The C-C motif chemokine ligands CCL5, CCL11, and CCL24 induce the migration of circulating fibrocytes from patients with severe asthma

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The C-C motif chemokine ligand 5 (CCL5), CCL11, and CCL24 are involved in the pathogenesis of asthma, and their function is mainly associated with the airway recruitment of eosinophils. This study tested their ability to induce the migration of circulating fibrocytes, which may contribute to the development of irreversible airflow obstruction in severe asthma. The sputum fluid phase (SFP) from patients with severe/treatment-refractory asthma (PwSA) contained elevated concentrations of CCL5, CCL11, and CCL24 in comparison with the SFP from patients with non-severe/treatment-responsive asthma (PwNSA). The circulating fibrocytes from PwSA expressed the receptors for these chemokines at increased levels and migrated in response to recombinant CCL5, CCL11, and CCL24. The SFP from PwSA induced the migration of autologous fibrocytes, and its activity was significantly attenuated by neutralization of endogenous CCL5, CCL11, and CCL24. These findings suggest that CCL5, CCL11, and CCL24 may contribute to the airway recruitment of fibrocytes in severe asthma.

INTRODUCTION

The fibrocytes are bone marrow–derived mesenchymal progenitor cells[1–4] that may contribute to the development of irreversible structural alterations in asthmatic airways by promoting subepithelial fibrosis and by expanding the population of contractile cells in the bronchial wall.[5–9] Increased numbers of fibrocytes have been detected in the peripheral blood and in the airways of asthmatic patients, particularly in those with a transient acute exacerbation of asthma[9] and in subjects with chronically severe/treatment-refractory disease.[7,8] The $CD34^+CD45RO^+$ fibrocytes that accumulate in the bronchial mucosa of asthmatic individuals actively synthesize new collagen and express the contractile protein α-smooth muscle actin (α-SMA).[5,6] These immature, myofibroblast-like, $CD34^+\alpha\text{-SMA}^+$ cells localize to areas of collagen deposition beneath the epithelial basement membrane[5,6,8] and also infiltrate the underlying smooth muscle in patients with severe disease.[8] The density of fibrocytes in the subepithelial zone correlates with the magnitude of subepithelial fibrosis and severity of asthma.[6,8] Fibrocytes that have recently migrated toward the bronchial epithelium and are present in high numbers beneath the epithelial basement membrane can be recovered from the asthmatic airways by bronchoalveolar lavage[6] and sputum induction,[9] suggesting that the bronchial epithelium is a major source of fibrocyte chemoattractants.

The C-C motif chemokine ligand 5 (CCL5; also known as regulated on activation normal T cell expressed and secreted), CCL11 (also known as eotaxin), and CCL24 (also known as eotaxin-2 or myeloid progenitor inhibitor factor-2) are all epithelial cell–derived chemokines involved in the pathogenesis of asthma.[10] The C-C motif chemokine receptors (CCRs) CCR3 and CCR5 are expressed at high levels in the bronchial mucosa of asthmatic individuals.[10] CCL5 and CCL11 bind to CCR3 and CCR5 with different affinities, while CCL24 selectively binds to CCR3 with high affinity.[11] Previous studies have mainly focused on the ability of these chemokines to induce the recruitment of eosinophils in asthmatic airways,[12–15] but recent observations have suggested that they may also promote the development of structural alterations in asthma through eosinophil-independent mechanisms.[16,17] It has been reported that human fibrocytes isolated in long-term cultures of

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peripheral blood mononuclear cells (PBMCs) from normal individuals express CCR3 and CCR5,[18] but there is no information on the levels of expression of these receptors in circulating fibrocytes from asthmatic donors and on their functionality. This study provides the first evidence that circulating fibrocytes from patients with severe/treatment-refractory asthma (PwSA) express CCR3 and CCR5 at higher levels than cells from patients with non-severe/treatment-responsive asthma (PwNSA). More importantly, it demonstrates that endogenous CCL5, CCL11, and CCL24 substantially contribute to the fibrocyte chemotactic activity of the induced sputum from PwSA, suggesting a major role of these chemokines in the airway recruitment of fibrocytes in severe disease.

