

Blood fibrocytes are recruited during acute exacerbations of chronic obstructive pulmonary disease through a CXCR4-dependent pathway

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Background: Chronic obstructive pulmonary disease (COPD) is characterized by peribronchial fibrosis. The chronic course of COPD is worsened by recurrent acute exacerbations.

Objective: The aim of the study was to evaluate the recruitment of blood fibrocytes in patients with COPD during exacerbations and, subsequently, to identify potential mechanisms implicated in such recruitment.

Methods: Using flow cytometry, we quantified circulating fibrocytes and characterized their chemokine receptor expression in 54 patients with COPD examined during an acute exacerbation (V1) and 2 months afterward (V2) and in 40 control subjects. The role of the chemokines CXCL12 and CCL11 in fibrocyte migration was investigated by using a chemotaxis assay. Patients were followed for up to 3 years after V1.

Results: We demonstrated a significantly increased number of circulating fibrocytes at V1 compared with control subjects. The number of circulating fibrocytes decreased at V2. A high

percentage of circulating fibrocytes during exacerbation was associated with increased risk of death. The percentage of fibrocytes at V2 was negatively correlated with FEV₁, forced vital capacity, FEV₁/forced vital capacity ratio, transfer lung capacity of carbon monoxide, and Pao₂. Fibrocytes highly expressed CXCR4 and CCR3, the chemokine receptors for CXCL12 and CCL11, respectively. Fibrocytes collected from patients with COPD at V1 had increased chemotactic migration in response to CXCL12 but not to CCL11 compared with those from control subjects. Plerixafor, a CXCR4 antagonist, decreased fibrocyte migration to plasma from patients with exacerbating COPD.

Conclusion: Blood fibrocytes are recruited during COPD exacerbations and related to mortality and low lung function. The CXCL12/CXCR4 axis is involved in such fibrocyte recruitment (Firebrob study; ClinicalTrials NCT01196832). (*J Allergy Clin Immunol* 2015;■■■:■■■-■■■.)

Key words: Chronic obstructive pulmonary disease, fibrocyte, mortality, lung function, chemotaxis

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Chronic obstructive pulmonary disease (COPD) is a very frequent airway disease that affects more than 200 million persons worldwide.¹ The main risk factor for COPD is tobacco smoking.¹ COPD is currently the fifth leading cause of death but might reach the fourth cause of death toward 2030.² The disease is characterized by chronic bronchial inflammation and remodeling of the distal airways and in particular bronchial and peribronchial fibrosis, leading to persistent airflow limitation.³ Current pharmacologic treatments act on symptoms and quality of life but do not improve mortality or the natural history of the disease, with the latter being characterized by a more rapid decrease in lung function.^{4,5}

The chronic course of COPD is also frequently worsened by acute exacerbations (acute exacerbations of chronic obstructive pulmonary disease [AECOPDs]) most often related to viral or bacterial infections.⁶ These AECOPDs are associated with various sputum cellular profiles, including neutrophils and eosinophils.⁷ AECOPDs affect nearly 80% of patients with COPD over a 3 year-period, and the frequency of exacerbation is mainly related to the occurrence of previous exacerbations.⁸ AECOPDs generate enormous health care costs, especially related to hospitalizations. AECOPDs dramatically affect quality of life and worsen the disease: lung function decreases more rapidly in patients with frequent exacerbations, with an increased risk of death.⁹ In particular, a high mortality rate has been

Abbreviations used

AECOPD: Acute exacerbation of chronic obstructive pulmonary disease
 APC: Allophycocyanin
 CAT: COPD Assessment Test
 COPD: Chronic obstructive pulmonary disease
 FVC: Forced vital capacity
 NANT: Nonadherent non-T
 SGQLQ: St Georges Quality of Life Questionnaire
 TLCO: Transfer lung capacity of carbon monoxide

reported in patients with COPD admitted to the hospital for AECOPDs¹⁰ and reaches up to 45% within the 4 subsequent years.¹¹ Severe AECOPDs are even considered an independent prognostic factor for mortality.¹⁰ However, the mechanisms underlying these latter findings remain totally unknown.

Fibrocytes are progenitor cells derived from bone marrow.¹² These cells circulate in the bloodstream and are recruited into injured tissues, where they influence tissue inflammation and remodeling.¹² Indeed, fibrocytes could differentiate into fibroblasts and myofibroblasts¹³ in lung tissues. An increased peripheral blood fibrocyte count has been observed in patients with chronic obstructive asthma,¹⁴ idiopathic pulmonary fibrosis,¹⁵ or Hermansky-Pudlak syndrome.¹⁶ In addition, an increase in circulating fibrocyte counts has also been observed during acute exacerbations of idiopathic pulmonary fibrosis¹⁵ and asthma¹⁷ compared with the stable state, suggesting that exacerbation could play a role in fibrocyte recruitment. To date, the migration of fibrocytes during and after an AECOPD has not been investigated.

Here we report the result of a translational clinical trial in which we studied peripheral blood fibrocyte counts in patients with COPD during an exacerbation and 2 months after an exacerbation in comparison with those in control subjects and patients with nonexacerbating COPD. We also characterized chemokine receptors and investigated the migratory properties of these fibrocytes from patients with COPD and control subjects to unravel the implicated mechanisms.

METHODS

A full description of the methods used in this study is presented in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Study populations

A total of 65 patients with AECOPDs, 9 patients with nonexacerbating COPD, and 50 control subjects were prospectively recruited from the University Hospital of Bordeaux. AECOPDs were identified according to the Global Initiative for Chronic Obstructive Lung Disease criteria¹ as a change in the patient's baseline dyspnea, cough, and/or sputum that is beyond normal day-to-day variations, is acute in onset, and might warrant a change in regular medication. Patients with nonexacerbating COPD were selected from the Cohorte Obstruction Bronchique et Asthme (COBRA; sponsored by the French National Institute of Health and Medical Research [INSERM]) cohorts and did not experience any exacerbation during a preceding minimal period of 12 months. Healthy volunteers had normal lung function test results and no history of lung disease. All subjects provided written informed consent to participate in the study after the nature of the procedure had been fully explained. The local research ethics committee ("Comité de Protection des Personnes") of the southwest area (France) and the French National Agency

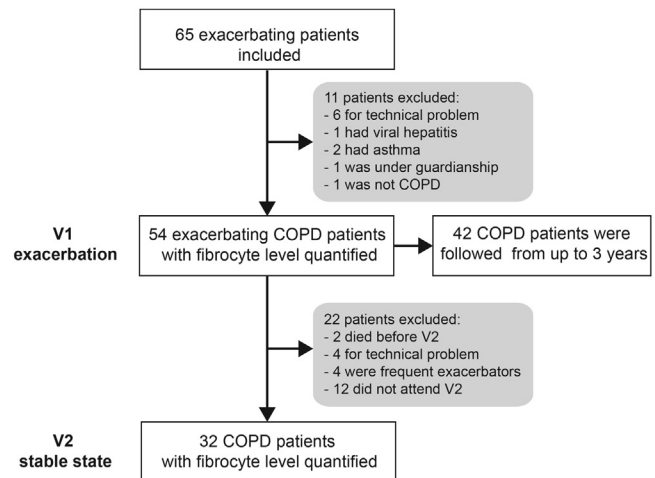


FIG 1. Study design. Numbers of patients who were included and had their fibrocyte counts quantified.

for Medicines and Health Products Safety approved the research protocol in March 2010.

