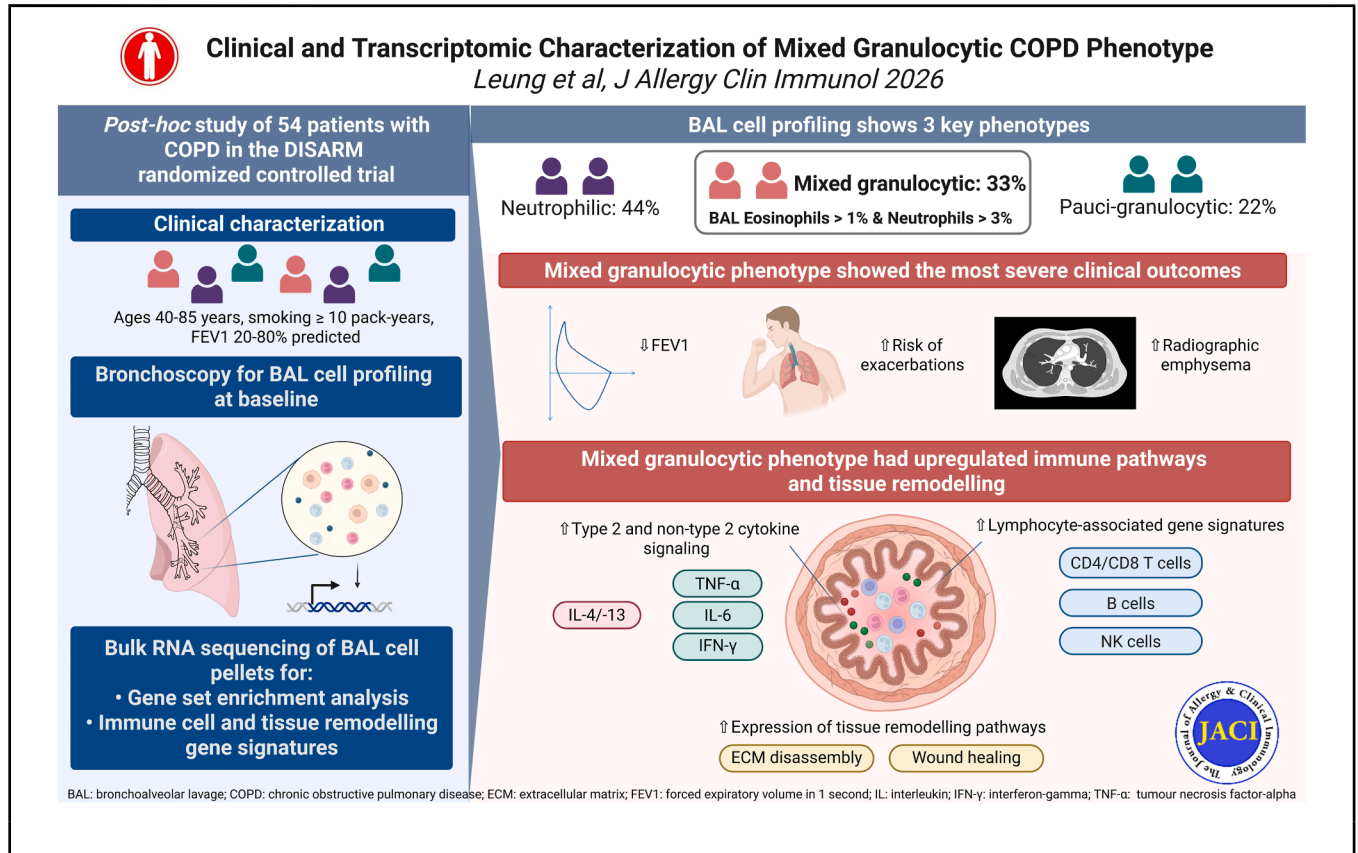


Clinical and transcriptomic characterization of mixed granulocytic phenotype in chronic obstructive pulmonary disease



Clarus Leung, MD, Jung-Wan Yoo, MD, PhD, Hye Yun Park, MD, PhD, Haad Bhutta, BSc, Puneet Sidhu, BASc, Elizabeth Guinto, PhD, et al

GRAPHICAL ABSTRACT



Capsule summary: Mixed granulocytic chronic obstructive pulmonary disease identifies a high-risk phenotype with worse outcomes, type 1 and type 2 immune activation, and increased lung tissue remodeling, supporting airway-based stratification to guide targeted treatment.

Clinical and transcriptomic characterization of mixed granulocytic phenotype in chronic obstructive pulmonary disease



Clarus Leung, MD,^{a,b} Jung-Wan Yoo, MD, PhD,^{b,c} Hye Yun Park, MD, PhD,^{b,d} Haad Bhutta, BSc,^b Puneet Sidhu, BASc,^{b,e} Elizabeth Guinto, PhD,^b Graeme J. Koelwyn, PhD,^{b,e} Milad Vahedi, DVSc,^{b,e} Xuan Li, MSc,^b Rachel L. Eddy, PhD,^{b,f} Jonathon A. Leipsic, MD,^{b,f} Fernando Sergio Leitao Filho, MD,^{b,g} Julia S. Yang, MSc,^b Stephen Milne, PhD,^{b,h,i,j} Hiroto Takiguchi, MD,^k Kentaro Akata, MD,^l Seung Won Ra, MD,^m Ji-Yong Moon, MD, PhD,ⁿ Hyun Kuk Kim, MD,^o Yuji Cho, MD,^c Kei Yamasaki, MD,^p Stephan F. van Eeden, MD, PhD,^{a,b} Tawimas Shaipanich, MD,^{a,b} Stephen Lam, MD,^{a,b,q} Janice M. Leung, MD,^{a,b} and Don D. Sin, MD^{a,b}

Vancouver and Burnaby, British Columbia, Canada; Jinju, Seoul, Ulsan, and Busan, South Korea; Sao Paulo, Brazil; Westmead and Camperdown, Australia; and Isehara and Fukuoka, Japan

Background: In chronic obstructive pulmonary disease (COPD), patients with peripheral airways infiltrated by eosinophils and neutrophils (“mixed granulocytic COPD”) show worse outcomes than those without.

Objectives: We sought to examine the expression of immune response and lung tissue remodeling pathways in patients with mixed granulocytic COPD.

Methods: In this *post hoc* study of the DISARM (A Study to Investigate the Differential Effects of Inhaled Symbicort and Advair on Lung Microbiota) randomized controlled trial, mixed granulocytic COPD was defined by eosinophils >1% and neutrophils >3% of the total leukocyte count in bronchoalveolar lavage (BAL). We compared clinical outcomes of mixed

granulocytic COPD with 2 other phenotypes (neutrophilic, pauci-granulocytic). We then examined canonical pathways using gene set enrichment analysis and expression of immune cell and tissue remodeling gene signatures in BAL across phenotypes.

Results: Among 54 patients, 33% had mixed granulocytic COPD. Patients with mixed granulocytic COPD had the lowest FEV₁, the most radiographic emphysema, and the highest annualized exacerbation rates. Cell pellets of BAL in mixed granulocytic COPD showed upregulated gene expression of type 1 (TNFA, IL6, and IFNG signaling) and type 2 (IL4/13 signaling) immune responses relative to the neutrophilic and pauci-granulocytic phenotypes. Mixed granulocytic COPD was marked by increased expression of gene signatures for natural killer cells, B cells, and CD4 and CD8 naive and memory/effector T cells. Mixed granulocytic COPD showed the highest expression of tissue remodeling processes, which significantly associated with lower FEV₁.

Conclusions: Mixed granulocytic COPD is marked by complex immune responses in the peripheral airways and is associated with increased tissue remodeling that associates with more severe airflow limitation. (J Allergy Clin Immunol 2026;158:982-93.)

