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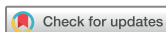
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B Cell-Adaptive Immune Profile in Emphysema-Predominant Chronic Obstructive Pulmonary Disease



To the Editor:

Subjects who fall into the same Global Initiative for Obstructive Lung Disease (GOLD) category of chronic obstructive pulmonary disease (COPD) severity are remarkably heterogeneous, and this diversity is often difficult to handle from a therapeutic standpoint (1). Computed tomography (CT) has been instrumental in identifying COPD subphenotypes, such as airway disease (AD) and parenchymal destruction (emphysema), the relative contribution of which varies from patient to patient. Importantly, emphysema is detected by CT scan in 20% of the smokers who do not meet the spirometric criteria of COPD (2). Recent studies have highlighted some major differences between emphysema and AD, such that they are now believed to be two specific

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Author Contributions: F.P. conceived the project and designed the experiments. J.-L.S., B.B., M.K., F.D.M., G.B., J.P.d.-T., R.S.J.E., S.G., and F.P. conducted experiments and/or contributed to data analysis and interpretation. All authors contributed to the writing and editing of the manuscript.

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endotypes (3) that can overlap with each other, and not manifestations of the same disease.

Cigarette smoke, the major risk factor for COPD in developed countries, causes pulmonary inflammation that persists long after smoking cessation, suggesting self-perpetuating adaptive immune responses similar to those that occur in autoimmune diseases. Increases in the number and size of B cell-rich lymphoid follicles (LFs) have been shown in patients in severe stages of COPD (4), and increased B-cell products (autoantibodies) have been observed in the blood and lungs of patients with COPD (5, 6). Oligoclonal rearrangement of the immunoglobulin genes has been observed in B cells isolated from COPD LFs, suggesting that a specific antigenic stimulation drives B-cell proliferation. Consistently, we have shown that in the COPD lung, there is an overexpression of BAFF (B-cell activation factor of the TNF family), which is a key regulator of B-cell homeostasis in several autoimmune diseases (7) and is involved in the growth of LFs in COPD. However, a network analysis of lung transcriptomics showed that a prominent B-cell molecular signature characterized emphysema preferentially but was absent in AD independently of the degree of airflow limitation (8). In the current study, we investigated the correlation between B-cell responses in lung tissue from patients with COPD and healthy smokers, and the extent of emphysema versus airflow limitation.

Methods

We collected formalin-fixed paraffin-embedded lung sections from 52 subjects undergoing lung volume reduction surgery or

transplant for treatment of severe emphysema, or lung resection for a solitary peripheral nodule (the lung tissue studied was at least 10 cm away from the nodule). The subjects were classified as 1) active or former smokers with GOLD stages 1–2 or GOLD stages 3–4 COPD, or 2) healthy smokers without COPD (SC; see Table 1). None of the subjects had evidence of respiratory tract infection at the time of the surgery. The lung sections were immunostained for 1) 1:200 murine anti-CD20 (B-cell marker) and 1:100 rat anti-BAFF; 2) 1:50 rat anti CD45R (hematopoietic origin cell marker expressed on B cells), 1:100 rabbit anti-CD138 (plasma cell marker), and 1:50 murine anti-CD10 (immature B-cell and follicle center B-cell [centrocyte] marker); 3) 1:50 rat anti-CD45R, 1:50 rabbit anti-IgD, and 1:50 murine anti-CD24 (naive B cells); and 4) 1:50 rat anti-CD45R, 1:50 murine anti-IgG, and 1:50 rabbit anti-CD27 (memory B cells). All of the antibodies were obtained from Abcam. Appropriate isotype-matched, nonimmune control antibodies were used for each staining. For each sample, at least 20 randomly selected, nonconsecutive, high-magnification fields were evaluated using a Leica epifluorescence microscope. The numbers of parenchymal, vascular, and bronchial LFs (defined as aggregates containing more than 40 contiguous mononuclear cells that demonstrated the characteristic topographical arrangement of B cells) (7), BAFF⁺ B cells, BAFF⁺ alveolar type I and type II cells, CD138⁺, CD10⁺, CD24⁺, IgD⁺, IgG⁺, and CD27⁺ B cells were counted and normalized by alveolar tissue area using

Table 1. Selected Demographics, Comorbidities, and Medication Use of the Study Participants