Study design

This clinical trial was sponsored by the University Hospital of Bordeaux. The study has been registered under as no. NCT01196832 at ClinicalTrials.gov.

The study design is summarized in [Fig 1](#). Two visits were scheduled for patients with exacerbating COPD: visit 1 (V1; inclusion) during an exacerbation and visit 2 (V2; stable state) 2 months $\pm$ 7 days after an exacerbation. There was only 1 visit for control subjects and patients with nonexacerbating COPD.

Identification and characterization of circulating fibrocytes

Nonadherent non-T (NANT) cells were obtained, as described previously, with some modifications.¹⁴ Briefly, PBMCs were first separated from whole blood by means of Ficoll-Hypaque (Dutscher, Brumath, France) density gradient centrifugation. After erythrocyte lysis and incubation for 1 hour at 37°C, the nonadherent mononuclear cells were further depleted with an anti-CD3 mAb (Miltenyi Biotec, Paris, France).

Fibrocytes were then identified by using flow cytometry as positive for both the cell-surface marker CD45 and intracellular collagen I (see [Fig E1, A-E](#), in this article's Online Repository at www.jacionline.org), as described previously by Moeller et al.¹⁵ Expression of the CD34 progenitor cell marker and chemokine receptors was also assessed by means of flow cytometry with specific fluorescent antibodies directed against CD34, CCR3, CCR7, CXCR4 (BD Biosciences, San Jose, Calif), and CCR2 (R&D Systems, Lille, France). Blood fibrocyte morphology was checked by using phase-contrast microscopy (Nikon, Champigny sur Marne, France; see [Fig E1, F](#)).

Fibrocyte migration

Fibrocyte chemotaxis was assessed by using a modified Boyden chamber assay.¹⁸ When indicated, NANT cells were pretreated for 1 hour at 37°C with an antagonist of CXCR4 (25 μ g/mL plerixafor; Sigma-Aldrich, Saint Quentin-Fallavier, France) or an antagonist of CCR3 (3.8 μ g/mL SB 328437, R&D Systems). Recombinant human CXCL12- α or CCL11 (both from R&D Systems) or plasma was added to the bottom compartment of each well. After 12 hours, the content of the bottom compartment was removed to assess fibrocyte migration by using flow cytometry, as described above.

Measurement of plasma CXCL12 and CCL11 concentrations

Plasma concentrations of CXCL12- α (R&D Systems), CXCL12- β (Sigma-Aldrich), and CCL11 (R&D Systems) were measured by using an ELISA.

Statistical analysis

Values are presented as means $\pm$ SDs or medians (95% CIs). Statistical significance ($P < .05$) was analyzed by using Fisher exact tests for comparison of proportions, by using t tests and multivariate ANOVA for variables with parametric distribution, and by using the Kruskal-Wallis test with multiple comparison z tests, Mann-Whitney tests, Wilcoxon tests, and Spearman correlation coefficients for variables with nonparametric distribution. Survival in patients with exacerbating COPD was compared by using Kaplan-Meier analysis.

RESULTS

Enrollment and baseline characteristics

Fig 1 shows the number of patients who were enrolled, excluded, and followed for up to 3 years. From a total of 65 enrolled patients, we successfully quantified fibrocyte counts in 54 patients with exacerbating COPD (V1) and 32 patients with COPD in a stable state (V2). We also enrolled 50 control subjects, and fibrocyte counts were quantified in 40 of them. The 2 groups were well matched for age, sex ratio, and body mass index (Table I). However, as expected, patients with COPD were significantly different from control subjects in terms of smoking habits, hospitalization, breath type, and lung function (FEV₁, forced vital capacity [FVC], FEV₁/FVC ratio, transfer lung capacity of carbon monoxide [TLCO], and Pao₂; Table I). We also quantified fibrocyte counts in 9 patients with nonexacerbating COPD (see Table E1 in this article's Online Repository at www.jacionline.org for patients' characteristics).

Circulating fibrocytes and COPD exacerbation

The percentage of blood fibrocytes (CD45⁺Col1⁺ cells) in PBMCs was higher in patients with COPD during exacerbation (median, 10.2% [95% CI, 9.9% to 15.5%] of PBMCs, $n = 54$) compared with that in patients with nonexacerbating COPD (median, 2.4% [95% CI, 0.8% to 6.3%] of PBMCs, $n = 9$; $P < .01$) or control subjects (median, 3.1% [95% CI, 3.1% to 5.1%] of PBMCs, $n = 40$; $P < .001$; Fig 2, A). Similar results were obtained with fibrocyte count expressed as the number of cells per milliliter of blood (Fig 2, B). Both the percentages (Fig 2, C) and absolute numbers (Fig 2, D) of circulating CD34⁺ fibrocytes were increased in patients with exacerbating COPD compared with those in control subjects. In control subjects, when subgroups were defined on the basis of smoking habits (current and former smokers vs never smokers), no significant difference in fibrocyte counts between the subgroups could be evidenced (data not shown). In patients with exacerbating COPD, when subgroups were defined on the basis of treatment for the exacerbation (antibiotics and oral corticosteroids), ventilation mode (spontaneous breathing, noninvasive ventilation, or orotracheal intubation), or hospitalization, no significant difference in fibrocyte counts between the different subgroups could be evidenced (data not shown).

Two months after exacerbation, in the stable state (V2), both percentages (Fig 2, E) and absolute numbers (Fig 2, F) of