Key words: COPD, eosinophilic, neutrophilic, immune response, emphysema, tissue remodeling, BAL

Chronic obstructive pulmonary disease (COPD) is a progressive lung disease characterized by persistent inflammation of the peripheral airways. Persistent airway inflammation contributes to airway tissue remodeling and to the formation of emphysema. These major histopathologies and others result in airflow limitation that determines the severity of COPD.¹ Reduced lung function in COPD has been linked to the differential expression of aberrant tissue repair genes that favor the degradation of tissues in the small conducting airways.²

The phenotypes of airway inflammation during stable disease are heterogeneous and have been broadly dichotomized into neutrophil- or eosinophil-associated subtypes.³ Previously, we showed that patients who have COPD with airway eosinophilia have higher gene expression of type 2 airway inflammation in epithelial brushings than those without airway eosinophilia.⁴ Patients who have COPD with eosinophilia can have an overlap of airway neutrophilia (often classified as a mixed granulocytic

From ^athe Division of Respiratory Medicine, Department of Medicine, ^bthe Centre for Heart Lung Innovation, St Paul's Hospital, ^cthe Department of Radiology, University of British Columbia, and ^dthe British Columbia Cancer Research Institute, Vancouver; ^ethe Division of Pulmonary Medicine, Department of Internal Medicine, Gyeongsang National University Hospital, Gyeongsang National University School of Medicine, Jinju; ^fthe Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, and ^gthe Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Internal Medicine, Konkuk University Medical Center, Konkuk University School of Medicine, Seoul; ^hthe Faculty of Health Sciences, Simon Fraser University, Burnaby; ⁱthe Division of Pulmonary Medicine, Federal University of Sao Paulo; ^jthe Department of Respiratory and Sleep Medicine, Westmead Hospital, and ^kthe Ludwig Engel Centre for Respiratory Research, The Westmead Institute for Medical Research; ^lthe Sydney Medical School, The University of Sydney, Camperdown; ^mthe Division of Pulmonary Medicine, Department of Medicine, Tokai University School of Medicine, Isehara; ⁿthe Division of Infection Control and Prevention, University of Occupational and Environmental Health, Kitakyushu City, and ^othe Department of Respiratory Medicine, University of Occupational and Environmental Health Japan, Fukuoka; ^pthe Ulsan University Hospital, University of Ulsan College of Medicine; and ^qthe Department of Internal Medicine, College of Medicine, Inje University Haeundae Paik Hospital, Busan.

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Corresponding author: Don D. Sin, MD, Centre for Heart Lung Innovation, St Paul's Hospital, Room 166, 1081 Burrard St, Vancouver, BC, Canada V6Z 1Y6. E-mail: don.sin@hli.ubc.ca.

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Abbreviations used

BAL:	Bronchoalveolar lavage
COPD:	Chronic obstructive pulmonary disease
CT:	Computed tomography
FVC:	Forced vital capacity
GSEA:	Gene set enrichment analysis
ICS:	Inhaled corticosteroids
IRR:	Incidence rate ratio
LABA:	Long-acting beta agonist
NK:	Natural killer

phenotype),^{5,6} and these patients have lower lung function and more abnormal lung parenchymal structure compared to others.⁷ However, very little is known about the inflammatory mediators that modulate this phenotype. We do not know whether the mixed granulocytic phenotype is marked by increased activity of other immune cells. Furthermore, how eosinophilia and neutrophilia of the peripheral airways relate to lung tissue remodeling and severity of COPD is unknown.

Here, we compared the clinical features of the mixed granulocytic phenotype with 2 other common COPD phenotypes (neutrophilic and pauci-granulocytic). We then investigated the expression of genes and pathways related to airway inflammation, immune cells, and lung tissue remodeling in bronchoalveolar lavage (BAL) fluid across the 3 phenotypes. We reasoned that cell pellets from BAL fluid collected during research bronchoscopies provide transcriptomic insights into mechanisms that may mediate histopathologic changes in the small airways and lung parenchyma.⁸ Using a cohort of patients with COPD, a subset of whom were randomized to inhaled corticosteroid (ICS) and long-acting beta agonist (LABA) treatment for 12 weeks, our secondary objective was to directly evaluate the effects of ICS/LABA on the changes in lung tissue remodeling gene expression in the BAL fluid. We hypothesized that patients with mixed granulocytic COPD have an upregulation of complex immune pathways that are associated with increased lung tissue remodeling compared to the pauci-granulocytic and neutrophilic phenotypes.

METHODS**Participants**

Our study included male and female participants between the ages of 40 and 85 years with a clinical diagnosis of COPD according to a modified GOLD (Global Initiative for Chronic Obstructive Lung Disease) criteria:⁹ post-bronchodilator FEV₁/forced vital capacity (FVC) <70%, a smoking history of ≥10 pack-years, and either a post-bronchodilator FEV₁ between 20% and 80% of predicted values or evidence of emphysema on computed tomography (CT) scans of the thorax (see [Table E1](#) in this article's Online Repository at www.jacionline.org).

Study design

DISARM (A Study to Investigate the Differential Effects of Inhaled Symbicort and Advair on Lung Microbiota) was a randomized controlled trial that compared the effects of 2 different ICS/LABA combinations versus LABA on the airway microbiome of patients with COPD.¹⁰ All participants underwent a 4-week run-in period during which they discontinued their

home ICS medication. Following the run-in period, participants underwent a baseline research bronchoscopy. CT lung scans were done to quantify radiographic emphysema and airway wall measurements. Participants were then randomly assigned (1:1:1) to receive either ICS/LABA (budesonide/formoterol 400/12 µg twice daily or fluticasone propionate/salmeterol 250/50 µg twice daily); or LABA only (formoterol 12 µg twice daily). After treatment for 12 weeks, participants underwent a follow-up bronchoscopy (see [Fig E1](#) in this article's Online Repository at www.jacionline.org). Participants were monitored for 52 weeks beginning 30 days after the follow-up bronchoscopy to ascertain the number and dates of moderate or severe respiratory exacerbations, defined by the need for systemic treatment, emergency department visits, or hospitalization.¹¹ [Fig E2](#) in this article's Online Repository (available at www.jacionline.org) shows the number of patients who were included in the analyses. This study is registered at ClinicalTrials.gov (NCT02833480) and received ethics approval from the University of British Columbia and the Providence Health Care Research Ethics Committee (H14-02277). All participants provided written informed consent. Additional details are included in the Methods section of this article's Online Repository (available at www.jacionline.org).

Bronchoscopy and specimen collection

We conducted the same bronchoscopy protocol at baseline (week 4) and at follow-up (week 17).⁴ Recovery volumes of BAL, total cell counts, cell concentrations, and anatomic location of BAL sampling at baseline are reported in [Table E2](#) in this article's Online Repository at www.jacionline.org. Cell populations in BAL were characterized using the cytopspin method, and cell counting was performed by 2 independent, trained observers who were blinded to the clinical information of the participants. Cell pellets from the BAL samples underwent bulk RNA sequencing. For our secondary analyses, profiling of the airway epithelial microbiome was performed and described in the Methods section of this article's Online Repository.

Definition of COPD subtypes based on BAL cell percentages

Gold-standard BAL thresholds for COPD phenotypes are not established. Therefore, we classified the COPD participants into 3 phenotypes based on the expected eosinophil and neutrophil percentages in the BAL fluid of healthy adults.¹² BAL eosinophil and neutrophil percentages were ≤1% and ≤3% in pauci-granulocytic subgroup, ≤1% and >3% in the neutrophilic subgroup, and >1% and >3% in the mixed granulocytic subgroup. The BAL eosinophil threshold of 1% was selected based on our prior work demonstrating its clinical relevance.^{4,13} The BAL neutrophil threshold of 3% was defined according to established BAL cellular patterns in healthy adult nonsmokers.¹²

RNA sequencing of BAL fluid

Cell pellets from BAL fluid were processed for bulk RNA sequencing according to our previous protocol and standardized bioinformatic pipeline (see the Methods section of this article's Online Repository).¹⁴ First, we performed differential gene expression using the *limma-voom* workflow.¹⁵ Raw counts were transformed to log₂ counts per million (logCPM) with precision

weights estimated using *voom*, and linear models were fitted using *lmFit*. We performed comparisons between the mixed granulocytic and neutrophilic groups, as well as between the mixed granulocytic and pauci-granulocytic groups. Next, to understand the biologic mechanisms associated with the mixed granulocytic phenotype, we conducted gene set enrichment analysis (GSEA) using genes that were ranked by the moderated *t*-statistic from the *limma-voom* model.^{4,16,17} We assessed for enriched pathways using Hallmark gene sets (MSigDB) and Reactome pathways annotations,¹⁸ using a weighted enrichment statistic. Gene sets were restricted to those with 10 to 500 genes. Statistical significance was defined as false discovery rate <0.05. As sensitivity analyses, we repeated our differential gene expression and GSEA in a model that adjusted for BAL cell concentration and another that adjusted for pack-years of smoking to show that our findings were not driven by cellularity differences or smoking across subjects. Additional details are included in the Methods section of this article's Online Repository.