RESULTS

Increased levels of CCL5, CCL11, and CCL24 were present in the SFP from PwSA

The study population included 19 PwNSA and 16 PwSA (**Table 1**) who provided informed consent once the protocol was approved by the appropriate review board. Disease severity was classified on the basis of international guidelines,[19,20] using criteria for the selection of patients with mild-to-moderate asthma and severe/treatment-refractory asthma similar to those adopted in a previous study.[8] In particular, the PwSA satisfied the following criteria for severe/treatment-refractory disease:[20] continuous therapy with high dose of inhaled corticosteroids and long-acting bronchodilators for >1 year; presence of asthma symptoms requiring the use of relievers

Table 1 Demographic and clinical characteristics

	PwSA	PwNSA
Patients, *n*	16	19
Age, mean years (s.d.)	46.2 (9.9)	39.7 (11.3)
Gender, females/males	9/7	10/9
Atopy, *n*	10	11
Duration of asthma, mean years (s.d.)	23.5 (9.2)	16.7 (12.2)
FEV_1, mean % of predicted normal (s.d.)	76.4 (8.1)*	92.5 (8.6)
FEV_1 response to bronchodilator, mean % increase (s.d.)[a]	4.8 (13.7)*	9.9 (12.4)
$PC_{20}M$, geometric mean mg ml^{-1} (range)	0.28 (0.06-1.12)*	0.92 (0.15-3.20)
Features of partly controlled/uncontrolled asthma >2 months[b]		
Pre-bronchodilator FEV_1 <80% of predicted normal	9	0
Daytime symptoms >2 days per week, *n*	16	0
Any limitations of activities because of symptoms, *n*	16	0
Night-time symptoms or on awakening at least once per week, *n*	9	0
Reliever use >2 days per week, *n*	16	0
≥ three of the above features per week, *n*	13	0
Emergency room visit for asthma exacerbation in the past year, *n*	6	0
Current treatment		
On-demand treatment with rapid-acting bronchodilators alone, *n*	0	4
Long-acting bronchodilators, *n*	16	8
Inhaled corticosteroids, *n*	16	15
Daily dose of inhaled corticosteroids		
200–500 μg BDE, *n*	0	7
>500–1,000 μg BDE, *n*	0	8
>1,000 μg BDE, *n*	16	0
Oral corticosteroids, *n*	7	0

BDE, beclomethasone diproprionate equivalents; FEV_1, forced expiratory volume in one second; $PC_{20}M$, provocation concentration of inhaled methacholine causing a 20% decrease in FEV_1; PwNSA, patients with non-severe/treatment-responsive asthma; PwSA, patients with severe/treatment-refractory asthma.
[a]FEV_1 measured before and 10 min after the inhalation of 200 μg salbutamol.
[b]According to: Global Initiative for Asthma (GINA). Global strategy for asthma management and prevention. Updated 2010. Available at: http://www.ginasthma.com.[19]
*$P<0.05$ compared with PwNSA by the unpaired Student's *t*-test or the Mann–Whitney test.

(rapid-acting bronchodilators) on a daily or almost daily basis, with or without persistent airflow obstruction; and at least one episode of disease exacerbation requiring a course of oral corticosteroids in the previous year and/or maintenance treatment with oral corticosteroids. All PwNSA had a disease controlled by current treatment (rapid-acting bronchodilators on demand alone or low daily doses of inhaled corticosteroids with or without long-acting bronchodilators for mild asthma and medium daily doses of inhaled corticosteroids with long-acting bronchodilators for moderate asthma),[19] as demonstrated by normal lung function and absence of symptoms on most days in the previous 2 months (**Table 1**). All patients were non-smokers or ex-smokers with a past smoking history of $\leqslant 10$ pack-years. None of them reported the occurrence of respiratory or systemic infections in the previous 2 months. Sputum induction by hypertonic saline was performed according to a standard procedure.[21] Whole expectorated samples were processed to collect the sputum fluid phase (SFP) as previously described,[22,23] and the concentrations of immunoreactive CCL5, CCL11, and CCL24 were measured by enzyme-linked immunosorbent assay (ELISA).

The SFP from PwSA contained elevated concentrations of immunoreactive CCL5, CCL11, and CCL24 in comparison with the concentrations detected in the SFP from PwNSA (**Figure 1**). The median (interquartile range) values were 504.1 pg ml^{-1} (260.3 pg ml^{-1}) vs. 130.6 pg ml^{-1} (174.9 pg ml^{-1}) for CCL5, 263.2 pg ml^{-1} (128.6 pg ml^{-1}) vs. 87.3 pg ml^{-1} (133.7 pg ml^{-1}) for CCL11, and 298.9 pg ml^{-1} (108.7 pg ml^{-1}) vs. 110.1 pg ml^{-1} (97.7 pg ml^{-1}) for CCL24.