Selected Demographics	n	GOLD 1–2	GOLD 3–4	SC	All Subjects	P Value
Total number of participants		23	18	11	52	
Sex, % female	52	35%	56%	18%	49%	0.122
Age	52	63.45 (10.73)	60.17 (5.01)	63.73 (11.98)	62.35 (9.40)	0.479
Percentage of current smokers	52	30%	11%	27%	23%	0.273
Pack-years	49	52.36 (42.63)	51.35 (20.72)	54.00 (33.80)	52.34 (33.75)	0.982
FEV ₁ % predicted	52	76.95 (18.51)	29.89 (11.11)	91.27 (15.93)	63.43 (29.88)	<0.001
FEV ₁ /FVC	50	60.95 (8.02)	43.47 (14.25)	78.40 (7.72)	58.45 (16.50)	<0.001
DL _{CO} %	34	67.29 (23.35)	42.83 (22.41)	68.29 (16.77)	58.61 (24.37)	0.014
K _{co} %	34	79.00 (26.27)	50.08 (33.10)	87.43 (26.44)	70.27 (32.23)	0.016
Comorbidities						
Hypertension	52	36%	28%	42%	35%	0.757
Gastroesophageal reflux disease	52	14%	6%	8%	10%	0.837
Hyperlipidemia	52	9%	11%	25%	13%	0.449
Diabetes mellitus	52	18%	6%	0%	10%	0.272
Lung adenocarcinoma	52	41%	6%	50%	31%	0.009
Squamous cell lung cancer	53	36%	0%	25%	21%	0.009
Medications						
LABA/LAMA/SABA	52	32%	100%	17%	52%	<0.001
Inhaled corticosteroids	52	18%	78%	25%	40%	<0.001
Statins	52	36%	17%	50%	33%	0.158
Protonic pump inhibitors	52	27%	22%	42%	29%	0.542
ACE inhibitors/angiotensin receptor blockers	52	32%	17%	25%	25%	0.560
Calcium antagonist	52	23%	11%	25%	19%	0.607
Diuretics	52	14%	22%	17%	17%	0.893
Oral corticosteroids	52	5%	22%	8%	12%	0.248
β blockers	52	5%	11%	25%	12%	0.208

Definition of abbreviations: ACE = angiotensin-converting enzyme; GOLD = Global Initiative for Obstructive Lung Disease; LABA = long-acting β-agonist; LAMA = long-acting muscarinic antagonist; SABA = short-acting β-agonist; SC = smokers without chronic obstructive pulmonary disease. The values are expressed as mean (SD). P values for difference across the three groups represent one-way ANOVA for continuous measures and Fisher's exact test for categorical measures.

