients with COPD, H3K4me3 peaks identified in BCG-trained neutrophils[29] were

retrieved from Gene Expression Omnibus database. The variation of this epigenetic signature in neutrophils from patients with COPD and controls was used to calculate a gene set variation analysis (GSVA) score. Remarkably, neutrophils from patients with COPD had a significantly ($P = .029$) higher GSVA score than controls (Fig 2, *B*), thus demonstrating that in patients with COPD circulating neutrophils acquired a trained-like epigenetic phenotype. Remarkably, and consistent with the results of neutrophils from BCG-vaccinated individuals, 9 of the genes annotated in the JAK-STAT Kyoto Encyclopedia of Genes and Genomes pathway showed increased H3K4me3 and increased expression in neutrophils from patients with COPD versus controls (Fig 2, *C*).

Induction and maintenance of the epigenetic reprogramming underlying trained immunity requires an increased energy demand, as well as increased amount of metabolites, such as acetyl-CoA, α−ketoglutarate, and succinate, derived primarily from the glycolytic pathway and the tricarboxylic acid cycle.[30] The metabolic activity of neutrophils from patients with COPD and controls was modeled using Compass, a flux balance analysis-based algorithm that uses transcriptomic data to characterize cell metabolism. Compass analysis of neutrophil transcriptome showed that neutrophils from patients with COPD have an increased metabolic activity compared to controls (Fig E4 in this article's Online Repository at www.jacionline.org). Notably, *in silico* analysis predicted an increased activity of glycolysis, tricarboxylic acid cycle, and fatty acid oxidation in neutrophils from patients with COPD (Fig 3, *A*). Supporting the *in silico* prediction, analysis of differentially expressed genes in COPD neutrophils versus controls showed increased expression of genes involved in the glycolysis and gluconeogenesis metabolic pathway. Remarkably, of 56 genes included in the glycolysis and gluconeogenesis pathway, the expression of 44 genes was upregulated in COPD neutrophils. In particular, upregulation of 4 glucose metabolism-related genes, namely *HK1* and *HK3*, *ACSS1,* and *GALM*, was statistically significantly (Fig 3, *B*), whereas the other 40 genes were upregulated, although not statistically so (Fig 3, *C*). These data indicate that COPD neutrophils undergo transcriptional modulation of metabolically relevant genes.

To analyze whether neutrophils from patients with COPD also exhibit a trained phenotype at a broader transcriptional level than just at the level of metabolically relevant genes or of the genes included in the JAK-STAT pathway, the variation of the published gene signature associated with BCG-induced trained immunity in neutrophils[29] was evaluated in the transcriptomic profiles of neutrophils of patients with COPD and controls. Consistent with a trained epigenetic phenotype, neutrophils from patients with COPD exhibit also a trained transcriptional signature, as demonstrated by the significant ($P < .0001$) increase of the GSVA score in neutrophils from patients with COPD versus controls (Fig 3, *D* and *E*). Taken together, data from epigenetic, transcriptomic, and *in silico* characterization of cell metabolism strongly indicate that neutrophils from patients with COPD resemble the main features of trained neutrophils.

Transcriptional changes identified in COPD neutrophils are present in other chronic respiratory diseases

Neutrophils participate in the initiation and aggravation of several chronic lung inflammation.[31] Other than COPD, severe asthma, cystic fibrosis, and idiopathic pulmonary fibrosis are all associated with continuously high neutrophil levels.[32-34] For these reasons, we sought to determine whether the 718 genes identified as upregulated in neutrophils from patients with COPD compared to controls represent a transcriptional signature shared by circulating innate immune cells in individuals with chronic respiratory diseases. To this end, we retrieved the gene expression profiles of whole blood and of PBMCs from individuals with various chronic respiratory disorders from the Gene Expression Omnibus database (see Supplemental Methods in the Online Repository for details). Using GSVA we then calculated a score for each donor to summarize the expression pattern of the gene set identified in our COPD cohort. Fig 4 shows that PBMCs of children affected by asthma[35] (Fig 4, *A*), whole blood of asthma patients (Fig 4, *B*), and neutrophils of patients with cystic fibrosis[36] (Fig 4, *C*), but not PBMCs of patients with idiopathic pulmonary fibrosis[37] (Fig 4, *D*) exhibit an increased expression of the neutrophil gene signature identified in our COPD cohort.