Gene signature scoring

We characterized the expression of gene markers specific for CD4 and CD8 naive and memory/effector T cells, B cells, and natural killer (NK) cells because of their potential pathologic roles in mediating various inflammatory phenotypes in COPD.¹⁹ Gene markers specific to each immune cell type were selected from a published single-cell RNA-sequencing lung atlas (see Table E3 and the Methods section in this article's Online Repository at www.jacionline.org).²⁰ Immune cell gene signatures were summarized using mean *z*-scores to average the expression of genes within each signature.²¹

We also quantified 2 Gene Ontology biological processes relevant to lung tissue remodeling (GO:0061041 Regulation of Wound Healing,²² GO:0022617 Extracellular Matrix Disassembly)²³ (see Table E4 and the Methods section in this article's Online Repository at www.jacionline.org). Gene signatures were calculated using gene set variation analysis with a gaussian kernel cumulative distribution function applied to the log₂ expression values from the *limma-voom* model.²⁴ Gene set variation analysis was selected to capture subject-level process activity in an unsupervised, nonparametric manner that is less sensitive to outlier genes and differences in expression scale.²⁴ The scores were then analyzed in relation to COPD phenotypes and clinical outcomes.

Statistical analyses

The objective of the analyses was to evaluate how mixed granulocytic COPD differs from other phenotypes (neutrophilic, pauci-granulocytic) in clinical features and gene expression relevant to airway inflammation, immune cells, and lung tissue remodeling in the bronchoalveolar space. First, we compared clinical and imaging characteristics among patients with mixed granulocytic COPD and other phenotypes using a 1-way ANOVA test (for normally distributed variables) or Kruskal-Wallis test (for non-normally distributed variables). As secondary analyses, we also compared airway microbiome profiles among groups. We determined the number of moderate or severe respiratory exacerbations experienced over 12 months of follow-up. We fitted a Poisson regression model and determined the annualized exacerbation rates (events per patient-year) for each subgroup using marginal predictions from the model. We accounted for

variable follow-up time due to deaths and administrative censoring. Incidence rate ratios (IRRs) comparing mixed granulocytic versus pauci-granulocytic, mixed versus neutrophilic, and neutrophilic versus pauci were derived using linear combination of model coefficients and shown in a Forest plot.

Next, we performed differential gene expression and GSEA to investigate canonical pathways in mixed granulocytic COPD using 2 independent differential expression comparisons (mixed granulocytic vs neutrophilic, mixed granulocytic vs pauci-granulocytic). To understand shared transcriptional patterns between comparisons, we performed a rank-based log fold-change correlation analysis and presented the GSEA results for the 2 comparisons using a plot of the overlapping upregulated and downregulated pathways. We compared the expression of immune cell and lung tissue remodeling gene scores across the groups. The relationships between lung tissue remodeling gene expression and FEV₁ were assessed in linear regression models. Finally, as secondary analysis, we examined the changes in expression of gene signatures among patients randomized to ICS/LABA versus LABA for 12 weeks. Statistical analyses were performed using Stata (version 19.5, StataCorp, College Station, Tex) and Prism (version 10.6.1, GraphPad, La Jolla, Calif). Statistical significance was defined as 2-sided *P* < .05.

RESULTS

Mixed granulocytic airway inflammation is present in one-third of patients with COPD

Among 54 patients with COPD, 18 patients (33%) were classified as the mixed granulocytic phenotype, 24 patients (44%) with the neutrophilic phenotype, and 12 patients (22%) with the pauci-granulocytic phenotype. Four patients in the DISARM study only met the BAL eosinophil > 1% threshold and were not classified into any of the 3 phenotypes. The percentages of neutrophils, eosinophils, macrophages, and lymphocytes in the BAL by COPD subtypes are shown in Fig 1, A and Table E5 in this article's Online Repository at www.jacionline.org. The percentage of neutrophils moderately correlated with the percentage of eosinophils in BAL (Spearman ρ = 0.43; *P* = .002), (Fig 1, B). Representative cytospin images of the phenotypes are shown in Fig 1, C.

Patients who have COPD with mixed granulocytic airway inflammation have worse clinical outcomes than others

Compared to the pauci-granulocytic group, the neutrophilic and mixed granulocytic groups had more severe airflow obstruction (lower FEV₁ and FEV₁/FVC) (Fig 2, A and B, Table I). Patients in the mixed granulocytic group also had more radiographic emphysema than the pauci-granulocytic group, as noted by a more negative mean Hounsfield unit value at the 15th percentile (Fig 2, C). The severity of radiographic emphysema was similar between the lobar location of BAL sampling and other lobes of the lung (see Fig E3 in this article's Online Repository at www.jacionline.org). There was no difference in CT-derived airway lumen area (mm²) or wall thickness (%) across groups (see Fig E4 in this article's Online Repository at www.jacionline.org). Patients with neutrophilic COPD accumulated the highest pack-years of smoking but there was no difference in current smoking status among the 3 phenotypes (Table I).