TABLE I. Patients' characteristics

	Patients with COPD	Control subjects	P value
No.	54	40	
Age (y)	65.6 $\pm$ 7.5	63.4 $\pm$ 7.4	.17
Sex (M/F)	37/17	25/15	.66
Body mass index (kg/m ²)	28.0 $\pm$ 7.3	25.9 $\pm$ 3.8	.11
Current smoker (yes/no)	15/39	2/38	.006
Former smoker (yes/no)	39/15	18/22	.01
Pack years (no.)	46.1 $\pm$ 19.9	7.1 $\pm$ 11.1	<.0001
In the previous year, no. of subjects with:			
0 or 1 unscheduled visit/y	15	NR	
≥ 2 unscheduled visits/y	16	NR	
Unknown number of unscheduled visits	23	NR	
COPD exacerbation			
Hospitalization (yes/no)	35/19	0/40	<.0001
Ventilation mode			
Spontaneous ventilation (yes/no)	24/30	40/0	<.0001
Noninvasive ventilation (yes/no)	22/32	0/40	<.0001
Orotacheal intubation (yes/no)	8/46	0/40	.02
Cause, no. (%)			
Respiratory tract infections	42 (77.8)	NR	
Unknown	12 (22.2)	NR	
Treatment			
Use of antibiotics (yes/no)	44/10	NR	
Use of oral corticoids (yes/no)	41/13	NR	
Stable state			
COPD duration (y)	6.1 $\pm$ 5.1	NR	
Severity of airflow limitation, no. (%)			
GOLD 1 (mild)	4 (7.4)	NR	
GOLD 2 (moderate)	16 (29.6)	NR	
GOLD 3 (severe)	14 (25.9)	NR	
GOLD 4 (very severe)	20 (37.1)	NR	
FEV ₁ (% predicted)	45.1 $\pm$ 22.4	109.9 $\pm$ 15.4	<.0001
FEV ₁ /FVC ratio (%)	46.7 $\pm$ 14.1	78.7 $\pm$ 4.9	<.0001
FVC (percent predicted)	76.6 $\pm$ 23.0	112.1 $\pm$ 17.1	<.0001
TLCO (percent predicted)	50.5 $\pm$ 29.2	99.6 $\pm$ 19.8	<.0001
Pao ₂ (mm Hg)	72.1 $\pm$ 14.6	91.9 $\pm$ 14.7	<.0001

Values are presented as means $\pm$ SDs, where shown. Severity of airflow limitation is assessed by using the GOLD classification.¹ P values were calculated with the use of a 2-sided independent t test for variables with a parametric distribution, the Fisher exact test for comparison of proportions, and the Mann-Whitney U test for comparison of nonparametric variables.

F, Female; GOLD, Global Initiative for Chronic Obstructive Lung Disease; M, male; NR, not relevant.

fibrocytes were significantly reduced compared with those assessed at V1 ($P < .01$). Moreover, there was a significant increase in the percentage of fibrocytes at V2 in a subgroup of patients with 2 or more unscheduled visits for COPD the year before V1 compared with those with 1 or no unscheduled visits (*post hoc* analysis, see Fig E2 in this article's Online Repository at www.jacionline.org).

Relationships between fibrocytes, survival, and both functional and clinical parameters

Survival data were collected in patients with COPD for a median period of 1.4 years and up to 3 years after V1. Kaplan-Meier survival analysis was performed in 2 subgroups of patients based on the percentage of fibrocytes assessed at V1. Patients with more than 28% fibrocytes had a significantly reduced life

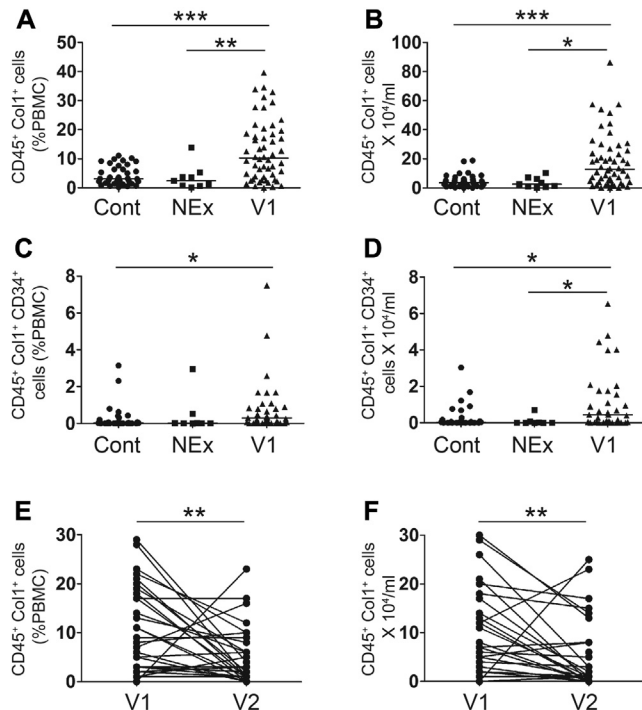


FIG 2. Increased blood fibrocyte counts during COPD exacerbation. **A** and **B**, Circulating fibrocytes (CD45⁺Col1⁺ cells) expressed as a percentage of PBMCs (Fig 2, A) and counts in blood (Fig 2, B) from control subjects (Cont, n = 40), patients with nonexacerbating COPD (NEx, n = 9), and patients with exacerbating COPD (V1, n = 54) are shown. **P* < .05, ***P* < .01, and ****P* < .001, nonparametric Kruskal-Wallis test. **C** and **D**, CD45⁺CD34⁺Col1⁺ cells expressed as a percentages of PBMCs (Fig 2, C) and counts in blood (Fig 2, D) from control subjects (Cont, n = 29), patients with nonexacerbating COPD (NEx, n = 8), and patients with exacerbating COPD (V1, n = 41). **P* < .05, nonparametric Kruskal-Wallis test. Fig 2, A-D, Medians are represented as horizontal lines. **E** and **F**, Comparison of fibrocytes (CD45⁺Col1⁺ cells) in patients with exacerbating COPD at the time of exacerbation (V1) and 2 months after exacerbation resolution (V2). ***P* < .01, Wilcoxon matched pairs test.

expectancy compared with patients with less than 28% fibrocytes (Fig 3, A). There was no statistical difference between the 2 subgroups in terms of age, sex ratio, FEV₁, and Pao₂ (data not shown). The subgroup of patients with more than 28% fibrocytes consisted of 6 patients with acute exacerbation all requiring hospitalization, whereas the subgroup of patients with less than 28% fibrocytes consisted of 36 patients with acute exacerbation (20 requiring hospitalization and 16 without hospitalization).

We also determined correlation coefficients between the percentage of fibrocytes assessed at the second visit (ie, V2 at 2 months after exacerbation in the stable state) and various functional parameters. The percentage of fibrocytes was negatively and significantly correlated to FEV₁ (percent predicted; Fig 3, B), FVC (percent predicted; Fig 3, C), and Pao₂ (in millimeters of mercury; Fig 3, D). By contrast, there was no significant correlation between the percentage of circulating fibrocytes from exacerbating patients with age, body mass index, and pack years smoked (data not shown).