Interestingly, the GSVA score was elevated in one cohort of patients with COPD (Fig 4, *E*) but not in a different one (Fig 4, *F*). The key difference between the 2 cohorts is the sample type: in the cohort where gene set variation was upregulated in patients with COPD versus controls, transcriptional profiling was performed on whole blood (Fig 4, *E*). In contrast, in the cohort where no difference between patients with COPD and controls was detected, profiling was conducted on PBMCs[38] (Fig 4, *F*). This finding is particularly noteworthy, because it underscores the predominant contribution of neutrophils to the identified gene set.

Overall, this analysis underscores how different chronic inflammatory lung diseases share common conditions that drive similar transcriptional alterations at the systemic level in circulating neutrophils.

Circulating neutrophils from patients with COPD show a heightened response to stimuli

At the functional level, trained neutrophils purified from subjects who are BCG-vaccinated[29] are characterized by an increased cellular response to a second stimulation.[30] To verify that neutrophils from patients with COPD exhibit a trained phenotype not only at the transcriptional and epigenetic levels but also in their functional properties, cytokine production, phagocytosis, and O_2^- production in response to *in vitro* stimulation was assessed in an independent cohort of 7 patients with COPD and 7 sex- and age-matched controls (independent cohort) (Table I). Patients and controls were recruited according to the same criteria as the initial cohort. Lung function parameters of the subjects included in the RNA-seq and the independent cohort recruited for functional studies were comparable (Table E1 in this article's Online Repository at www.jacionline.org).

According to Moorlag et al[29] *CXCL8* and *IL1B* were selected as representative of the poised genes. In fact, ChIP- and RNA-seq data showed that *CXCL8* and *IL1B* promoters are characterized by elevated H3K4me3 in COPD (Fig 5, *A-D*), but are expressed at comparable levels in COPD and control neutrophils (Fig 5, *E* and *F*). Comparable expression of *CXCL8* and *IL1B* mRNA was confirmed in the independent cohort (Fig 5, *G* and *H*). Remarkably, on *in vitro* stimulation with LPS or R848 (resiquimod), neutrophils from patients with COPD released significantly higher amounts of CXCL8 (407.0 ± 43.65 ng/mL) and IL1B (13.36 ± 2.46 pg/mL) compared to neutrophils from controls (CXCL8: 223.6 ± 22.34 ng/mL; IL1B: 5.57 ± 0.83 pg/mL) (Fig 5, *I* and *J*).