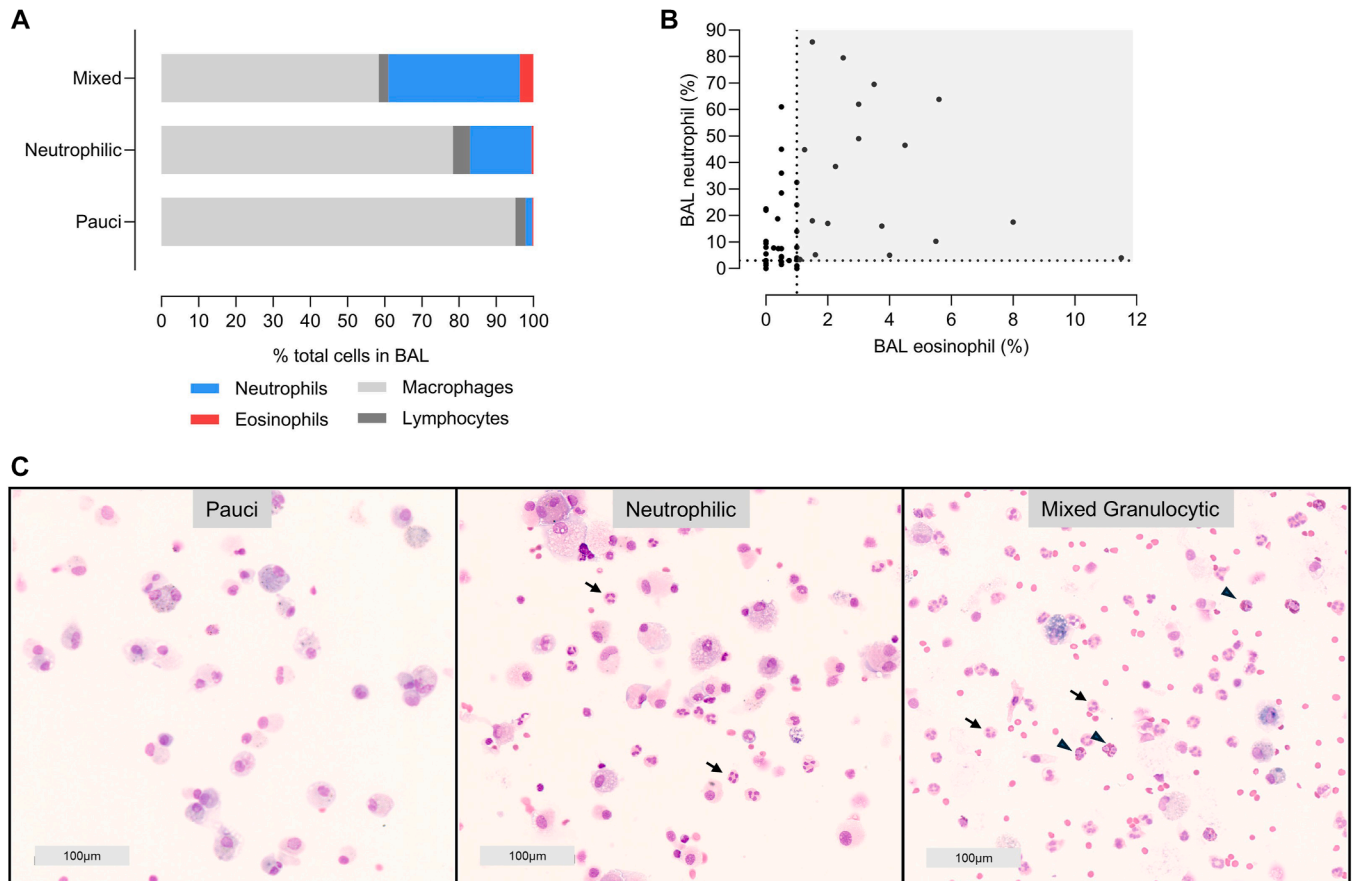


FIG 1. Mixed granulocytic COPD is characterized by the presence of neutrophils and eosinophils in BAL. **(A)** Percentages of cell differential in BAL across COPD subgroups. Data are reported in Table E5. **(B)** Correlation between BAL neutrophil percentages and BAL eosinophil percentages in COPD. Spearman $\rho = 0.43$; $P = .002$. Shaded region represents patients with COPD who were included in the mixed granulocytic group. **(C)** Representative images of cells in BAL fluid across COPD subgroups. Slides were prepared using the cytopsin method and stained with Hemacolor Stain (Millipore Sigma, USA) to visualize cellular morphology. Neutrophils are marked by the black arrows. Eosinophils are marked by the black arrowheads. Images were taken at 21.5 $\times$ magnification.

The annualized exacerbation rate was the highest in the mixed granulocytic group: 0.0275 events per person-year (Fig 2, D). The mixed granulocytic group was significantly more likely to experience an exacerbation than the neutrophilic group: IRR = 2.35; 95% CI = 1.17-4.72; $P = .017$ (Fig 2, E). The likelihood of exacerbation was similar between the mixed granulocytic and the pauci-granulocytic groups, as well as between the neutrophilic and the pauci-granulocytic groups (Fig 2, E). The prevalence of frequent exacerbation (≥ 2 over 12 months) was 50% in the mixed granulocytic group, 21% in the neutrophilic group, and 17% in the pauci-granulocytic group, although differences across groups did not meet statistical significance (see Fig E5 in this article's Online Repository at www.jacionline.org).

Types 1 and 2 airway immune responses are upregulated in mixed granulocytic COPD compared to pauci-granulocytic and neutrophilic groups

We performed analyses of differentially expressed genes and enriched pathways using GSEA in 2 comparisons: mixed

granulocytic versus pauci-granulocytic COPD and mixed granulocytic versus neutrophilic COPD. Results of our differential gene expression analyses are shown in Fig E6 and Tables E6 and E7 in this article's Online Repository (available at www.jacionline.org). There was a strong positive correlation in the $\log_2$ fold-change values of genes between the 2 comparisons (Spearman correlation: 0.77; $P < .0001$) (Fig 3, A). Among genes concordantly upregulated in mixed granulocytic COPD, those with the highest positive correlation scores included *CCL4L1* (a CCR5-binding chemokine paralog of CCL4 that recruits NK cells and CD8 T cells and contributes to type 1 airway inflammation),^{25,26} *OSM* (a cytokine in the IL6 family that drives tissue remodeling),²⁷ and *CXCL8* (a neutrophil-recruiting chemokine).²⁵

Our GSEA between mixed granulocytic and pauci-granulocytic COPD showed 32 significant Hallmark pathways and 390 significant Reactome pathways (Tables E8 and E9 in this article's Online Repository at www.jacionline.org). Our GSEA between mixed granulocytic and neutrophilic COPD showed 29 significant Hallmark pathways and 461 significant Reactome pathways (Tables E10 and E11 in this article's Online Repository