Fibrocyte expression of chemokine receptors

Expression of chemokine receptors was further evaluated in fibrocytes by using flow cytometry. CXCR4, CCR2, and CCR3

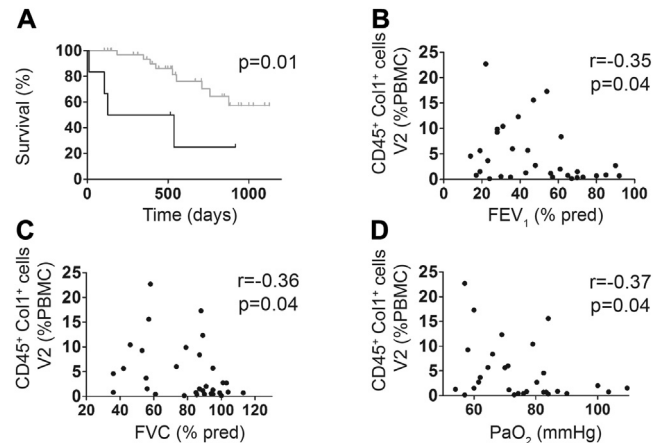


FIG 3. Consequences of increased fibrocyte percentages on survival and lung function in patients with COPD. **A**, Kaplan-Meier survival analysis comparing patients with exacerbating COPD and greater than 28% CD45⁺Col1⁺ cells among PBMCs measured at the time of exacerbation (n = 6; black curve) with patients with less than 28% CD45⁺Col1⁺ cells among PBMCs (n = 36; gray curve). **B-D**, Relationships between FEV₁ (Fig 3, B), FVC (Fig 3, C), Pao₂ (Fig 3, D), and the percentage of CD45⁺Col1⁺ cells in PBMCs in patients with exacerbating COPD at V2. Correlation coefficient (*r*) and significance level (*P* value) were obtained by using nonparametric Spearman analysis.

were expressed by a large proportion of fibrocytes (Fig 4, A, C and E), whereas CCR7 was only found in a small proportion of fibrocytes (Fig 4, G). There were more of both CXCR4⁺ and CCR3⁺ fibrocytes in patients with exacerbating COPD than in control subjects (Fig 4, B and F), whereas CCR2⁺ and CCR7⁺ fibrocyte numbers were similar in both populations (Fig 4, D and H).

Role of the CXCL12/CXCR4 and CCL11/CCR3 axes in fibrocyte migration

Because we found more CXCR4⁺ and CCR3⁺ fibrocytes in the blood of patients with exacerbating COPD, we investigated the role of both CXCR4 and CCR3 in plasma-induced fibrocyte migration in an *in vitro* assay. The CXCR4 antagonist plerixafor significantly reduced plasma-induced recruitment of fibrocytes obtained from patients with exacerbating COPD but not that of fibrocytes obtained from healthy subjects (Fig 5, A). By contrast, plasma-induced migration of fibrocytes from either patients with exacerbating COPD or control subjects was not affected by SB 328437, an antagonist of CCR3 (see Fig E3, A, in this article's Online Repository at www.jacionline.org). We also compared the plasma concentration of some ligands of CXCR4 and CCR3. Plasma concentrations of CXCR4 ligands (ie, CXCL12-α [Fig 5, B] and CXCL12-β [see Fig E4 in this article's Online Repository at www.jacionline.org]) and a CCR3 ligand (ie, CCL11; see Fig E3, B) did not differ significantly between groups. Therefore we examined the migratory response of fibrocytes to increasing concentrations of CXCL12-α and CCL11. CXCL12-α (Fig 5, C), but not CCL11 (see Fig E3, C), significantly increased fibrocyte migration in a concentration-dependent manner. Interestingly, 100 ng/mL CXCL12-α triggered a significantly higher migration of fibrocytes from patients with exacerbating COPD compared with that seen in fibrocytes from control subjects (Fig 5, C), suggesting that CXCL12-α

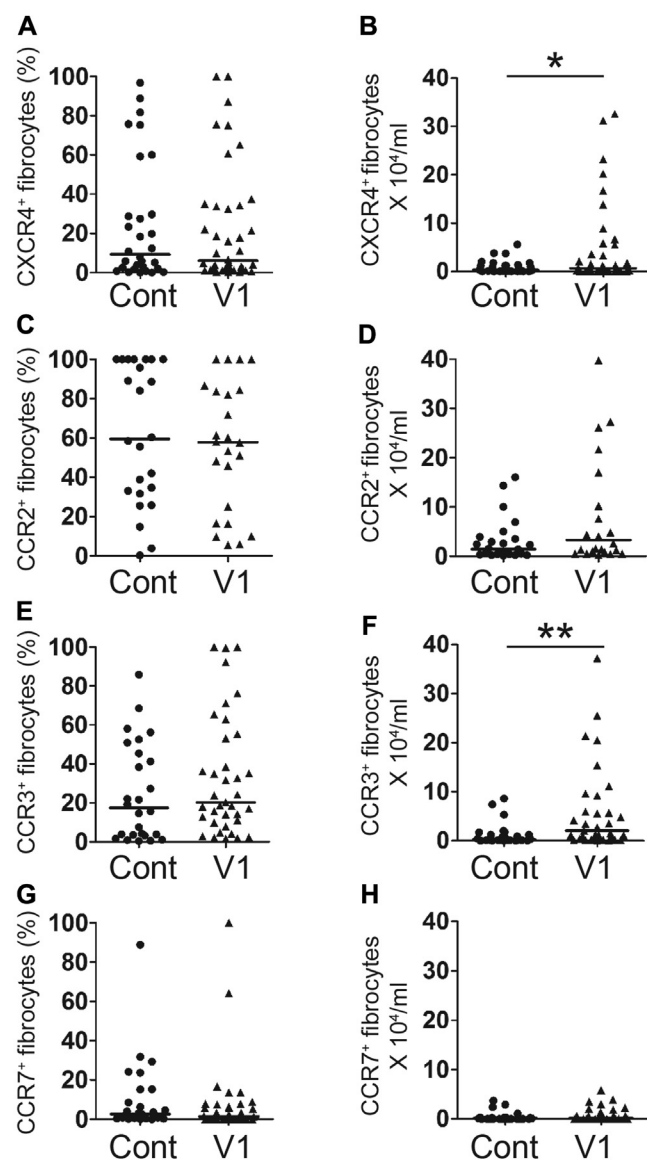


FIG 4. Analysis of fibrocyte marker expression by means of flow cytometry. Expression of CXCR4 (A and B), CCR2 (C and D), CCR3 (E and F), and CCR7 (G and H) in fibrocytes from control subjects (Cont) and patients with exacerbating COPD (V1). Fibrocytes are CD45⁺Col1⁺ cells. Results were expressed as percentages of PBMCs (Fig 4, A, C, E, and G) and counts in blood (Fig 4, B, D, F, and H). **P* < .05 and ***P* < .01, Mann-Whitney tests.

specifically contributes to fibrocyte recruitment during COPD exacerbations. This upregulated response was completely abolished by pretreatment with the CXCR4 antagonist plerixafor (Fig 5, D). Not surprisingly, the CCR3 antagonist SB 328437 had no significant effect on fibrocyte migration from patients with exacerbating COPD induced by CCL11 (see Fig E3, D).

DISCUSSION

For the first time, these results indicate that the percentage of circulating fibrocytes is significantly increased in patients with COPD during an exacerbation and associated with an increased risk of death. The remaining circulating fibrocyte percentage decreases 2 months after exacerbation resolution and is negatively correlated to various functional parameters.