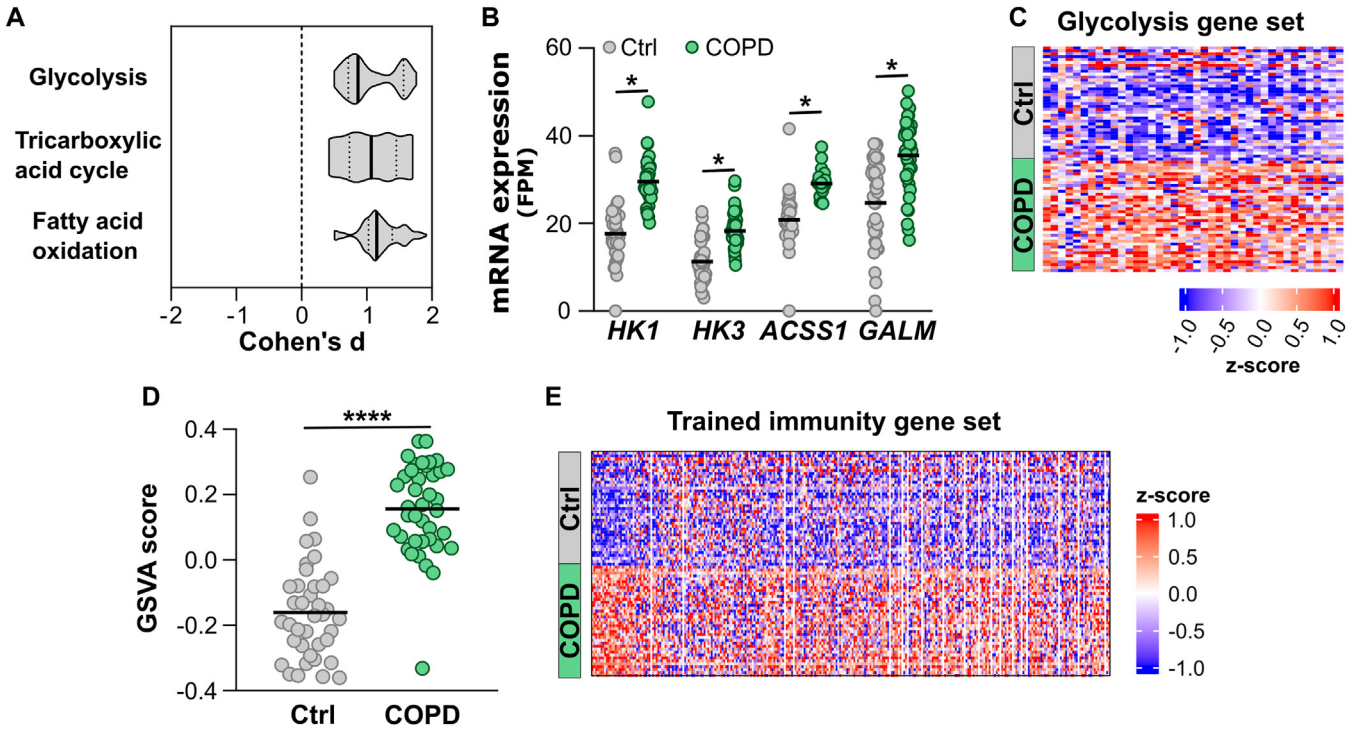

FIG 3. Metabolic and transcriptional reprogramming in neutrophils from patients with COPD resemble trained neutrophils. **(A)** Activity of metabolic reactions significantly modulated (Benjamini-Hochberg-corrected, $P < .05$) in COPD neutrophils according to Compass analysis. Modulation of metabolic reactions is expressed as Cohen *d*-score. **(B)** Expression of *HK1*, *HK3*, *ACSS1*, and *GALM* in neutrophils from patients with COPD (n = 41, *green dots*) and control donors (n = 38, *gray dots*). Data are shown as FPM mapped reads determined by RNA-seq analysis (*$P < .05$ according to the Wald test). **(C)** Heatmap of the expression levels of genes from the glycolysis gene set in neutrophils from patients with COPD (n = 41) and controls (n = 38). Data are shown as *z*-score of the FPM. **(D)** GSVA analysis of the trained immunity gene set retrieved from BCG-treated neutrophils RNA-seq data[29] in the transcriptomic profile of neutrophils from patients with COPD (n = 41, *green dots*) and control donors (n = 38, *gray dots*) (****$P < .0001$ according to Mann-Whitney test). **(E)** Heatmap of the expression levels of genes from the trained immunity gene set in neutrophils from patients with COPD (n = 41) and controls (n = 38). Data are shown as *z*-score of the FPM.

Building on *in silico* predictions suggesting that COPD neutrophils exhibit enhanced glycolysis, along with transcriptomic data revealing the upregulation of 4 glucose metabolism-related genes in these cells, we investigated glucose consumption in the supernatants of LPS- and R848-stimulated neutrophils. Under resting conditions, neutrophils from both patients with COPD and healthy controls displayed comparable glucose consumption after overnight culture. However, on stimulation with LPS (Fig 5, *K*) or R848 (Fig 5, *L*), only COPD neutrophils exhibited a significant increase in glucose uptake. Taken together, these data suggest that COPD neutrophils are likely reprogrammed toward an enhanced metabolic state, characterized by increased glycolytic activity.