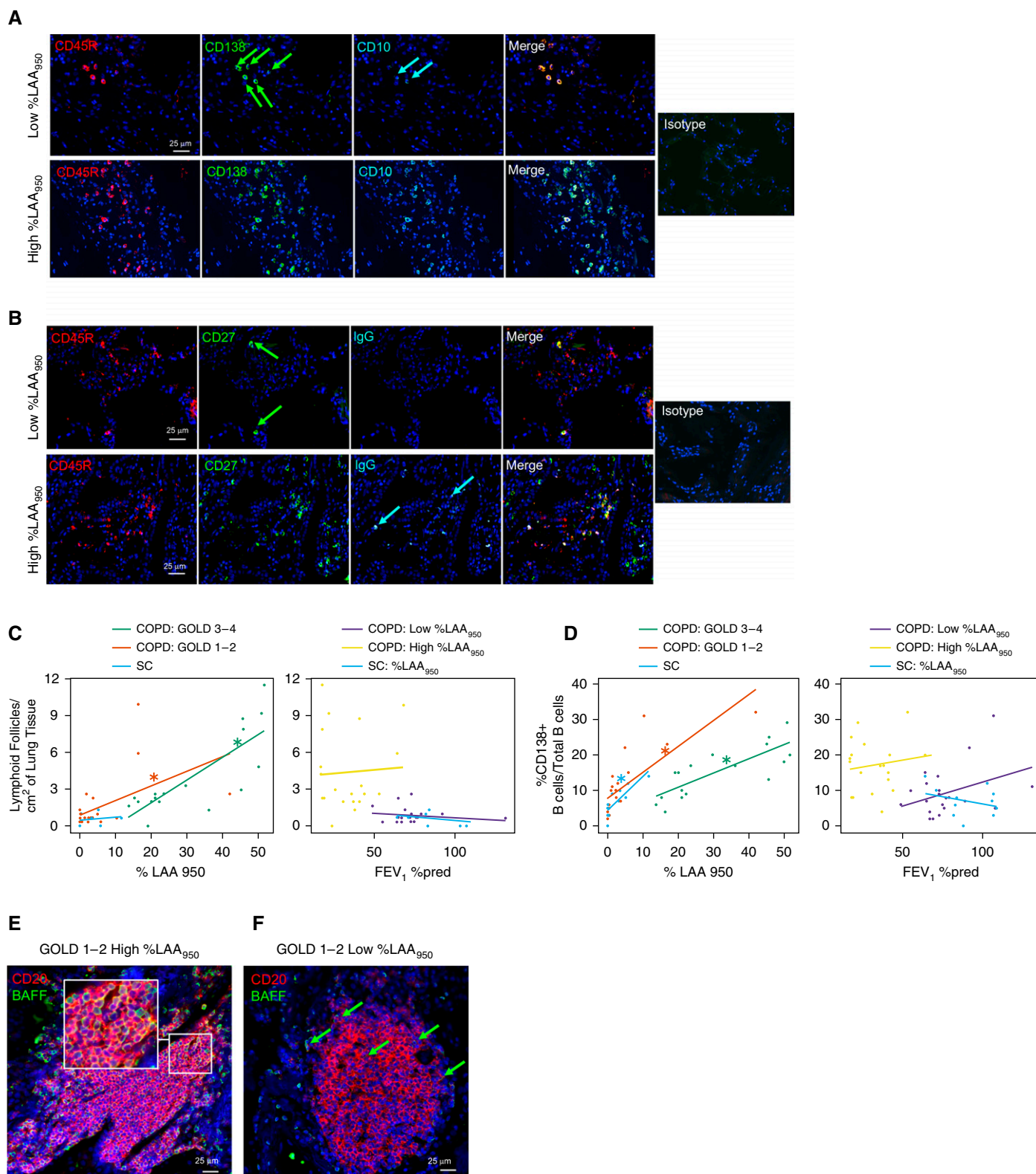


Figure 1. Increases in B cell–adaptive immune responses are associated with the extent of emphysema and not with airflow limitation. (A) Triple immunofluorescence staining for CD45R (B-cell marker), CD138 (plasma-cell marker), CD10 (immature B-cell and follicle center B-cell [centrocyte] marker), and the merge panel. The green arrows indicate CD138⁺ B cells, and cyan arrows indicate CD10⁺ B cells. (B) Triple immunofluorescence staining for CD45R, CD27 (memory B cells), IgGs, and the merge panel. The green arrows indicate CD27⁺ B cells, and the cyan arrows indicate

Table 2. Adjusted Coefficients for the Associations of Emphysema (%LAA₉₅₀) and FEV₁% Predicted with Cellular Parameters of B-Cell Activation in Separate (Left Columns) and Mutually Adjusted (Right Columns) Linear Regression Models