PwSA had increased numbers of circulating fibrocytes expressing high levels of α-SMA and type III collagen (COL3) upon stimulation with autologous SFP

Circulating fibrocytes were purified from PBMCs by using a previously described[24] two-step procedure that is based on the known phenotypic characteristics of these cells.[5,6,8,9,18] It involves the immunomagnetic selection of CD34$^+$ cells and the subsequent isolation of the fraction coexpressing CD45RO, CD11b, and CD13 by sorting the cells triple-stained with specific fluorochrome-conjugated monoclonal antibodies (mAbs) on a BD FACSAria II (BD Biosciences, St Jose, CA). The purity of the isolated fibrocytes is then ascertained by staining for intracellular type I collagen (COL1).[24] The enumeration of circulating fibrocytes in healthy individuals by using this procedure for cell isolation provides results very similar to those obtained with other methods.[7–9,25] In the present study, the numbers of CD34$^+$CD45RO$^+$CD11b$^+$CD13$^+$COL1$^+$ cells isolated from the peripheral blood of PwSA were significantly higher than those isolated from the peripheral blood of PwNSA (**Figure 2a**). The medians (interquartile range) values were 4.6×10^4 per ml (2.2×10^4per ml) and 1.7×10^4per ml (2.1×10^4per ml), respectively. The cells from both the groups of patients expressed α-SMA when cultured for 48 h in medium containing 20% autologous serum, as assessed by western blot analysis of cell lysates using a specific mAb against α-SMA (**Figure 2b**). Replacement of the serum with 20% autologous SFP further increased α-SMA expression to a greater extent in circulating fibrocytes from PwSA than in circulating fibrocytes from PwNSA (**Figure 2b**). The constitutive expression of COL3 was also significantly increased in circulating cells from PwSA by stimulation with 20% autologous SFP for 48 h, as assessed by western blot analysis of cell lysates using a polyclonal Ab against the α1 chain of COL3 (COL3A1; **Figure 2b**). The SFP-induced increase in the expression of α-SMA and COL3 in circulating fibrocytes from PwSA occurred in a time- and dose-dependent manner (**Figure 3a,b**). These experiments confirmed the nature of the isolated cells and also demonstrated that the SFP from PwSA contained factor(s) capable of promoting the contractile and profibrotic properties of circulating fibrocytes once they have migrated in the bronchial mucosa of these patients.

Circulating fibrocytes from PwSA exhibited increased expression of CCR3 and CCR5 and migrated in response to recombinant CCL5, CCL11, and CCL24

Possible differences in the levels of expression of CCR3 and CCR5 on the surface of freshly isolated circulating fibrocytes from PwSA and PwNSA were evaluated by flow cytometry. The percentages of fibrocytes that specifically stained with an anti-CCR3 or an anti-CCR5 mAbs were significantly higher in PwSA than in PwNSA (**Figure 4a–d**). The mean fluorescence

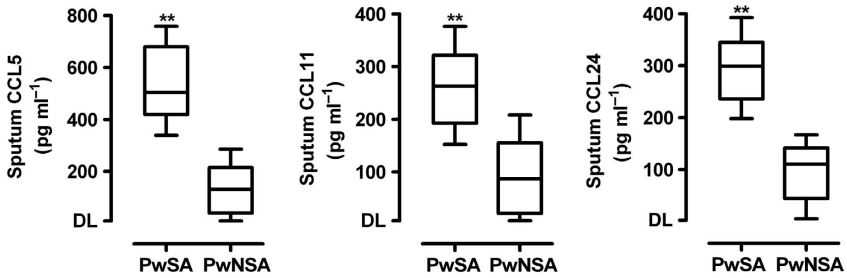

Figure 1 Patients with severe/treatment-refractory asthma (PwSA, $n = 16$) had higher sputum concentrations of the C-C motif chemokine ligand 5 (CCL5), CCL11, and CCL24 than patients with non-severe/treatment-responsive asthma (PwNSA, $n = 19$). Chemokine concentrations were measured by enzyme-linked immunosorbent assay and the detection limits (DLs) were 9.6 pg ml^{-1} for CCL5, 7.8 pg ml^{-1} for CCL11, and 5.2 pg ml^{-1} for CCL24. Samples with chemokine levels below the detection limits were assigned a value of zero for statistical analysis. Horizontal lines indicate the medians; boxes indicate the interquartile ranges; whiskers indicate the minimum and maximum values. $^{**}P < 0.01$ compared with PwNSA by the Mann–Whitney test.