We next assessed whether COPD neutrophils display enhanced phagocytosis capacity toward *Candida albicans*. Forty minutes after heat-killed *C albicans* challenge, the phagocytic index of COPD neutrophils, expressed as percentage of neutrophils that phagocytosed *C albicans,* was significantly higher (63.50 ± 3.52%) than that of control neutrophils (46.43 ± 6.31%) (Fig 5, *M*). BCG-trained neutrophils have been shown to have enhanced ability to generate ROS, which plays a major role in microbial killing by neutrophils. Consistently, O_2^- released by phorbol 12-myristate 12-acetate–activated COPD neutrophils was significantly increased (2.68 ± 0.29 nmol) as compared to that released by control ones (1.67 ± 0.34 nmol) (Fig 5, *N*). Taken together, data demonstrate that circulating neutrophils from patients with COPD are *bona fide* trained neutrophils.

Epigenetic reprogramming correlates with disease severity in neutrophils from COPD patients

Next, we addressed whether the epigenetic reprogramming of COPD neutrophils is associated with the severity of the disease. To this aim, we generated a score (hereafter referred to as "H3K4me3 score") and searched for potential correlations with clinical features.

This score represents the aggregated trimethylation levels of 1033 H3K4me3 peaks that are upregulated in neutrophils from patients with COPD compared to controls (Fig 1, *C* and *D*). It facilitates the assessment of global trimethylation variability across different samples and was generated through variance analysis of the upregulated H3K4me3 peaks using the GSVA package, as detailed in the Supplemental Methods in the Online Repository.

The H3K4me3 score was then correlated with patients' clinical data. Remarkably, an inverse correlation between the H3K4me3 score and FEV_1% of predicted ($r = 0.648$, $P = .031$) (Fig 6, *A*) and a direct correlation with Modified British Medical Research Council Questionnaire ($\rho = 0.593$, $P = .05$) (Fig 6, *B*) indicated that the degree of H3K4me3 increases along with the disease

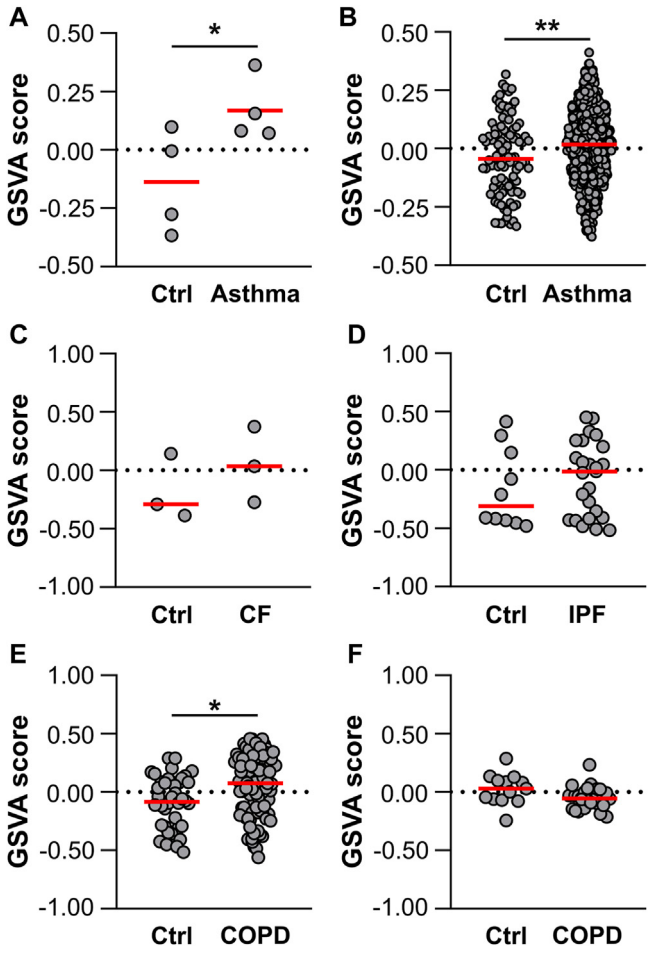

FIG 4. Transcriptional changes identified in COPD neutrophils are present in other chronic respiratory diseases. Expression of the 718 genes upregulated in neutrophils from patients with COPD compared to control donors was evaluated in the transcriptomic profile of **(A)** PBMCs from children with asthma,[35] **(B)** whole blood of patients with asthma, **(C)** neutrophils from patients with cystic fibrosis (*CF*),[36] (D) PBMCs from patients with idiopathic pulmonary fibrosis (*IPF*),[37] **(E)** whole blood and **(F)** PBMCs of patients with COPD,[38] and relative controls. Data are shown as GSVA score obtained as described in Supplemental Methods in the Online Repository. $*P < .05$, $**P < .01$, according to unpaired *t*-test or Wilcoxon test, as appropriate.