Dependent Variable	Predictor	Separate Models: %LAA ₉₅₀ or FEV ₁ %pred As Predictor			Mutually Adjusted Models: Both %LAA ₉₅₀ and FEV ₁ %pred As Predictors		
		Adjusted Coefficient*	95% CI	Adjusted P Value	Adjusted Coefficient*	95% CI	Adjusted P Value
No. of lymphoid follicles/cm ² of lung tissue, log [†]	%LAA ₉₅₀	0.023	0.014, 0.032	<0.001	0.021	0.010, 0.032	<0.001
	FEV ₁ %pred	-0.009	-0.014, -0.003	0.004	-0.002	-0.008, 0.004	0.505
No. of BAFF ⁺ B cells/cm ² of alveolar tissue, log [†]	%LAA ₉₅₀	0.018	0.010, 0.026	<0.001	0.016	0.006, 0.026	0.003
	FEV ₁ %pred	-0.007	-0.012, -0.002	0.005	-0.002	-0.007, 0.004	0.517
No. of BAFF ⁺ alveolar cells/cm ² of alveolar tissue, log [†]	%LAA ₉₅₀	0.010	0.005, 0.016	0.001	0.008	0.001, 0.016	0.031
	FEV ₁ %pred	-0.005	-0.008, -0.001	0.007	-0.002	-0.006, 0.002	0.321
Percentage of CD10 ⁺ B cells/total B cells	%LAA ₉₅₀	0.326	0.236, 0.415	<0.001	0.338	0.228, 0.449	<0.001
	FEV ₁ %pred	-0.093	-0.159, -0.027	0.007	0.012	-0.048, 0.072	0.683
Percentage of CD27 ⁺ B cells/total B cells	%LAA ₉₅₀	0.278	0.093, 0.464	0.004	0.239	0.010, 0.467	0.041
	FEV ₁ %pred	-0.112	-0.217, -0.007	0.038	-0.037	-0.161, 0.086	0.546
Percentage of CD138 ⁺ B cells/total B cells	%LAA ₉₅₀	0.377	0.238, 0.516	<0.001	n/a	n/a	n/a
	FEV ₁ %pred	-0.049	-0.144, 0.047	0.311	n/a	n/a	n/a
Percentage of IgG ⁺ B cells/total B cells	%LAA ₉₅₀	0.152	0.080, 0.224	<0.001	n/a	n/a	n/a
	FEV ₁ %pred	-0.020	-0.065, 0.026	0.395	n/a	n/a	n/a

Definition of abbreviations: BAFF = B-cell activation factor of the TNF family; CI = confidence interval; FEV₁%pred = FEV₁% predicted; n/a = not applicable; %LAA₉₅₀ = low-attenuation areas below a threshold of -950 Hounsfield units.

Models included all patients with chronic obstructive pulmonary disease (without stratification by Global Initiative for Obstructive Lung Disease stage) and smoking control subjects.

*Also adjusted for sex, age, smoking status, and presence of lung cancer. Pack-years were excluded from the models owing to missing data for three participants. Results were confirmed in a sensitivity analysis after further adjustment for pack-years.

[†]Dependent variables were first log-transformed in base 10 to achieve normalization. Participants with no lymphoid follicles were transformed to the base 10 log of 0.1.

MetaMorph software. The analysis of the CT scans was performed by two independent experts in chest CT scans. The Chest Imaging Platform software (<https://chestimagingplatform.org>) was used to quantify emphysema as the percentage ratio of low-attenuation areas below a threshold of -950 Hounsfield units (%LAA₉₅₀) (9).

Statistical analysis. Associations between BAFF⁺ B cells, BAFF⁺ alveolar cells, DL_{CO}, K_{CO} and CD10⁺, CD24⁺, CD27⁺, CD138⁺, IgD⁺, and IgG⁺ B cells were tested with Spearman's rank correlation tests. To determine whether %LAA₉₅₀ and FEV₁% predicted (FEV₁%pred) were independently associated with the B-cell-related parameters measured, multivariate linear regression models were used that included, among other covariates, either %LAA₉₅₀ or FEV₁%pred as the independent predictor. For cellular parameters that showed a significant association with both %LAA₉₅₀ and

FEV₁%pred, mutually adjusted models that included both predictors were assessed. The models included all subjects from both COPD groups (stages 1-2 and 3-4) as well as smoking control subjects.

The independent relationship between selected cellular parameters and emphysema and FEV₁ was displayed and tested with Spearman's correlation after the participants were stratified into groups according to GOLD stage (GOLD 1-2 and GOLD 3-4) and emphysema level (above or below the median %LAA₉₅₀), respectively. Smoking control subjects were kept as a separate group in these graphs.

Results

As expected, %LAA₉₅₀ and FEV₁%pred correlated inversely with each other ($r = -0.766$; $P < 0.001$), and they were both associated