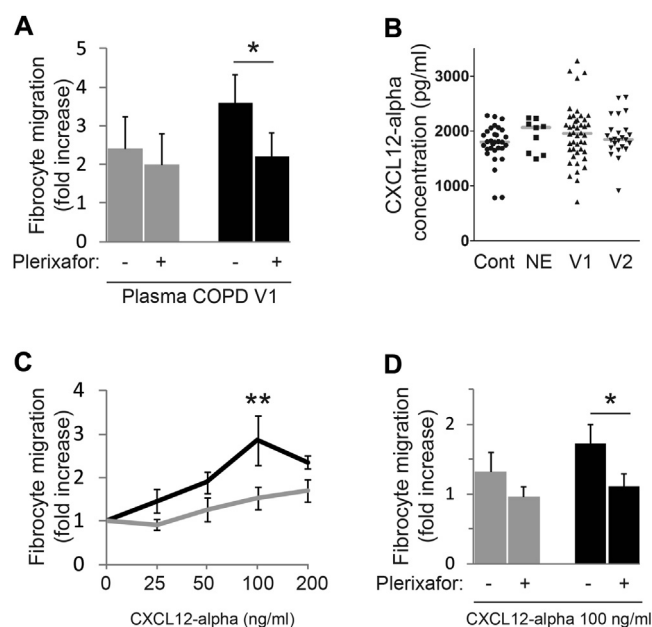


FIG 5. Analysis of fibrocyte chemotaxis. **A**, Migration of fibrocytes from control subjects (*n* = 8, gray bars) and patients with exacerbating COPD (*n* = 6, black bars) in response to plasma from patients with exacerbating COPD in the presence or absence of 25 μ g/mL plerixafor. **P* < .05, paired *t* test. **B**, Plasma CXCL12- α levels in individual subjects. Cont, Control subjects; NE, patients with nonexacerbating COPD; V1, patients with exacerbating COPD during an AECOPD; V2, patients with exacerbating COPD in the stable state. **C**, Migration of fibrocytes from control subjects (*n* = 8, gray lines) and patients with exacerbating COPD (*n* = 5, black lines) in response to CXCL12. ***P* < .01, 2-way ANOVA with Bonferroni tests. **D**, Migration of fibrocytes from control subjects (*n* = 5) and patients with exacerbating COPD (*n* = 7) in response to CXCL12 in the presence or absence of 25 μ g/mL plerixafor. **P* < .05, paired *t* test. Results were expressed as means $\pm$ SEMs (Fig 5, A, C, and D) or with symbols indicating individual subject values and horizontal gray lines representing medians (Fig 5, B).

We have demonstrated that the migration induced by the CXCL12/CXCR4 axis is upregulated in fibrocytes from patients with exacerbating COPD.

In this study we paid special attention to quantifying fibrocytes by using the most accurate available methodology. Although fibrocytes are described as the unique cell expressing both CD45 and the extracellular matrix collagen-1,¹² these markers can also be present in other cell types (ie, macrophages and monocytes). Our quantification approach takes advantage of the method previously described by Wang et al,¹⁴ quantifying fibrocytes in the NANT cell portion of the blood. We checked that the T-cell fraction and adherent fraction contain mainly CD3⁺ (ie, T cells) and CD14⁺ (ie, monocytes) cells, respectively (data not shown). Therefore removing the T-cell fraction and the adherent fraction allowed us to optimize fibrocyte detection. Moreover, removing the T-cell population also allowed us to avoid any variation in the percentage of fibrocytes caused by modifications of the T-lymphocyte subsets in patients with exacerbating COPD. As described previously by Moeller et al,¹⁵ we used the minimum criteria to identify circulating fibrocytes (ie, coexpression of collagen-1 and CD45). Nevertheless, we also found an increase in CD34⁺ fibrocyte counts in patients with exacerbating COPD compared with counts in control subjects, showing that coexpression of collagen-1 and CD45 was the reliable minimum criteria for fibrocyte identification in our study.

During episodes of acute exacerbation, fibrocyte counts in the blood of patients with COPD are increased and return to a level similar to those in control subjects 2 months later. These findings are reminiscent of data obtained in patients with idiopathic pulmonary fibrosis¹⁵ and asthmatic patients,¹⁷ in whom fibrocyte counts were significantly increased during acute disease exacerbation. Nevertheless, fibrocyte counts are also significantly increased in the blood of patients with stable idiopathic pulmonary fibrosis¹⁵ or patients with stable chronic obstructive asthma^{14,17} compared with those in control subjects. In the present study, by contrast, we found no difference in the percentage of blood fibrocytes in patients with exacerbating COPD in the stable state compared with either patients with nonexacerbating COPD or control subjects. We believe that this latter observation might constitute an interesting specificity to assess COPD pathophysiology and provide a framework to unravel the consequences of acute exacerbations in patients with COPD.

In patients with fibrotic interstitial lung disease, CXCL12 expression is enhanced in plasma and lungs, which is associated with a high CXCR4⁺ fibrocyte pool.¹⁹ In contrast, the CCR7/CCL19 axis plays a critical role for fibrocyte recruitment in the blood of patients with chronic obstructive asthma.¹⁷ However, the chemotactic mechanism for recruitment of circulating fibrocytes might differ for asthmatic patients without airflow obstruction during acute exacerbation. Indeed, there were more CXCR4⁺ fibrocytes in patients with chronic obstructive asthma, and the expression of CXCL12 was also increased in their airways.¹⁷ In the present study we have shown that the CXCR4/CXCL12 axis is also important for fibrocyte chemotaxis during an AECOPD. However, there was no increase in blood CXCL12 levels, and the augmented CXCL12-induced migratory activity of fibrocytes from patients with exacerbating COPD did not seem to be caused by an upregulation of CXCR4 expression. Whether a modification of the signaling pathway downstream of CXCR4 or a differential incorporation of CXCR4 into membrane lipid rafts²⁰ is implicated in this migratory response deserves further study.

By using a large cohort of well-characterized patients with COPD in a stable state, a variety of clinical, functional, or biological parameters have been previously related to mortality, including age; the body mass index, airflow obstruction, dyspnea, and exercise index (BODE index); IL-6 concentrations; neutrophil counts; and levels of fibrinogen, C-reactive protein, CCL18, and surfactant protein D.²¹ Here we have observed that a high percentage of circulating fibrocytes during an AECOPD is associated with a lower survival. However, it is noteworthy that this result is a secondary outcome of the present clinical trial. Whether this level is actually independent of the previously identified biomarkers needs to be confirmed in larger cohorts. Interestingly however, the percentage of circulating fibrocytes 2 months after the AECOPD was not related to mortality but rather to a more pronounced bronchial obstruction and a more degraded hematosis.