severity. None of the other clinical parameters tested (forced vital capacity [%] of predicted, FEV_1/forced vital capacity, Charlson comorbidity index, and diffusing capacity of the lung CO [%]) correlated with the H3K4me3 score. In addition, none of the other clinical parameters, including age, body mass index, gender, or smoking habits, affected the correlations between H3K4me3 and FEV_1% of predicted and Modified British Medical Research Council Questionnaire, as shown in the correlation matrix for identifying confounders (Fig E5 in this article's Online Repository at www.jacionline.org).

DISCUSSION

This study demonstrates that circulating neutrophils from patients with COPD exhibit epigenetic, transcriptomic, metabolic, and functional characteristics similar to those described in neutrophils exhibiting an enhanced responsiveness to stimuli, a condition that is referred to as "trained."[39] The concept of trained immunity has been introduced to describe a state in which innate immune cells that, after an initial stimulus, develop the ability to mount faster and stronger inflammatory response on reactivation.[29]

In line with this concept, following *in vitro* stimulation, neutrophils from patients with COPD showed enhanced production of CXCL8, IL1B, and ROS (O_2^-) along with increased phagocytic activity, suggesting a trained phenotype. This phenotype is consistent with previous studies reporting that neutrophils from both stable and exacerbating patients with COPD have altered functions, such as degranulation, ROS production, and phagocytosis,[4,5] and have been "primed" for increased reactivity.[5,6] While from a functional perspective terms such as "primed" and "trained" imply similar outcomes in terms of increased responsiveness,[40] trained immunity is distinct in its association with long-term innate memory and the capacity to revert to a baseline state postactivation.[40] In support of this, freshly purified blood neutrophils from stable patients with COPD exhibited no differences from control neutrophils in baseline activation markers, ROS, and cytokine levels, confirming that they were in an inactivated state.

The acquisition of a trained phenotype has been well characterized at the molecular level, and has been shown to depend on neutrophils epigenetic and metabolic reprogramming.[28] Trained neutrophils, characterized in subjects who are BCG-vaccinated, undergo epigenetic reprogramming, as evidenced by H3K4me3 epigenetic modifications that render genes more responsive to activation.[29] COPD neutrophils shared this H3K4me3 signature with BCG-trained neutrophils, with 95% of the H3K4me3 peaks annotated at promoters of genes poised for rapid activation on stimulation. Remarkably, high levels of H3K4me3 at the promoter of *CXCL8* and *IL1B*, taken as a paradigm of poised genes,[29] did not impact the level of expression of these proinflammatory cytokines under resting conditions but were associated with CXCL8- and IL1B–enhanced production by *in vitro* stimulated COPD neutrophils, showing that *CXCL8* and *IL1B* are ready to be increasingly expressed in response to a second challenge. The set of genes identified as poised was enriched in biological pathways collectively associated with innate immune cell activation, namely response to infection, phagocytosis, cytokine signaling, and production.

As mentioned above, epigenetic reprogramming in trained immunity depends on the metabolic switch toward aerobic glycolysis. Metabolic dysregulation has been shown to occur in patients with COPD, and likely contributes to disease pathogenesis and progression.[41] However, the roles of these metabolic reprogrammings have not been fully understood yet.[42] *In silico* analysis predicted metabolic reprogramming of COPD neutrophils that aligns with trained immunity, marked by increased glycolysis, fatty acid oxidation, and cholesterol metabolism. Elevated expression of *HK1, HK3*, *ACSS1*, and *GALM* and increased glucose consumption on activation corroborate a shift toward aerobic glycolysis, which, although characteristic of neutrophils, may intensify in pathological states such as COPD. In fact, aerobic glycolysis operates within a dynamic, context-dependent metabolic framework influenced by immune factors, pathogen interactions, and inflammatory states. Metabolic reprogramming thus appears central to neutrophil activation in COPD, likely contributing to disease progression.[41]