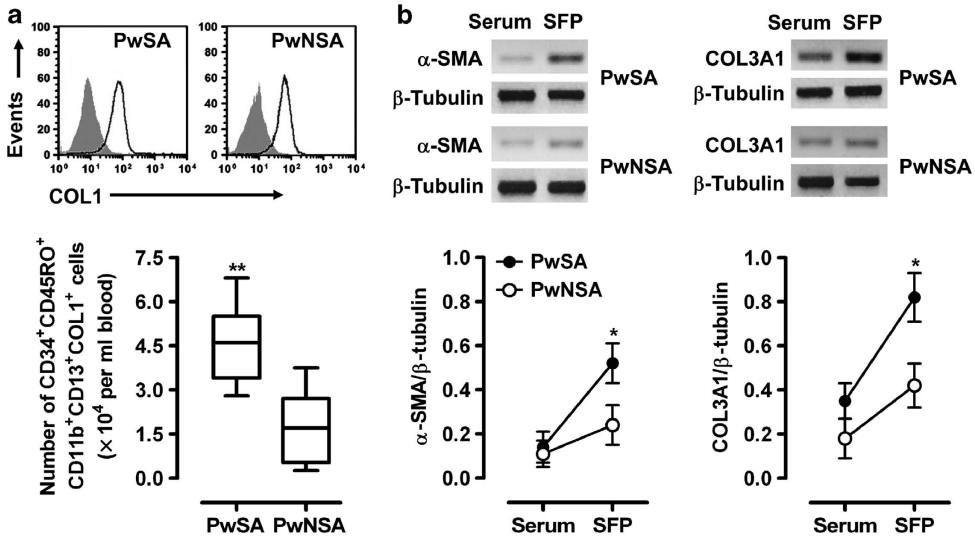

Figure 2 The CD34$^+$CD45RO$^+$CD11b$^+$CD13$^+$ cells isolated from the peripheral blood of patients with severe/treatment-refractory asthma (PwSA) and patients with non-severe/treatment-responsive asthma (PwNSA) were authentic fibrocytes, as demonstrated by their high level of expression of intracellular type I collagen (COL1) and ability to express α-smooth muscle actin (α-SMA) and type III collagen (COL3). (**a**) Representative flow cytometry graphs of isolated cells stained with a specific fluorescein isothiocyanate-conjugated anti-COL1 monoclonal antibody (solid line) or the isotype-matched control (gray area) and enumeration of the circulating fibrocytes. In the lower panel, horizontal lines indicate the medians; boxes indicate the interquartile ranges; whiskers indicate the minimum and maximum values. $^{**}P<0.01$ compared with PwNSA by the Mann–Whitney test (PwSA, $n=16$; PwNSA, $n=19$). (**b**) Western blot analysis of the expression of α-SMA and COL3 (α1 chain (COL3A1)) in cells cultured for 48 h in 20% autologous serum or 20% sputum fluid phase (SFP). Representative blots are reported in the upper panels. β-Tubulin was the loading control used for signal normalization and quantification of the relative densitometric units. The quantitative data in the lower panels are expressed as the means and s.d. $^*P<0.05$ compared with cells cultured in 20% serum by the paired Student's *t*-test or the Wilcoxon matched-pairs signed rank test ($n=3$–4).