The present study has some limitations, which deserve further comment. The initial cause of AECOPDs has not been initially explored. From the literature, viral and bacterial infections are responsible for 40% and 21% of AECOPDs, respectively.²² Furthermore, we did not perform bronchoalveolar lavage during AECOPDs. Bronchoalveolar lavage samples from patients with AECOPDs might show increased CXCL12 levels to confirm the notion that this chemokine could be important in fibrocyte

recruitment to the lungs. Moreover, we did not systematically measure airway wall thickness using a computed tomographic scan or pulmonary arterial pressure²³ in the present study because circulating fibrocyte counts have been shown to be increased during pulmonary hypertension only in very young patients and never in patients with COPD.²⁴ One might suggest that a large proportion of the enrolled patients were not tested at V2, which could limit the results from the stable state. However, only 12 from the 54 enrolled patients were lost at follow-up, whereas the remaining 10 patients were either frequent exacerbators, deceased before V2, or had attended V2 but their fibrocyte quantification failed. In addition, we limit the *in vitro* experiments to the analysis of migration, and thus we did not explore other fibrocyte functions, such as production of extracellular matrix components or proinflammatory mediators. Such further results might precisely define the role of fibrocytes in COPD exacerbation.

In conclusion, this study demonstrates that circulating fibrocytes are recruited during COPD exacerbations and are related to mortality and lung function. From the *in vitro* experiments, the CXCL12/CXCR4 axis appears to be involved in such fibrocyte recruitment. Thus targeting CXCR4 to limit the recruitment of fibrocytes could be of potential interest. Nevertheless, further studies are required to assess the efficacy of CXCR4 antagonists *in vivo* on both mortality and lung function decline, which represent the 2 most relevant COPD outcomes.

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Clinical implications: There are higher numbers of circulating fibrocytes in patients with exacerbating COPD. A high fibrocyte count during a COPD exacerbation is associated with an increased risk of death.

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METHODS

Study populations

Subjects aged more than 40 years were eligible for enrollment if they had a clinical diagnosis of COPD exacerbation according to the GOLD guidelines,^{E1} which was defined as change in the patient's baseline dyspnea, cough, and/or sputum that is beyond normal day-to-day variations, is acute in onset, and might warrant a change in regular medication. Patients with COPD with exacerbation have been recruited during hospitalization in the intensive care unit, in the respiratory department, or as outpatients in the clinical investigation center of the University Hospital of Bordeaux. Fifty healthy volunteers without any history of lung disease and with normal lung function testing (ie, FEV₁ and FVC >80% of predicted value and FEV₁/FVC ratio >0.70) were recruited. The main exclusion criteria for patients with COPD and healthy subjects were history of asthma, lung fibrosis, idiopathic pulmonary hypertension, and chronic viral infections (hepatitis and HIV). Both patients with exacerbating COPD and control subjects were enrolled within the Firebrob study. Additionally, patients with COPD without any exacerbations during a preceding minimal period of 12 months were also recruited from the Cohorte Obstruction Bronchique et Asthme (COBRA; Bronchial Obstruction and Asthma Cohort; sponsored by the French National Institute of Health and Medical Research [INSERM]) cohort as control outpatients in the Clinical Investigation Centre of the University Hospital of Bordeaux by using the same exclusion criteria. They are designated as patients with nonexacerbating COPD in the following text.

All subjects provided written informed consent to participate to the study after the nature of the procedure had been fully explained. All clinical data were collected in the Clinical Investigation Center (CIC1401) from the University Hospital of Bordeaux. The study protocol was approved by the local research ethics committee and the French National Agency for Medicines and Health Products Safety.

Design of the Firebrob study

The study was a clinical trial conducted from May 2011 to May 2015. Patients were recruited from (1) the Intensive Care Unit of Pellegrin Hospital, (2) the Intensive Care Unit of Saint André Hospital, (3) the Respiratory Department of Haut-Lévêque Hospital, and (4) the Clinical Investigation Centre of the University Hospital of Bordeaux. The study was sponsored by the University Hospital of Bordeaux (ie, CHU de Bordeaux) and funded by Nycomed and Takeda. All authors were academic and made the decision to submit the manuscript for publication and vouch for the accuracy and integrity of the contents. The study has been registered at ClinicalTrials.gov as no. NCT01196832 (ie, Firebrob study).

The study design is summarized in [Fig 1](#). There were 2 visits for patients with exacerbating COPD: a visit during an exacerbation (inclusion [V1]) and a visit 2 months $\pm$ 7 days after an exacerbation (stable state [V2]). The inclusion visit (V1) consisted of patient information, signature of the informed consent form, and venous blood sample (50 mL) for fibrocyte analysis. The second visit (V2) consisted of a clinical (COPD Assessment Test [CAT] and St Georges Quality of Life Questionnaire [SGQLQ]) and functional (plethysmography, TLCO, and arterial gas) evaluation and venous blood samples (50 mL) for fibrocyte analysis. Subjects who experienced a COPD exacerbation (as defined by the study protocol) between V1 and V2 had to be seen 2 months after the last exacerbation for V2. There was only 1 visit for control subjects and patients with nonexacerbating COPD, during which the informed consent form was signed, a clinical (CAT and SGQLQ) and functional (plethysmography, TLCO, and arterial gas) evaluation was performed, and a blood sample was taken for fibrocyte analysis.

The primary outcome was the percentage of blood fibrocytes among PBMCs. Secondary outcomes included characterization of fibrocyte chemokine receptor expression.

Circulating fibrocytes

NANT cells were obtained, as described previously, with some modifications.^{E2} PBMCs were first separated from whole blood by means of

Ficoll-Hypaque (Dutscher) density gradient centrifugation. After the first centrifugation at 150g for 15 minutes, the top plasma layer was harvested and kept at -80°C for further analysis. Mononuclear cells at the interface were harvested and washed once with PBS. Erythrocyte lysis was performed by adding 20 mL of hypotonic 0.2% NaCl solution for 30 seconds, followed by adding 20 mL of 1.6% NaCl to end with an isotonic solution. Mononuclear cells were again washed with PBS; resuspended in Dulbecco modified Eagle medium (Sigma-Aldrich) containing 4.5 g/L glucose and L-glutamine; supplemented with 20% FBS (Invitrogen, Cergy Pontoise, France), penicillin/streptomycin (Invitrogen), and MEM nonessential amino acid solution (Sigma-Aldrich); and incubated for 1 hour at 37°C . The nonadherent mononuclear cell fraction was taken and washed in cold PBS containing 0.5% BSA (Sigma-Aldrich) and 2 mmol/L EDTA (Invitrogen). T cells were further depleted with anti-CD3 mAb (Miltenyi Biotec, Paris, France). At least 0.2×10^6 NANT cells were distributed in each FACS tube and fixed overnight with Cytotfix/Cytoperm (eBioscience, Paris, France).