The long-term effect of training despite the short neutrophil lifespan has been demonstrated to rely on modification of immune

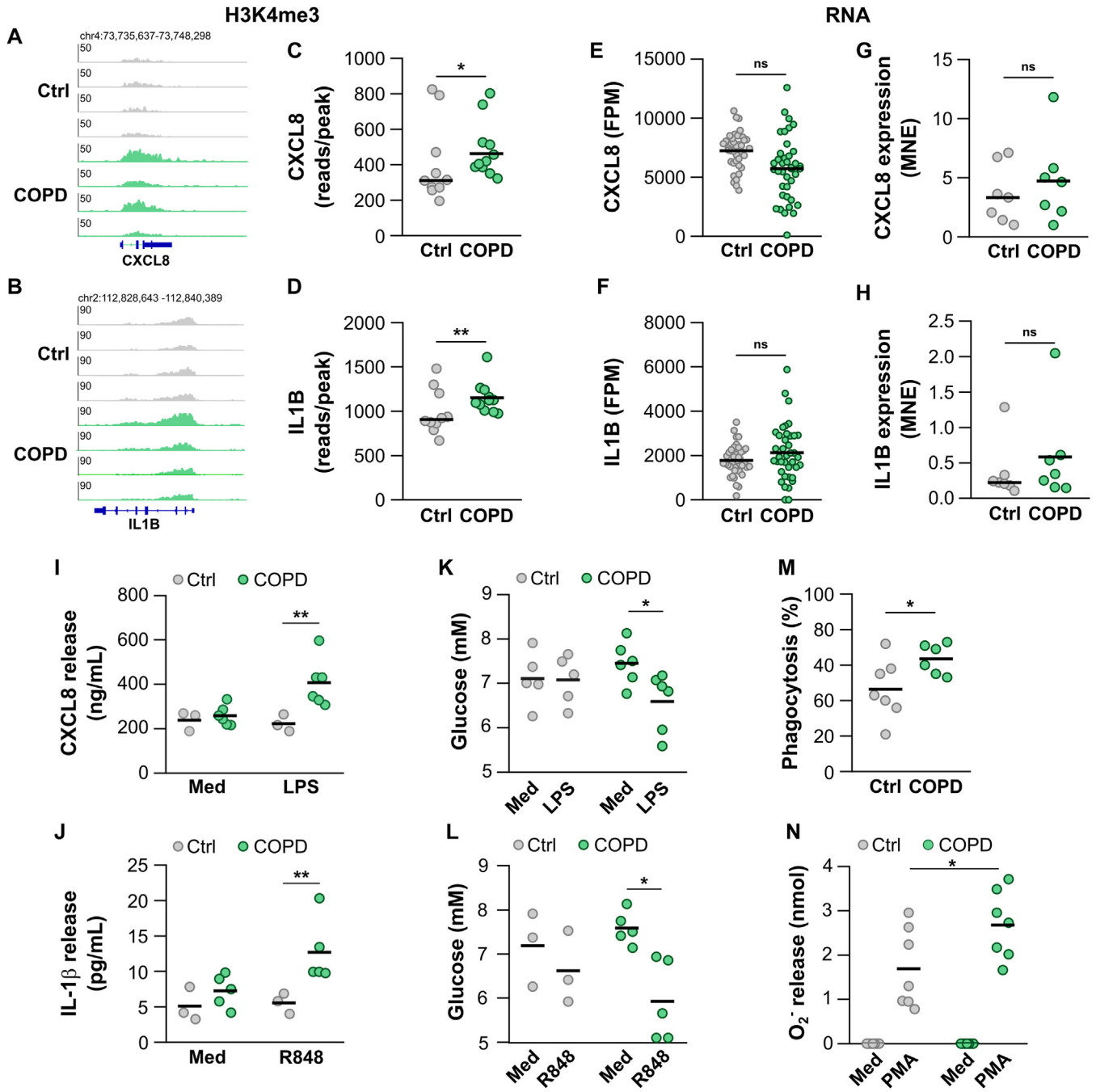

FIG 5. Neutrophils from patients with COPD are characterized by a trained phenotype. Integrative Genomics Viewer snapshots of the H3K4me3 peaks in neutrophils from 4 representative controls (*gray*) and 4 representative patients with COPD (*green*) near *CXCL8* **(A)** and *IL1B* **(B)** loci. H3K4me3 **(C,D)** and RNA **(E, F)** expression levels of *CXCL8* and *IL1B* were quantified for each donor. (*$P < .05$, **$P < .01$, according to the Wald test implemented in DESeq2). *CXCL8* **(G)** and *IL1B* **(H)** mRNA expression in freshly purified neutrophils of COPD (n = 7) and control donors (n = 7) from the independent cohort. Gene expression is shown as mean normalized expression (*MNE*) units after normalization over *GAPDH*. Production of CXCL8 **(I)** and IL1B **(J)** was measured in cell-free supernatants of resting (*med*) and LPS-stimulated (10 ng/mL) or R848-stimulated (5 μmol/L) neutrophils for 20 hours (patients with COPD: n = 6, control donors: n = 3; **$P < .01$ according to 2-way ANOVA followed by Sidak multiple comparison test). Glucose concentration was measured in cell-free