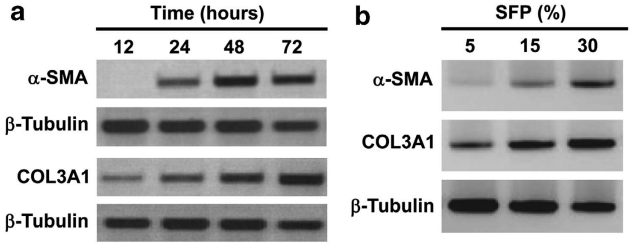

Figure 3 The increase in the expression of α-smooth muscle actin (α-SMA) and type III collagen (COL3) induced by autologous sputum fluid phase (SFP) in circulating fibrocytes from patients with severe/treatment-refractory asthma occurred in a time- and dose-dependent manner. (**a**) Western blot analysis of the expression of α-SMA and COL3 (α1 chain (COL3A1)) in cells cultured for 12–72 h in 20% autologous SFP. (**b**) Western blot analysis of the expression of α-SMA and COL3A1 in cells cultured for 48 h in presence of the indicated amounts of autologous SFP. The blots are representative of three experiments with fibrocytes from different patients. β-Tubulin was the loading control.

intensities (s.d.) of CCR3$^+$COL1$^+$ cells were 112 (22) in PwSA and 64 (19) in PwNSA ($P<0.01$ by the unpaired Student's *t* test or the Mann–Whitney test). The corresponding values for CCR5$^+$COL1$^+$ cells were 183 (30) and 78 (27) ($P<0.01$ by the unpaired Student's *t* test or the Mann–Whitney test).

In agreement with these findings, the proportion of circulating fibrocytes that migrated in response to stimulation with the same concentration of recombinant CCL5 (high-affinity CCR5 ligand and potent CCR3 agonist), CCL11 (high-affinity CCR3 ligand and weak CCR5 agonist), and CCL24 (high-affinity CCR3 ligand) was significantly higher when the cell donors where PwSA, as assessed in a standard chemotaxis assay (**Figure 5a**). The molecular mass of the recombinant proteins was 8.4, 10.6, and 7.8 kDa, respectively. Therefore, CCL5 was more potent than CCL11, and both CCL5 and CCL11 were more potent than the selective CCR3 agonist CCL24 in increasing the migration of circulating fibrocytes from PwSA over the spontaneous migration (**Figure 5a**).

The existence of significant differences in the migratory response of fibrocytes from PwSA and PwNSA to CCL5, CCL11, and CCL24 was confirmed by two-way analysis of variance of the concentration–response curves obtained by stimulating the cells from the same donors with increasing concentrations of all three chemokines (**Figure 5b**). According to the results of this analysis, the curves were significantly different ($P<0.01$ for CCL5 and CCL11; $P<0.05$ for CCL24) and the concentrations significantly affected the results ($P<0.01$ for CCL5 and CCL11; $P<0.05$ for CCL24). Comparison of the specific migratory responses at each chemokine concentration with the Bonferroni *post-hoc* test gave the results presented in **Figure 5b**. It is worth noting that the cells from PwSA already showed an appreciable migratory response at the biologically relevant concentrations of each chemokine comprised between 0.2 ng ml^{-1} and 2 ng ml^{-1} (**Figure 5b**).

Importantly, all recombinant proteins elicited a migratory response in the circulating fibrocytes from PwSA only when they were exclusively added to the bottom compartment of the chemotaxis chamber (**Figure 5c**), indicating that the exogenous