Identification and characterization of circulating fibrocytes

Fibrocytes were identified by means of flow cytometry as double positive for the surface marker CD45 and the intracellular marker collagen I (see [Fig E1](#)). Fixed blood NANT cells were washed in permeabilization buffer (eBioscience) and incubated either with mouse anti-human collagen I antibody (Millipore, St-Quentin-en-Yvelines, France) or matched IgG₁ isotype control (Santa Cruz Biotechnology, Heidelberg, Germany), followed by fluorescein isothiocyanate-conjugated anti-mouse antibodies (Beckman Coulter, Villepinte, France). Next, the cell pellet was incubated with either allophycocyanin (APC)-conjugated anti-CD45 antibodies (BD Biosciences) or matched APC-conjugated IgG₁ isotype control (BD Biosciences). The cell suspension was analyzed with a BD FACSCanto II flow cytometer (BD Biosciences). Offline analysis was performed with FACS Diva (BD Biosciences) and FlowJo (TreeStar, Ashland, Ore) software. The negative threshold for CD45 was set by using a matched APC-conjugated IgG₁ isotype control, and all subsequent samples were gated for the CD45⁺ region (see [Fig E1](#)). Cells gated for CD45 were analyzed for collagen-I expression, with negative control thresholds set by using fluorescein isothiocyanate-stained cells. Specific staining for collagen-I was determined as an increase in positive events over this threshold. Fibrocyte numbers were expressed as both a percentage of total PBMC counts^{E3} and the absolute number of cells.^{E4} Antigen CD34 progenitor cell marker and chemokine receptor expressions were also assessed by means of flow cytometry with specific fluorescent antibodies directed against CD34, CCR3, CCR7, CXCR4 (BD Biosciences), and CCR2 (R&D Systems) and their corresponding irrelevant antibodies. Blood fibrocyte morphology was checked by using phase-contrast microscopy (Nikon, Champigny sur Marne, France).

Fibrocyte migration

Fibrocyte migration was assessed by using a modified Boyden chamber assay. The transwell inserts (pore size, 8 μm ; Dutscher) and wells were coated for 1 hour at room temperature with polylysine-ethylene glycol (SuSoS, Dübendorf, Switzerland) to prevent cell adhesion. The inserts and wells were rinsed with PBS. In total, 0.3×10^6 NANT cells resuspended in 0.2 mL of Dulbecco modified Eagle medium containing 4.5 g/L glucose and L-glutamine supplemented with Insulin Transferrin Solution (Dutscher), penicillin/streptomycin, and MEM nonessential amino acid solution were added to the upper compartments of each well. When indicated, NANT cells were pretreated for 1 hour at 37°C with 25 $\mu\text{g}/\text{mL}$ plerixafor (Sigma-Aldrich), an antagonist of CXCR4,^{E5} or 3.8 $\mu\text{g}/\text{mL}$ SB 328437 (R&D Systems), an antagonist of CCR3,^{E6} before being added to the upper compartment. Recombinant human CXCL12- α (25-200 ng/mL, R&D Systems), recombinant human CCL11 (25-200 ng/mL, R&D Systems), or plasma (50% diluted from blood) from patients with exacerbating COPD or control subjects was added to the bottom compartment of each well. After 12 hours, the content of the bottom compartment was removed to assess fibrocyte

migration by using flow cytometry with double labeling with CD45–collagen I, as described above. Flow cytometric counts for each condition were obtained during a constant predetermined time period to obtain absolute values of migratory cells (1 minute).^{E7} The fraction of migratory fibrocytes was defined as the number of CD45⁺Col1⁺ cells counted in the bottom chamber divided by the number of CD45⁺Col1⁺ cells added in the upper compartment. These values were normalized to the fraction of migratory fibrocytes obtained in the basal condition (medium only).

Measurement of plasma CXCL12 and CCL11 concentrations

Concentrations of plasma CXCL12- α (R&D Systems), CXCL12- β (Sigma-Aldrich), and CCL11 (R&D Systems) were measured by means of ELISA, according to the manufacturer's instructions.

Statistical analysis

The primary outcome was the percentage of blood fibrocytes among PBMCs. Secondary outcomes were number of deaths, CAT score, SGQLQ score, FEV₁, FVC, FEV₁/FVC ratio, FEF_{25–75}, Pao₂, and TLCO. Statistical analysis was performed with Prism 6 software (GraphPad Software, La Jolla, Calif). Values are presented as means $\pm$ SDs. Statistical significance ($P < .05$) was analyzed by using Fisher exact tests for comparison of proportions, by using 2-sided independent t tests and multivariate ANOVA for variables with a parametric distribution, and by using the Kruskal-Wallis test with multiple comparison z tests, Mann-Whitney U tests, paired Wilcoxon tests, and Spearman correlation coefficients for variables with a nonparametric

distribution. Survival in patients with exacerbating COPD with high versus low blood fibrocyte counts was compared by using Kaplan-Meier analysis.

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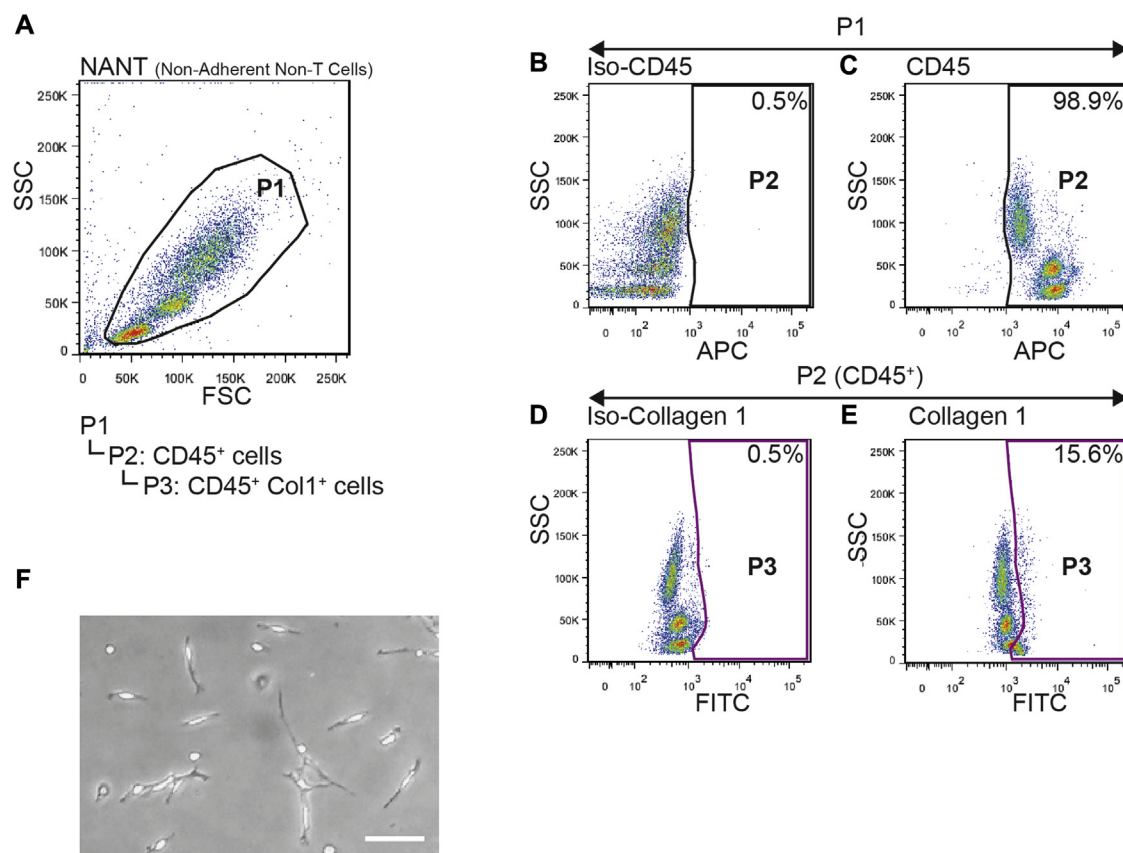