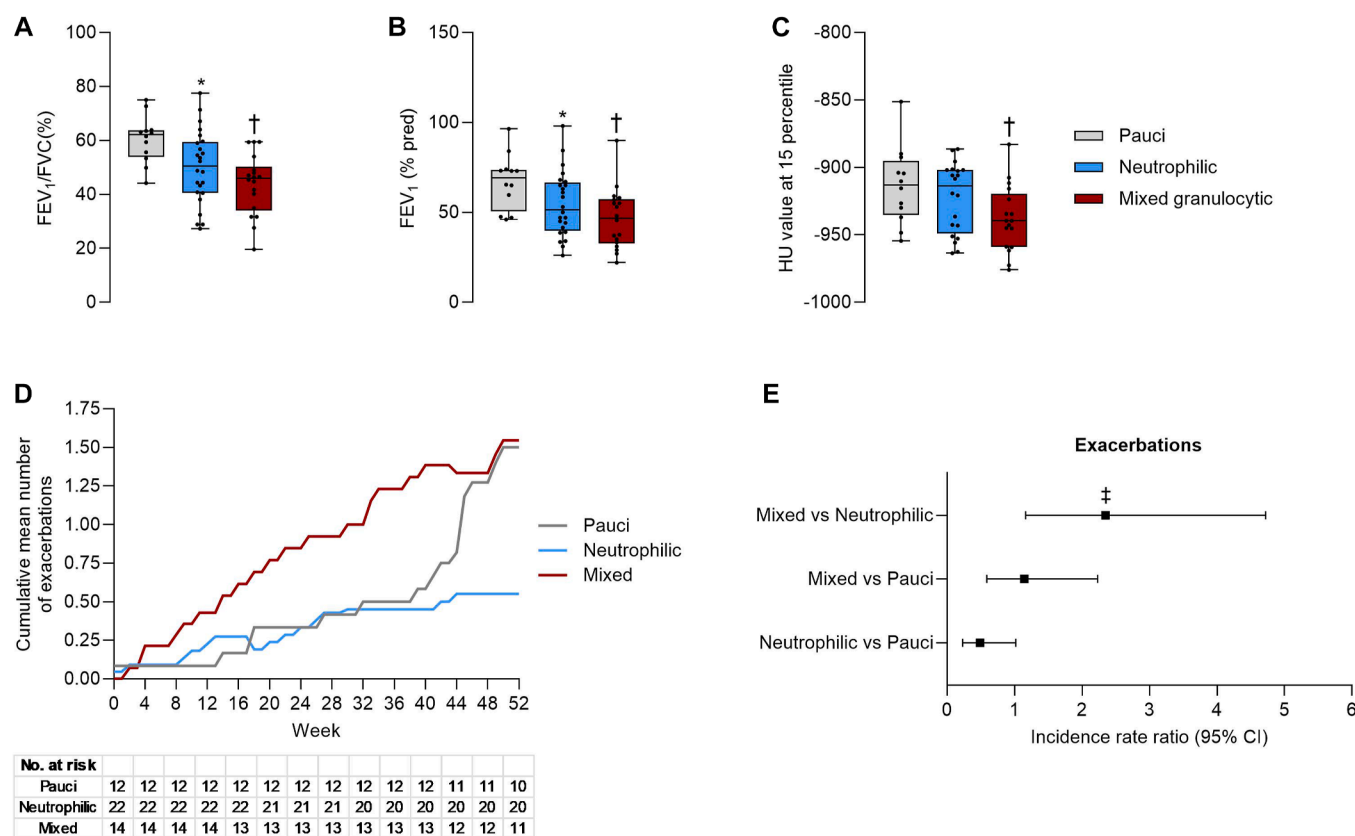


FIG 2. Patients with mixed granulocytic COPD have more severe clinical features. **(A)** Mixed granulocytic group has lower prebronchodilator FEV₁/FVC than the pauci-granulocytic group ($43.7 \pm 11.2\%$ vs $60.4 \pm 8.8\%$; $P = .0005$). Neutrophilic group has lower prebronchodilator FEV₁/FVC than the pauci-granulocytic group ($50.0 \pm 13.6\%$ vs $60.4 \pm 8.8\%$; $P = .017$). **(B)** Mixed granulocytic group has lower prebronchodilator FEV₁ than the pauci-granulocytic group ($46.5 \pm 16.8\%$ predicted vs $67.0 \pm 15.3\%$ predicted; $P = .002$). Neutrophilic group has lower prebronchodilator FEV₁ than the pauci-granulocytic group ($54.5 \pm 18.0\%$ predicted vs $67.0 \pm 15.3\%$ predicted; $P = .043$). **(C)** Mixed granulocytic group has more radiographic emphysema (lower Hounsfield unit [HU] value at 15th percentile) than the pauci-granulocytic group (median [Q1, Q3]: $-939.6 [-958.7, -923.5]$ HU vs $-912.9 [-933.4, -898.2]$ HU; $P = .027$). *Pauci versus neutrophilic: $P < .05$; †Pauci versus mixed: $P < .05$. **(D)** Annualized exacerbation rate (events per patient-year) is 0.024 (95% CI: 0.012-0.036) in the pauci-granulocytic group, 0.012 (95% CI: 0.005-0.018) in the neutrophilic group, and 0.028 (95% CI: 0.015-0.040) in the mixed granulocytic group. To calculate exacerbation rates, participants were monitored for 52 weeks beginning 30 days after the follow-up bronchoscopy. Of 54 participants, 48 (12 pauci, 22 neutrophilic, 14 mixed) were included in this analysis. Six participants were not included because they did not undergo a follow-up bronchoscopy. We accounted for variable follow-up time due to administrative censoring and deaths. Numbers below the figure indicate the number of participants contributing data ("at risk") at each time point. **(E)** In the pairwise comparisons of IRRs, the mixed granulocytic group had significantly higher exacerbation rates than the neutrophilic group (IRR = 2.35; 95% CI: 1.17-4.72; ‡ $P = .017$). Exacerbation rates were similar between the mixed granulocytic group and the pauci-granulocytic group (IRR = 1.14; 95% CI: 0.59-2.23; $P = .69$). Exacerbation rates were similar between the neutrophilic group and the pauci-granulocytic group (IRR = 0.49; 95% CI: 0.23-1.02; $P = .056$).

at www.jacionline.org). The top upregulated and downregulated pathways that overlap in the 2 comparisons are shown in Fig 3, B. There was upregulation of multiple inflammatory response pathways including TNFA signaling via NFKB, IL6/JAK/STAT3 signaling, IFNG response, and IL4 and IL13 signaling. In our exploratory analyses, we found that higher pathway activity in IL4/13 signaling and TNFA signaling via NFKB correlated with more severe airflow obstruction (lower FEV₁ and FEV₁/FVC) (see Fig E7 in this article's Online Repository at www.jacionline.org). There was downregulation of fatty acid metabolism and adipogenesis pathways.

In our sensitivity analyses, the results remained similar after adjusting for BAL cell concentration (see Fig E8 and Table E12 in

this article's Online Repository at www.jacionline.org), and for pack-years of smoking (Table E13 in this article's Online Repository at www.jacionline.org).

Expression of other immune cell signatures is increased in mixed granulocytic COPD

Our GSEA findings were further supported by increased expression of immune cell gene signatures in mixed granulocytic COPD relative to other phenotypes. The mixed granulocytic group showed significantly higher expression of NK-cell, B-cell, CD4 memory/effector T-cell, CD8 memory/effector T-cell, and CD4 naive T-cell gene signatures than the pauci-granulocytic

TABLE I. Clinical characteristics of COPD patients by phenotype

	Pauci-granulocytic (n = 12)	Neutrophilic (n = 24)	Mixed granulocytic (n = 18)	P value
Demographics				
Age (y)	62.9 ± 9.8	65.4 ± 7.4	66.1 ± 7.6	.55
Male sex	10 (83.3)	20 (83.3)	15 (83.3)	1.00
Body mass index (kg/m ²)	26.3 (4.8)	26.3 (6.4)	23.2 (4.4)	.14
Current smoker	8 (66.7)	11 (45.8)	6 (33.3)	.20
Smoking (pack-years)	32.0 ± 17.2	55.5 ± 21.4	41.1 ± 16.6	.003
Pulmonary function				
Prebronchodilator spirometry data				
FEV ₁ , % pred	67.0 ± 15.3	54.5 ± 18.0	46.5 ± 16.8	.009
FVC, % pred	85.9 ± 16.3	84.6 ± 17.7	82.5 ± 19.5	.87
FEV ₁ /FVC, %	60.4 ± 8.8	50.0 ± 13.6	43.7 ± 11.2	.002
Postbronchodilator spirometry data				
FEV ₁ , % pred	70.0 ± 14.6	60.8 ± 19.0	51.9 ± 17.2	.06
FVC, % pred	88.2 ± 15.6	89.5 ± 18.5	85.5 ± 20.5	.82
FEV ₁ /FVC, %	63.0 ± 10.2	51.8 ± 13.5	49.0 ± 12.0	.017
COPD characteristics				
Total SGRQ score*	41.9 (35.1, 47.9)	37.8 (28.5, 56.0)	44.6 (29.3, 53.7)	.82
Long-term oxygen use	1 (8.3)	0 (0)	1 (5.6)	.40
Baseline ICS use†	5 (41.7)	15 (62.5)	13 (72.2)	.24
Blood leukocytes				
Blood eosinophil (×10 ⁹ /L)	0.2 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	.22
Blood neutrophil (×10 ⁹ /L)	4.0 ± 1.3	4.6 ± 1.5	4.8 ± 2.1	.47
CT imaging characteristics				
LAA ⁻⁹⁵⁰ , %	3.88 (1.24, 8.95)	4.00 (0.90, 12.7)	10.16 (6.05, 21.08)	.08
HU value at 15 th percentile	-912.9 (-933.4, -898.2)	-913.8 (-947.1, -901.9)	-939.6 (-958.8, -923.6)	.06

Data presented as mean ± SD, n (%), or median (Q1, Q3). *P* values calculated as chi-square test for categorical variables, 1-way ANOVA for normally distributed continuous variables, and Kruskal Wallis test for non-normally distributed continuous variables. Demographics, pulmonary function, COPD characteristics, and blood leukocytes were collected at week 0. CT imaging characteristics were measured at week 4.

LAA⁻⁹⁵⁰, percentage of low attenuation areas <950; HU, Hounsfield units; *Pred*, predicted; *SGRQ*, St George's Respiratory Questionnaire.

*SGRQ measures impact on overall health, daily life, and perceived well-being in patients with COPD. Scores range from 0 to 100, with higher scores indicating more limitations.

†Prior to study run-in.

group (Fig 4, A-E). The mixed granulocytic group also showed a significantly higher B-cell signature than the neutrophilic group (Fig 4, B).

The increased immune response in mixed granulocytic COPD is not related to differences in airway microbiome

The upregulation of type 1 immune response is strongly associated with airway bacterial dysbiosis in COPD.^{28,29} This prompted us to investigate whether mixed granulocytic COPD is driven by certain airway microbiome characteristics. Here, we did not observe any differences in the relative abundance of bacterial genera (see Fig E9 in this article's Online Repository at www.jacionline.org), or alpha-diversity metrics as measured by observed operational taxonomic units, Shannon index, Pielou evenness, or Faith phylogenetic diversity (see Fig E10 in this article's Online Repository at www.jacionline.org).