Figure 1. (Continued). IgG⁺ B cells. Isotype control merge figures are shown on the right of both A and B. (C and D) Stratified graphs are presented for the association of low-attenuation areas below a threshold of -950 Hounsfield units (%LAA₉₅₀) and FEV₁% predicted (FEV₁%pred) with (C) the number of lymphoid follicles (LFs)/cm² of lung tissue and (D) the %CD138⁺ B cells/total B cells. For each graph, the relationship of the parameter of interest with %LAA₉₅₀ within different Global Initiative for Obstructive Lung Disease (GOLD) stages (1-2 vs. 3-4) is shown in the left panel, and the relationship with FEV₁%pred within different levels of emphysema is shown in the right panel. SC = smokers without chronic obstructive pulmonary disease (COPD). (E and F) Double-immunofluorescence pictures of formalin-fixed paraffin-embedded lung sections from 1) a patient with GOLD 1-2 COPD and severe emphysema (E), showing robust BAFF (B-cell activation factor of the TNF family) staining in most of the alveolar cells, LF B cells, and parenchymal B cells; and 2) a patient with GOLD 1-2 COPD and low emphysema (F), showing fewer BAFF⁺ alveolar cells, B cells within the LF, and parenchymal B cells. In E, the inset shows a detail of an LF, with the great majority of B cells expressing BAFF. The green arrows indicate BAFF⁺ B cells and alveolar cells.

with the number of LFs, BAFF⁺ B cells and alveolar cells, CD10⁺ B cells and plasma cells (Figure 1A), and memory and IgG⁺ B cells (Figure 1B) when tested separately in multivariate linear regression models (Table 2, left columns). However, when they were mutually adjusted in the same regression models (Table 2, right columns), only %LAA₉₅₀, and not FEV₁%pred, remained significantly and strongly associated with all of these cellular parameters. We did not find any significant association between the numbers of IgD⁺ and CD24⁺ cells and %LAA₉₅₀ and FEV₁%pred (data not shown). From the analysis of consecutive tissue sections, we observed that, interestingly, in subjects with the highest %LAA₉₅₀ values, most of the CD138⁺ B cells tended to cluster together and were also CD10⁺. Some of these cells were also expressing either CD27 or IgG, or both. In contrast, in the subjects with low %LAA₉₅₀ values, only a minority of CD138⁺ B cells were also positive for CD10.

Consistent with these results, as shown in Figure 1C, levels of %LAA₉₅₀ correlated significantly with the number of LFs both among subjects in GOLD stages 1–2 and among those in GOLD stages 3–4 (left panel). However, after stratification by emphysema levels, FEV₁%pred did not correlate with the number of LFs among subjects with low or high emphysema (right panel). Similarly, %LAA₉₅₀ was found to be associated with the percentage of plasma cells in each COPD group as well as among SC (Figure 1D, left panel), whereas no association was found between FEV₁%pred and the percentage of plasma cells in either of the emphysema groups or among SC (Figure 1D, right panel). In line with these results, %LAA₉₅₀, but not FEV₁%pred, was also shown to be significantly associated with the other B-cell subpopulations studied when stratified into the same groups (data not shown). As expected, LFs in lungs from subjects with high %LAA₉₅₀ were very rich in BAFF (Figure 1E), in contrast to the subjects with low %LAA₉₅₀, where low pulmonary LF BAFF levels were observed (Figure 1F). The numbers of BAFF⁺ B cells and alveolar cells were highly correlated with the numbers of LFs ($r=0.7$ and 0.6 , respectively), CD10⁺ B cells ($r=0.6$ and 0.7 , respectively), plasma cells ($r=0.4$ and 0.6 , respectively), memory B cells ($r=0.4$ and 0.5 , respectively), and IgG⁺ B cells ($r=0.3$ and 0.5 , respectively). The DL_{CO} and K_{CO} values were also strongly correlated with the numbers of LFs ($r=-0.5$), BAFF⁺ B cells ($r=-0.6$), and BAFF⁺ parenchymal cells ($r=-0.5$), and with CD10⁺ B cells ($r=-0.5$). In addition, K_{CO} was also correlated with the number of plasma cells and memory B cells ($r=-0.4$).

Discussion

These data are in line with previous findings that the presence of emphysema, and not the degree of airflow limitation, is correlated with a specific lung endotype dominated by B-cell responses (8). We now extend these findings to all COPD GOLD stages and SC, showing that an upregulation of the B-cell immune compartment in lung tissue is directly linked to %LAA₉₅₀ and not to FEV₁%pred. Our results support the hypothesis that an overactivation of the B-cell compartment, characterized by increases in naive, memory, and antibody-producing B cells and expression of BAFF by B cells and alveolar cells, is abundant in the emphysematous lung, either as a consequence or as a concurrent cause of the ongoing emphysematous process (10). Importantly, the cellular readouts of activation of the B-cell compartment were also significantly directly associated with the extent of emphysema in the

smokers without airflow limitation. This suggests that increases in B cell–adaptive immune responses are present before lung function starts to decline. We should acknowledge that the association between B cells and emphysema in our cross-sectional study does not provide proof of a causal association (cause–effect), and could be due to chance, bias, confounding, and/or reverse causation (effect–cause), the effects of which need to be explored in future studies analyzing broader cohorts of subjects.