supernatants of resting and LPS-stimulated (10 ng/mL) **(K)** or R848-stimulated (5 μmol/L) **(L)** neutrophils for 20 hours. *Green dots*: patients with COPD (n = 6 in **K**, n = 5 in **L**); *gray dots*: control donors (n = 5 in **K**, n = 3 in **L**). *$P < .05$ according to 2-way ANOVA followed by Sidak multiple comparison test. **(M)** *C albicans* phagocytosis measured in 6 patients with COPD and 6 controls. Data show the percentage of neutrophils that phagocytosed at least 1 yeast after 40 minutes. **(N)** O_2^- production of resting or phorbol 12-myristate 12-acetate (*PMA*)-activated (10 ng/mL) neutrophils for 1 hour (*$P < .05$ according to unpaired *t*-test). *Green dots*: patients with COPD (n = 7); *gray dots*: control donors (n = 7).

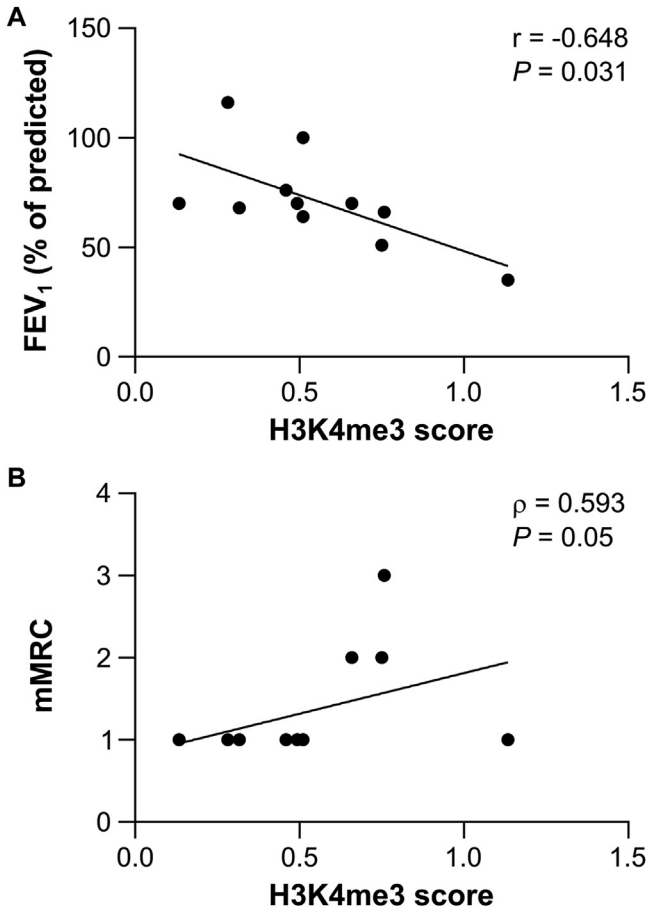

FIG 6. Correlation analysis between epigenetic signature and clinical data of COPD donors. **(A)** H3K4me3 score was generated from H3K4me3 ChIP-seq data of COPD neutrophils (n = 11) as described in Supplemental Methods in the Online Repository. Correlation analysis between the H3K4me3 score and FEV_1 (% of predicted) **(A)** or mMRC **(B)** is shown. The regression line is shown. *R* and *P* obtained from the Pearson **(A)** and Spearman **(B)** analyses are reported.