Lung tissue remodeling is increased in mixed granulocytic COPD

Chronic airway inflammation in mixed granulocytic COPD, characterized by upregulated IL6 and IL4/13 signaling, may have important implications for lung tissue injury, repair, and remodeling. Therefore, we investigated the expression of 2 prespecified remodeling pathways in the human Gene Ontology biological processes. We found that the mixed granulocytic COPD group

had a significantly higher expression of extracellular matrix disassembly and regulation of wound healing gene scores than the neutrophilic and the pauci-granulocytic groups (Fig 5, A and B).

Relevant here is that higher expression of the extracellular matrix disassembly and wound healing gene scores is associated with worse airflow limitation (lower FEV₁) in COPD (Fig 5, C and D). These associations remained significant in linear regression models that adjusted for pack-years of cigarette smoking.

Lung tissue remodeling gene scores do not change significantly with ICS

As secondary analyses, we explored the effects of randomized 12-week ICS/LABA versus LABA treatment on the expression of the extracellular matrix disassembly and wound healing gene scores in all patients with COPD. We found no difference in the changes of extracellular matrix disassembly and regulation of wound healing gene scores between ICS/LABA and LABA treatment groups (see Fig E11 in this article's Online Repository at www.jacionline.org). Gene scores at baseline and at follow-up after ICS/LABA and LABA treatment stratified by COPD groups are shown in Fig E12 in this article's Online Repository at www.jacionline.org.

DISCUSSION

Patients who have COPD with mixed granulocytic airway inflammation composed approximately one-third of our clinical

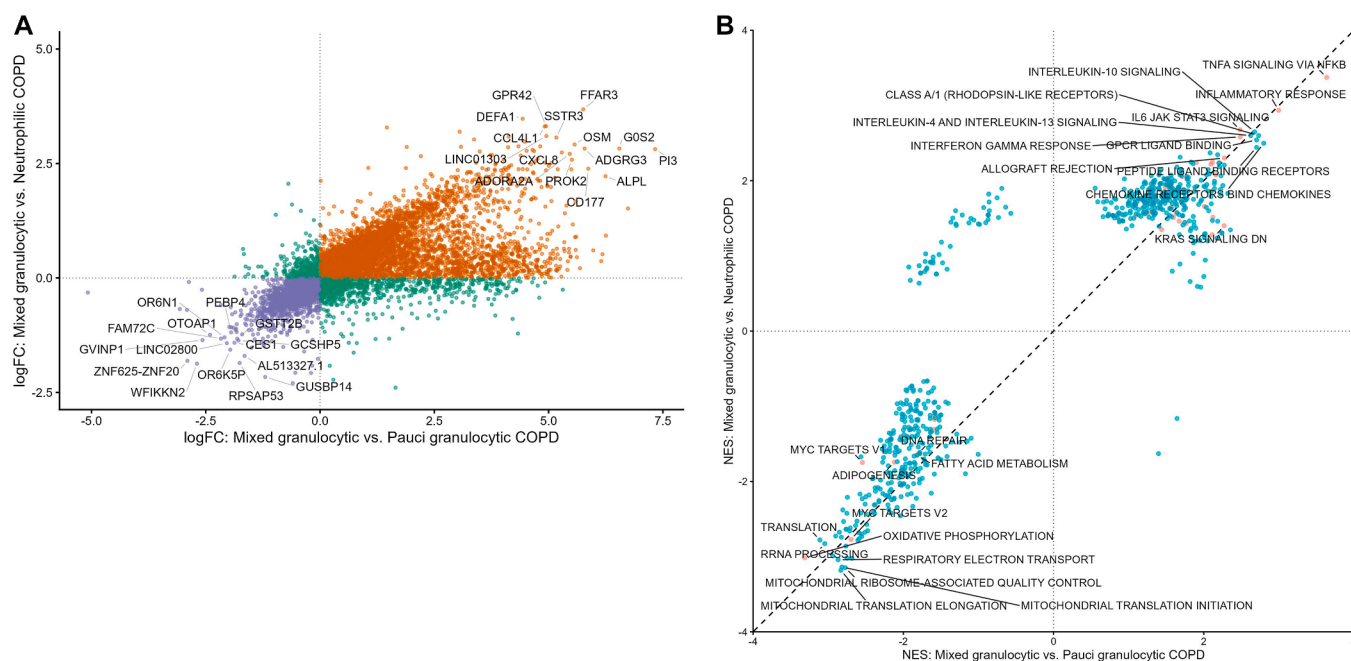


FIG 3. GSEAs show upregulated immune responses in mixed granulocytic COPD. **(A)** Concordance of differentially expressed genes between the 2 comparisons (mixed granulocytic vs pauci-granulocytic groups and mixed granulocytic vs neutrophilic groups). Data points represent genes plotted by its log₂ fold change (logFC) in mixed granulocytic relative to pauci-granulocytic group (x-axis) and in mixed granulocytic relative to neutrophilic group (y-axis). Genes are color-coded based on directional concordance between comparisons: concordantly upregulated in both (orange), concordantly downregulated in both (purple), and discordant (upregulated in one comparison and downregulated in the other) (green). The top concordantly upregulated and downregulated genes with the highest positive or negative correlation scores are labeled. **(B)** Concordance of upregulated and downregulated pathways between the 2 comparisons (mixed granulocytic vs pauci-granulocytic groups and mixed granulocytic vs neutrophilic groups). Scatterplot of normalized enrichment scores (NES) from GSEA comparing mixed granulocytic COPD with pauci-granulocytic (x-axis) and neutrophilic COPD (y-axis). Each point represents a pathway from Hallmark (MSigDB) or Reactome gene sets. Only pathways with false discovery rate <0.05 are shown. Pathways in the upper right quadrant are positively enriched in mixed granulocytic COPD relative to both comparator groups, whereas those in the lower left quadrant are negatively enriched in both comparisons. Selected labels denote the top positive and top negative pathways (by NES) within each gene set collection.

cohort and had more severe clinical features (lower FEV₁, more radiographic emphysema, and more exacerbations) than the neutrophilic and pauci-granulocytic phenotypes. By profiling the patterns of gene expression in cell pellets isolated from BAL samples, we showed that patients with mixed granulocytic COPD had significantly higher expression of heterogeneous (types 1 and 2) immune response than the neutrophilic and pauci-granulocytic phenotypes. Accordingly, the mixed granulocytic COPD phenotype also showed higher activity of multiple immune cells including NK cells, B cells, and CD4 and CD8 naive and memory/effector T cells. Lung tissue remodeling is a pathologic feature of this chronic, complex immune activation and may be a driver of lung function decline and emphysematous changes.

Airway inflammation in COPD is often dichotomized into separate non-type 2 (neutrophilic) and type 2 (eosinophilic) subtypes with distinct biologic mechanisms and therapeutic approaches.^{4,30,31} Here, we found that one-third of patients have an overlap of both subtypes and that airway eosinophilia alone is uncommon. This observation suggests that neutrophils and eosinophils have a reciprocal, or possibly synergistic, relationship in driving airway inflammation. Our pathway analyses support this idea by demonstrating the increased expression of IL4/13 signaling, IFNG response, IL6/JAK/STAT3 signaling, and

TNFA signaling in mixed granulocytic COPD compared to neutrophilic COPD. Therefore, the presence of airway eosinophilia, in addition to neutrophilia, is a marker of a complex immune environment that portends more severe disease. What drives the mixed granulocytic phenotype is unknown because there was no clear relationship with clinical characteristics such as age, pack-years of smoking, or airway microbiome. The absence of stable-state microbiome differences across COPD phenotypes does not preclude host-microbiome interactions, which may emerge most prominently during acute exacerbations.^{28,32}

Our finding of increased IL4/13 signaling in the bronchoalveolar spaces is consistent with our previous study showing increased epithelial signatures of type 2 inflammation and IL13 activation in a subset of COPD.⁴ Together, these observations provide a rationale for the use of type 2-targeting biologic treatments in COPD.^{33,34} Specifically, the upregulation of IL4/13 signaling and mucus pathology may explain why dupilumab (an anti-IL4R- α mAb that inhibits both IL4 and IL13) has been the most consistently effective biologic treatment in eosinophilic COPD.³³⁻³⁵ In this study, the mixed granulocytic group had a mean blood eosinophil count of around 300 cells/ μ L. Consequently, a portion of these individuals are likely eligible