FIG E1. Identification of fibrocytes by using flow cytometry and phase-contrast microscopy. Representative dot plots of flow cytometry for fibrocyte quantification from a patient with COPD exacerbation. **A**, Total NANT cell population selected on unstained cells. **B**, Isotype control for CD45 set on unstained cells. **C**, Positive population for CD45. **D**, Isotype control for collagen-1 set on a CD45-gated population. **E**, Positive population for both CD45 and collagen-1. *FITC*, Fluorescein isothiocyanate; *FSC-A*, forward scatter; *SSC-A*, side scatter. **F**, Representative image of cultured blood fibrocytes taken with a phase-contrast microscope, showing their morphology as spindle-shaped adherent cells. *Bar* = 20 μ m.

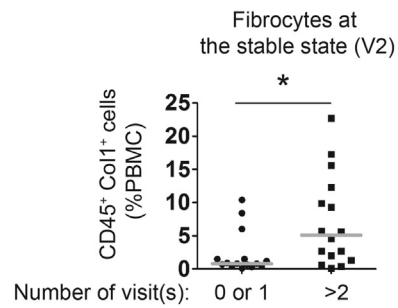


FIG E2. Percentage of CD45⁺Col1⁺ cells among PBMCs of patients with exacerbating COPD at V2 with 1 or no unscheduled visits ($n = 15$) or with 2 or more unscheduled visits ($n = 16$) the year before V1. Medians are represented as *gray horizontal lines*. * $P < .05$, Mann-Whitney test.

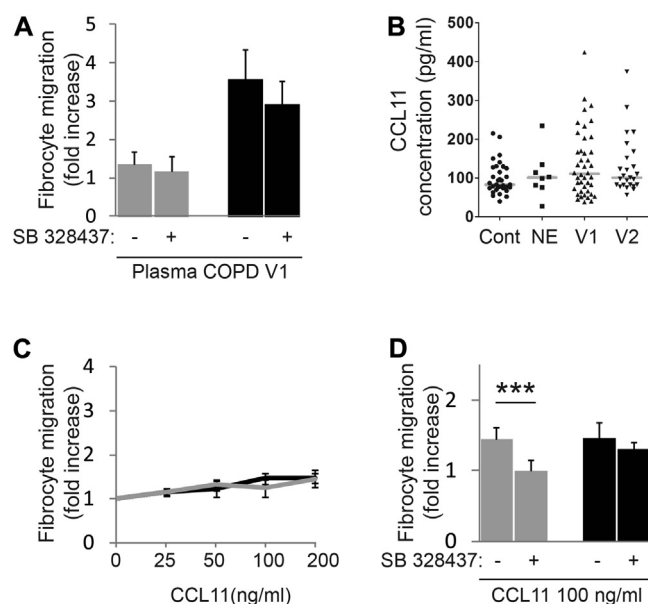


FIG E3. **A**, Migration of fibrocytes from control subjects ($n = 3$, gray bars) and patients with exacerbating COPD ($n = 6$, black bars) in response to plasma of patients with exacerbating COPD in the presence or absence of $3.8 \mu\text{g/mL}$ SB 328437 (paired t test). **B**, Plasma CCL11 levels in individual subjects. Cont, Control subjects; NE, patients with nonexacerbating COPD; V1, patients with exacerbating COPD during an AECOPD; V2, patients with exacerbating COPD in the stable state. **C**, Migration of fibrocytes from control subjects ($n = 4$, gray lines) and patients with exacerbating COPD ($n = 6$, black lines) in response to CCL11. **D**, Migration of fibrocytes from control subjects ($n = 4$) and patients with exacerbating COPD ($n = 5$) in response to CCL11 in the presence or absence of $3.8 \mu\text{g/mL}$ SB 328437. $P < .001$, paired t test. Results are expressed as means $\pm$ SEMs (Fig E3, A, C, and D) or with symbols indicating individual subject values and horizontal gray lines representing medians (Fig E3, B). *** $P < .001$.

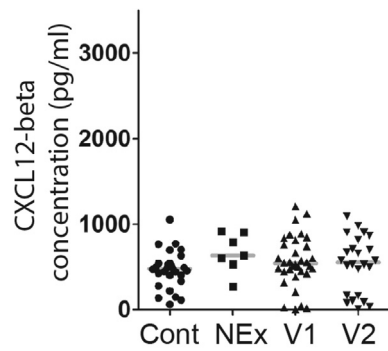


FIG E4. Individual CXCL12- β plasma concentrations. *Cont*, Control subjects; *NEx*, patients with nonexacerbating COPD; *V1*, patients with exacerbating COPD during an AECOPD; *V2*, patients with exacerbating COPD in the stable state. *Symbols* indicate individual subjects, and *horizontal gray lines* represent medians.

TABLE E1. Characteristics of patients with nonexacerbating COPD

	Patients with nonexacerbating COPD
No.	9
Age (y)	61.3 ± 6.4
Sex (M/F)	8/1
Body mass index (kg/m ²)	31.9 ± 8.1
Current smoker (yes/no)	3/6
Former smoker (yes/no)	6/3
Pack years smoked (no.)	39.7 ± 17.1
In the previous year, no. of subjects with:	
0 unscheduled visit/y	9
Hospitalization (yes/no)	0/9
Ventilation mode	
Spontaneous ventilation (yes/no)	9/0
Noninvasive ventilation (yes/no)	0/9
Orotracheal intubation (yes/no)	0/9
Stable state	
COPD duration (y)	6.8 ± 4.8
FEV ₁ (percent predicted)	68.0 ± 16.1
FEV ₁ /FVC ratio (%)	60.4 ± 7.7
FVC (percent predicted)	89.9 ± 16.9
TLCO (percent predicted)	75.4 ± 41.9
Pao ₂ (mm Hg)	76.6 ± 10.5

Values are presented as means ± SDs where shown.

F, Female; M, male.