cell progenitors in the bone marrow, thereby endowing neutrophils with heightened responsiveness.[43] Although bone marrow studies in patients with COPD are not indicated, indirect evidence supports the hypothesis of trained immunity at the progenitor level. For instance, chronic inflammation, as seen in COPD, has been associated with an increase in myelopoiesis.[44] Moreover, an increase in myelopoiesis and a concomitant decrease in lymphopoiesis occurs in subjects with COPD and correlates with the disease severity.[45] This trait had been observed also in BCG-trained mice where trained immunity induces a shift in progenitor cell differentiation toward myeloid lineages.[43] In a murine model of COPD, Kapellos et al[8] recently showed that the transcriptional reprogramming observed in circulating neutrophils is present within the bone marrow compartment with the majority of changes occurring in early granulocyte-monocyte progenitors (GMPs), thus supporting that altered transcription of neutrophil subsets in COPD is induced at the progenitor stage. This elevated and reprogrammed granulopoiesis is directly linked to alteration in the human blood compartment and correlates with disease clinical manifestations.[8]

Identifying the soluble mediator in COPD that influences developing neutrophils in the bone marrow remains a crucial yet unresolved question. To date, several cytokines have been shown to induce long-term trained immunity by reprogramming stem cells in the bone marrow, with IL1B and types I and II interferons playing key roles.[46] At the same time, >50 cytokines have been found to be elevated in the serum of patients with COPD,[47] making it particularly challenging to pinpoint the specific cytokine responsible for neutrophil precursor reprogramming. This complexity underscores the need for future investigations to unravel the key drivers of this process.

Despite the increased functional responsiveness of neutrophils, which should theoretically protect against infections, COPD is paradoxically associated with recurrent acute respiratory infections[48] and bacterial colonization,[49] underscoring a possible maladaptive response. Indeed, the persistent heightened responsiveness of innate immune cells due to epigenetically imprinted "memory" from prior stimuli, has now expanded to encompass its potential roles in both health and disease. Recent research revealed that the ability of innate immune cells to enhance their responsiveness extends beyond merely strengthening host defense mechanisms against infection. This ability also plays a role in chronic inflammation, autoimmune disorders, and even cancer progression highlighting its broader impact on both immune regulation and disease development.[50]

Studies on the phagocytic ability of neutrophils in COPD provided conflicting results, demonstrating, contrary to what we observed *in vitro*, either no impairment or reduction of *C albicans* phagocytosis.[51] However, these data are not necessarily in conflict because neutrophil responses *in vivo* may depend on several factors, such as the local immunologic and metabolic environment. In line with this concept, mouse alveolar macrophages trained by pre-exposure to LPS promoted bacterial clearance *in vitro*, whereas *in vivo* showed impaired bacterial clearance after *Streptococcus pneumoniae* infection and promoted increased lung inflammation.[52] Likewise, it is conceivable that maladaptive trained immunity may impair bactericidal properties while facilitating proinflammatory and pathological functions of neutrophils. This maladaptive hyper-responsiveness is suggested by a weak yet significant correlation between H3K4me3 epigenetic scores in neutrophils and clinical COPD severity metrics such as FEV_1% of predicted and Modified British Medical Research Council Questionnaire. Although preliminary due to a small cohort size, these findings suggest that trained neutrophils could indeed contribute to disease exacerbation through excessive inflammatory responses. This supports the growing notion that hyperinflammation in trained immunity may shift from protective to pathogenic,[53] highlighting an area for further research to assess therapeutic interventions targeting neutrophil reprogramming.

DISCLOSURE STATEMENT

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Key messages

- **Circulating neutrophils in COPD exhibit transcriptional and epigenetic features of neutrophils from individuals who are BCG-vaccinated and referred to as "trained."**
- **Transcriptional and epigenetic adaptations in COPD neutrophils enhance their responsiveness to stimuli, including elevated production of CXCL8, IL1B, ROS, and increased phagocytic activity.**

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