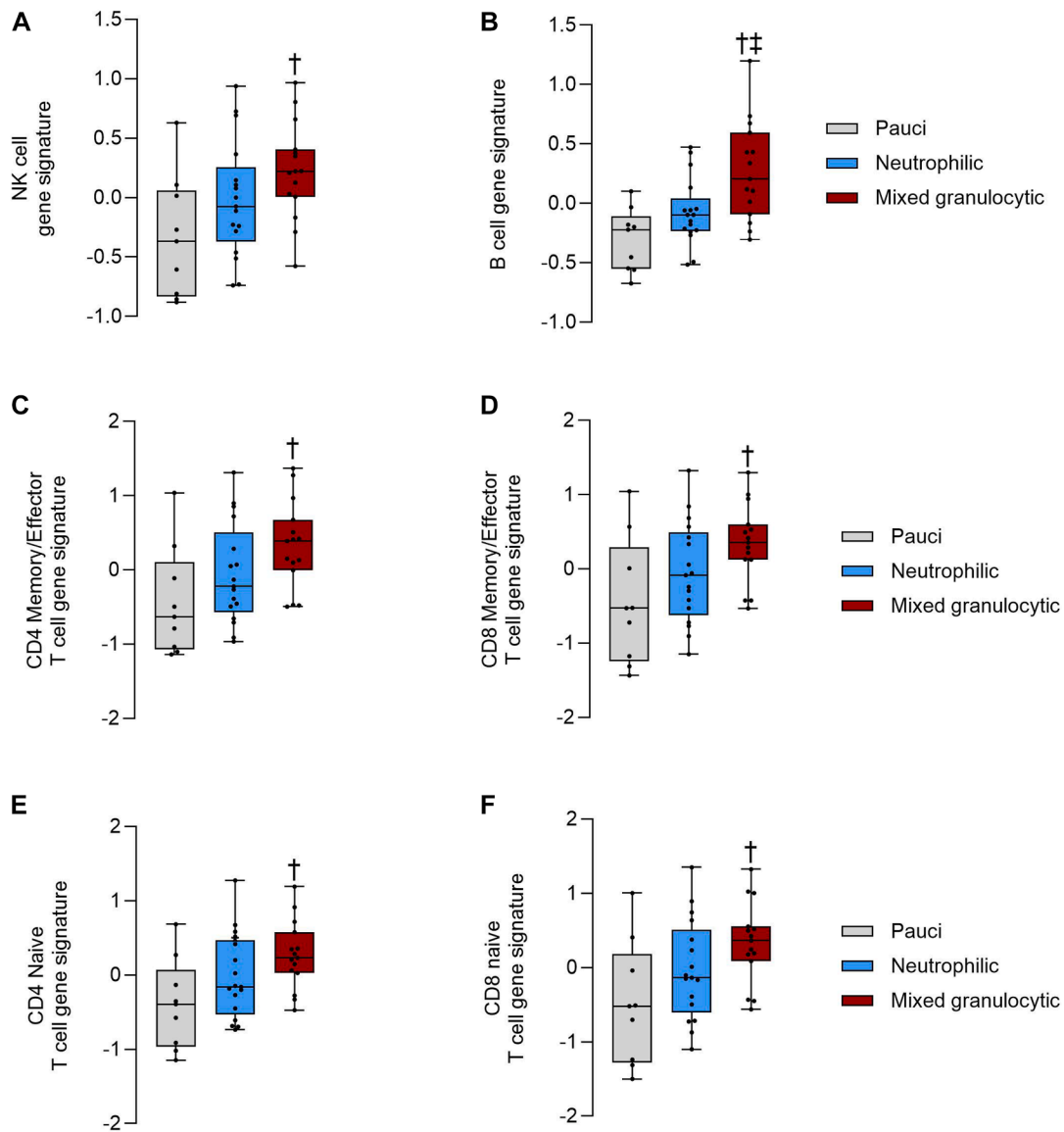


FIG 4. Expression of immune cell gene signatures is increased in mixed granulocytic COPD. **(A)** The mixed granulocytic group demonstrated higher expression of the NK-cell gene signature compared with the pauci-granulocytic group (median [Q1, Q3]: mixed vs pauci, 0.22 [0.01, 0.41] vs -0.37 [-0.81, -0.01]; $P = .006$). **(B)** The mixed granulocytic group demonstrated higher expression of the B-cell gene signature compared with both the pauci-granulocytic and neutrophilic groups (median [Q1, Q3]: mixed vs pauci, 0.21 [-0.09, 0.60] vs -0.22 [-0.55, -0.18]; $P = .0007$; mixed vs neutrophilic, 0.21 [-0.09, 0.60] vs -0.10 [-0.23, -0.05]; $P = .02$). **(C)** The mixed granulocytic group demonstrated higher expression of the CD4 memory/effector T-cell gene signature compared with the pauci-granulocytic group (median [Q1, Q3]: mixed vs pauci, 0.39 [-0.004, 0.67] vs -0.63 [-1.04, -0.11]; $P = .006$). **(D)** The mixed granulocytic group demonstrated higher expression of the CD8 memory/effector T-cell gene signature compared with the pauci-granulocytic group (median [Q1, Q3]: mixed vs pauci, 0.35 [0.12, 0.60] vs -0.53 [-1.18, 0.01]; $P = .02$). **(E)** The mixed granulocytic group demonstrated higher expression of the CD4 naive T-cell gene signature compared with the pauci-granulocytic group (median [Q1, Q3]: mixed vs pauci, 0.23 [0.03, 0.58] vs -0.39 [-0.91, -0.13]; $P = .01$). **(F)** The mixed granulocytic group demonstrated higher expression of the CD8-naive T-cell gene signature compared with the pauci-granulocytic group (median [Q1, Q3]: mixed vs pauci, 0.37 [0.09, 0.56] vs -0.52 [-1.24, -0.04]; $P = .009$). †Mixed versus pauci: $P < .05$; ‡mixed versus neutrophilic: $P < .05$.

for add-on dupilumab therapy in the setting of recurrent exacerbations despite maximal triple inhaled therapy.¹¹ From the results of the BOREAS (Pivotal Study to Assess the Efficacy, Safety, and Tolerability of Dupilumab in Patients With Moderate-to-Severe COPD With Type 2 Inflammation)³⁵ and NOTUS (Pivotal Study to Assess the Efficacy, Safety, and Tolerability of Dupilumab in

Patients With Moderate to Severe COPD With Type 2 Inflammation)³⁴ phase 3 trials, dupilumab treatment reduced the annualized rate of moderate or severe COPD exacerbations over 52 weeks by approximately 30% to 35%, supporting the clinical relevance of IL4 and IL13 biology in COPD. However, inhibiting IL4/IL13 signaling addresses only one major component of exacerbation