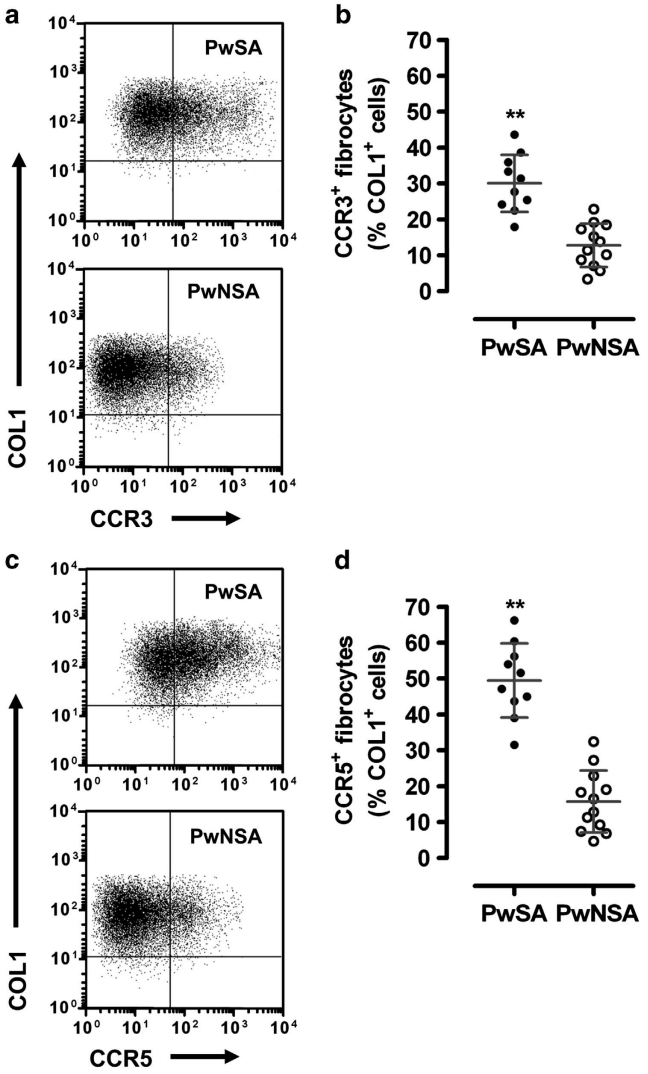

Figure 4 The percentages of circulating fibrocytes that expressed the C-C motif chemokine receptor 3 (CCR3) and CCR5 were significantly higher in patients with severe/treatment-refractory asthma (PwSA) than in patients with non-severe/treatment-responsive asthma (PwNSA). Representative flow cytometry dot plots of the isolated cells double stained with allophycocyanin-labeled anti-type I collagen (COL1) and fluorescein isothiocyanate-labeled anti-CCR3 monoclonal antibodies or with allophycocyanin-labeled anti-COL1 and fluorescein isothiocyanate-labeled anti-CCR5 monoclonal antibodies are presented in panels **a** and **c**, respectively. The horizontal and vertical lines mark fluorescence intensities greater than those observed with the isotype-matched controls. Individual data with the means and s.d. are presented in panels **b** and **d**. $^{**}P<0.01$ compared with PwNSA by the unpaired Student's *t*-test.

proteins elicited an authentic chemotactic effect instead of increasing the random motility of the fibrocytes (chemokinesis). In blocking experiments with specific anti-CCR3 and anti-CCR5 mAbs, the chemotactic response of circulating fibrocytes from PwSA to CCL24 was totally abrogated by inhibiting ligand binding to the CCR3 present on the cell surface, whereas the chemotactic response to CCL5 was substantially attenuated only by inhibiting CCL5 binding to both CCR3 and CCR5 (**Figure 6**). These results demonstrated that CCR3 and CCR5 were both functional receptors. Nonetheless, a residual chemotactic response to CCL5 was still evident after cell treatment with a combination of anti-CCR3 and anti-CCR5 mAbs (**Figure 6**), suggesting that fibrocytes from PwSA expressed an additional CCL5 receptor,[11] possibly CCR1.[4]

The fibrocyte chemotactic activity of the SFP from PwSA was significantly attenuated by neutralization of endogenous CCL5, CCL11, and CCL24

The addition of 50% autologous SFP to the chemotaxis assay medium markedly enhanced the migration of circulating fibrocytes from PwSA over the spontaneous migration observed in presence of the assay medium alone (**Figure 7a**). Conversely, similar amounts of SFP from PwNSA only slightly increased the spontaneous migration of autologous circulating fibrocytes (**Figure 7a**). The migratory response of circulating fibrocytes from PwSA increased in parallel with the increase in the amounts of autologous SFP present in the bottom compartment of the chemotaxis chamber (**Figure 7b**), and the effect was largely attributable to an authentic chemotactic activity of the SFP, as demonstrated by the results of the checkerboard-like analysis presented in **Figure 7c**. The chemotactic activity of the SFP from PwSA was attenuated to various extents by neutralization of endogenous CCL5, CCL11, or CCL24 with specific mAbs (**Figure 8**). In the experiments where the activities of all these chemokines were simultaneously blocked, the chemotaxis index was, on the average, reduced by 53.1%, dropping from a mean value (s.d.) of 4.9 (0.57) after pretreatment of the SFPs with a control IgG to a mean value of 2.3 (0.42) after pretreatment of the SFPs with a combination of all mAbs (**Figure 8**).