These observations may open new therapeutic paths for patients with COPD, as the complexity of B-cell maturation presents opportunities for therapeutic interventions. Currently, there is a lack of disease-modifying therapies for COPD, mainly because available therapies target patients with COPD as a whole and cluster them simply according to their airflow limitation. We believe that further characterization of a B-cell endotype associated with emphysema could 1) shift the notion that patients with COPD, even within the same GOLD stage, are pathobiologically similar and thus require similar clinical management; and 2) define the clinical phenotype (likely emphysema) that could benefit from therapies targeting B cells or B-cell products (e.g., BAFF), leading to earlier and more personalized therapeutic interventions that may greatly alleviate the burden of COPD. ■

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Network Analysis of Genome-Wide Association Studies for Chronic Obstructive Pulmonary Disease in the Context of Biological Pathways

To the Editor:

Chronic obstructive pulmonary disease (COPD) is a common respiratory disease projected to be the third leading cause of death by 2020 (1). The main risk factor is tobacco smoking, but other

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environmental exposures may also contribute (1). Furthermore, host factors including genetic abnormalities, abnormal lung development, and accelerated aging increase susceptibility to COPD (1). However, the causal mechanisms remain poorly understood (2).

As a result of genome-wide association studies, many interesting genetic variations, including SNPs, have been discovered. However, the interpretation of these large amounts of data within the context of biological systems, disease processes, and unknown gene functions remains difficult. Considering genes in a biological context may aid in the elucidation of SNP function. Network analysis provides a way of deciphering the biological relationships among SNPs, genes, and pathways by providing a framework that allows for the integration, analysis, and display of these complex data (3).

We used data from a recent meta-analysis to identify and extract all genetic variants published in pooled and meta-analysis studies related COPD risk (Prospero CRD4201705; May 2018). We extracted the 181 significant genetic variants (regardless of linkage disequilibrium) mapped to 99 genes that included 176 SNPs with reference SNP cluster identifier (rs) and other variants such as multiple SNP combinations, insertions and deletions, or length polymorphisms.

Genes and variants were represented in a SNP–gene network using Cytoscape version 3.6. Second, the genes were used to retrieve the biological pathways from WikiPathways Human curated collection (10 July 2018). Genes present in one or more pathways were displayed in a Cytoscape gene–pathway network. The SNP–gene and gene–pathway networks were then consolidated by merging them. This yielded a SNP–gene–pathway network that was used as a basic reference for the biological interpretation of the connected elements. Finally, genes were classified according to their function and potential effect, using the variant effect predictor analysis in Ensembl (4).

Our analysis produced four different visualizations. In Table 1, an overview of the main characteristics of the networks is reported. In each network title, the digital object identifier to the Network Data Exchange visualization is provided and the main features of the networks and nodes codes are reported, all of which are fully downloadable and interactive.

The networks consist of 181 variant nodes, 99 gene nodes, and 315 pathway nodes, and 735 connections between them. Of the original set of 99 genes, 74 genes are present in pathways from the curated WikiPathways collection. The basic version, Gene–pathway network, highlights the three elements: SNPs, genes, and pathways in different colors.

The Functional gene map visualization presents functional classes in the network. Here we show 13 nonoverlapping functional classes: Addiction, Cellular interaction, Cellular metabolism, Cellular structure, Detoxification, Development, Homeostasis organismal, Inflammation, Lung function, Metabolism organismal, Regulation, Tissue remodeling, and Unknown. Interestingly, some of the gene functional classes are dispersed, whereas in others all are connected. Cellular metabolism (forest green) shows dispersion: 15 genes are not connected in the major central network, and 7 of the 15 do not present any pathway connections. Comparatively, all five genes related to Detoxification cluster in a specific area (refer to online visualization, pink-nodes). Similarly, all 15 genes involved in Inflammation are intensely connected to genes and other pathways and are grouped in the