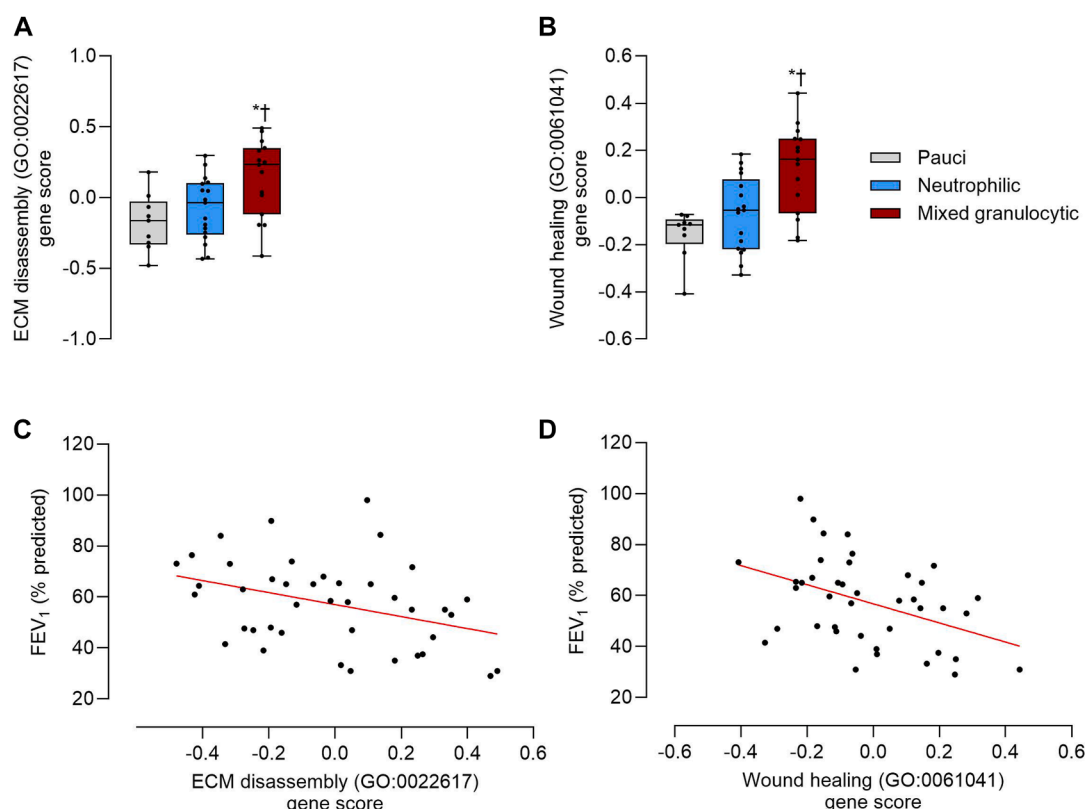


FIG 5. Expression of lung tissue remodeling gene scores is increased in mixed granulocytic COPD and is associated with worse lung function. **(A)** The mixed granulocytic group demonstrated higher expression of the extracellular matrix (ECM) disassembly gene score compared with both the pauci-granulocytic and neutrophilic groups (median [Q1, Q3]: mixed vs pauci, 0.23 [−0.12, 0.35] vs −0.16 [−0.32, −0.07]; $P = .005$; mixed vs neutrophilic, 0.23 [−0.12, 0.35] vs −0.04 [−0.25, 0.10]; $\dagger P = .02$). **(B)** The mixed granulocytic group demonstrated higher expression of the wound healing gene score compared with both the pauci-granulocytic and neutrophilic groups (median [Q1, Q3]: mixed vs pauci, 0.16 [−0.07, 0.25] vs −0.12 [−0.16, −0.11]; $P = .0008$; mixed vs. neutrophilic, 0.16 [−0.07, 0.25] vs −0.05 [−0.22, 0.05]; $\dagger P = .009$). **(C)** FEV₁ decreases with higher expression of the ECM disassembly gene score in BAL. The unadjusted linear regression line (red) is shown. In the unadjusted model, higher ECM disassembly gene score was associated with lower FEV₁ ($\beta = -23.50$; 95% CI: −42.62 to −4.39; $P = .017$). In the model adjusted for pack-years of cigarette smoking, this association remained significant ($\beta = -22.32$; 95% CI: −41.01 to −3.62; $P = .021$). **(D)** FEV₁ decreases with higher expression of the wound healing gene score in BAL. The unadjusted linear regression line (red) is shown. In the unadjusted model, higher wound healing gene score was associated with lower FEV₁ ($\beta = -37.50$; 95% CI: −63.07 to −11.94; $P = .005$). In the model adjusted for pack-years of cigarette smoking, this association remained significant ($\beta = -33.25$; 95% CI: −59.30 to −7.21; $P = .014$).

biology, with the residual exacerbation risk most likely mediated by non-type 2 pathways. Based on the present study, some of these non-type 2 pathways may include IFNG response, IL6/JAK/STAT3 signaling, and TNFA signaling. Previous attempts to therapeutically target these pathways in COPD have not resulted in consistent clinical benefit,^{36–38} and in the case of TNFA blockade, there was a signal toward harm with an increased incidence of pneumonia and malignancy.³⁹ This represents a critical mechanistic and therapeutic knowledge gap and supports the need to better define how type 2 and non-type 2 immune processes interact to drive airway inflammation in biologically complex COPD phenotypes. The mixed granulocytic group expressed higher lung tissue remodeling processes than other groups, suggesting an important relationship between lung remodeling and chronic airway inflammation. Lung tissue remodeling is likely mediated by multiple adaptive immune cells (rather than neutrophils and eosinophils alone) because we found increased

expression of their gene signatures in BAL. These immune cells are known to contribute to persistent inflammation, epithelial injury, and alveolar destruction in COPD.^{40–42}

Several subsets of T cells, including IL17 and IFNG-producing CD4+ T cells and CD8+ cytotoxic T cells, have been associated with corticosteroid-resistant airway inflammation in COPD.^{43,44} Here, we found that pathways relevant to wound healing and extracellular matrix disassembly, on average, were not significantly affected by ICS treatment over 12 weeks. A possible explanation is that 12 weeks may have been a short window to observe gene expression related to lung remodeling. However, the lack of change in the lung remodeling gene scores over 12 weeks can also suggest that ICS treatment does not effectively reduce or reverse lung tissue remodeling. We further speculate that this persistence of the pathologic activity in mixed granulocytic COPD may be related to multiple adaptive immune cells. This observation is consistent with prior studies that showed a lack of improvement

in radiographic emphysema and longitudinal lung function decline with ICS.⁴⁵ These study findings are clinically pertinent because ICS, a mainstay treatment used in COPD, may not change the trajectory of radiographic emphysema progression or lung function decline in this vulnerable group of patients with COPD.

Our study findings should be interpreted in context of several limitations in this *post hoc* study. First, sample sizes for paired BAL gene expression analyses before and after randomized intervention were relatively small because of the technical difficulties of performing this procedure in patients with COPD. As a result, we were unable to perform meaningful analyses of ICS treatment effect on gene expression across COPD phenotypes. Second, a few patients who could not tolerate ICS withdrawal during the run-in period were excluded but could represent a subtype with different inflammatory mechanisms. Finally, we used a BAL eosinophil threshold of 1% and neutrophil threshold of 3% to define mixed granulocytic airway inflammation based on data from our own work and thresholds in healthy nonsmokers,^{4,12,13} but the gold standard in COPD has not been defined. Future studies should validate these thresholds in independent cohorts and determine how alternative eosinophil and neutrophil cutoffs relate to clinical outcomes and treatment response.

In conclusion, we demonstrated that mixed granulocytic airway inflammation, marked by concurrent eosinophilia and neutrophilia, is present in one-third of patients with COPD. Mixed granulocytic COPD is a phenotype with poor clinical outcomes, driven by complex type 2 and non-type 2 immune responses in the lower airways and is associated with increased lung tissue remodeling processes that are largely unresponsive to ICS treatment.

DISCLOSURE STATEMENT

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Disclosure of potential conflicts of interest: H. Y. Park reports research grants from GlaxoSmithKline (GSK) and honoraria for lecturing or participating in advisory boards from GSK and Sanofi. R. L. Eddy reports consulting fees from VIDA Diagnostics Inc and a presentation honorarium from GSK. S. Milne reports honoraria for presentations from Chiesi Australia, Sanofi Australia, AstraZeneca (AZ), Research Review Australia, for participating in advisory boards from GSK, AZ, and Sanofi Australia. D. D. Sin has received honoraria for giving talks on COPD from GSK and AZ and received research funding from Uniquity. The rest of the authors declare that they have no relevant conflicts of interest.

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biospecimens, developing systems for analysis, and coordinating data collection and analysis. This work was supported by AstraZeneca, the Canadian Institutes of Health Research, and the British Columbia Lung Association.

Key messages

- Eosinophilia and neutrophilia in the BAL is observed in one-third of patients with COPD.
- This mixed granulocytic group has increased expression of inflammatory pathways compared to other phenotypes.
- This mixed granulocytic group has increased expression of lung tissue remodeling processes that are associated with lower lung function.

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