DISCUSSION

Airway infiltration with fibrocytes is a characteristic of allergen-exacerbated asthma[5,9] and chronically severe/treatment-refractory disease.[8] Recent studies[7–9,24,26] have uncovered some of the mechanisms that may promote the airway accumulation of fibrocytes in these conditions by demonstrating that the migratory and proliferative responses of circulating fibrocytes from PwSA or patients with allergen-exacerbated asthma to certain stimuli are substantially increased in comparison with those elicited in normal fibrocytes or fibrocytes from PwNSA. Further investigations in this field are warranted because fibrocytes may contribute to the development of the irreversible structural changes[5–9] that lead to fixed airway narrowing and progressive deterioration of lung function in the individuals with unstable and difficult-to-treat asthma.[27–30] This hypothesis is supported by the existence of a significant correlation between the number of circulating COL1-producing fibrocytes and the magnitude of the yearly decline in lung function in PwSA who already exhibit fixed airway narrowing.[7] The present study was addressed to investigate the fibrocytes chemotactic activity of CCR3 and CCR5 ligands that are known eosinophil chemoattractants and are produced in large amounts in the airways of symptomatic patients with chronically severe asthma in spite of treatment with high doses of inhaled corticosteroids and oral corticosteroids, as

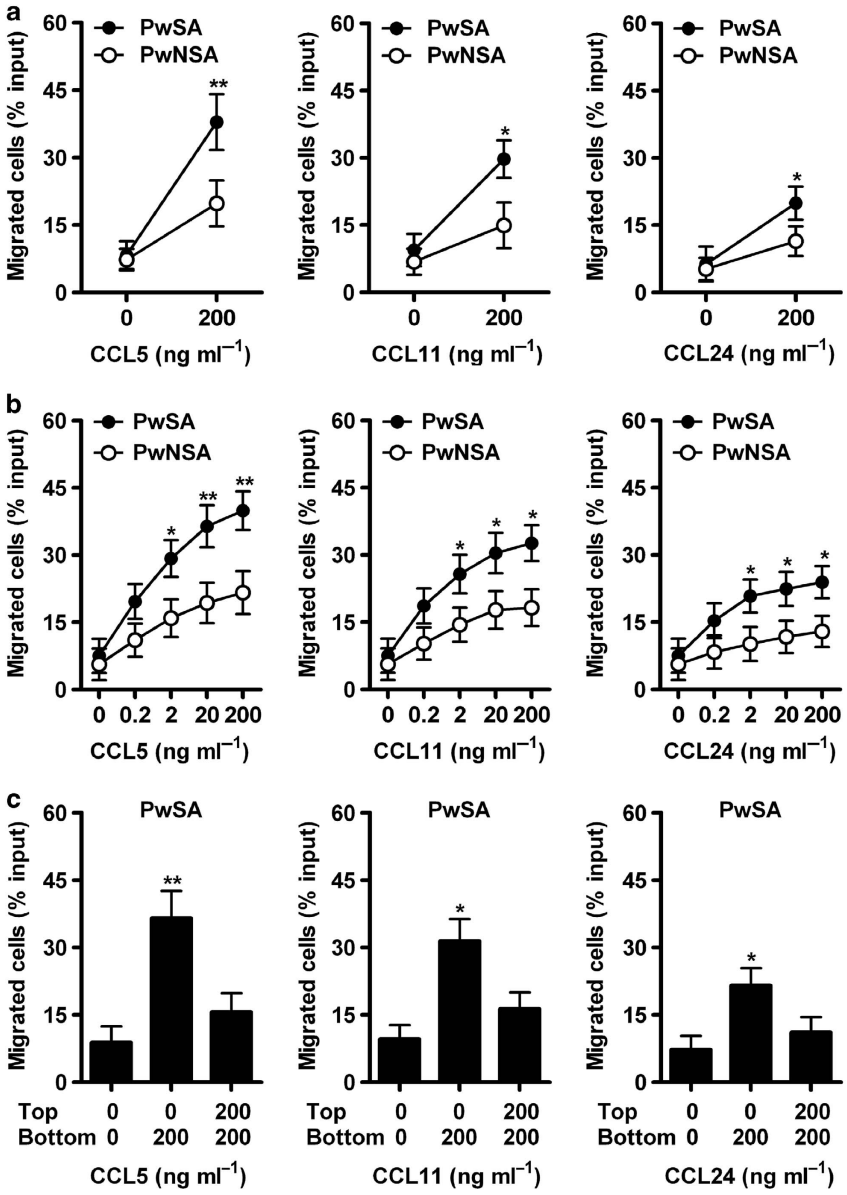

Figure 5 The circulating fibrocytes from patients with severe/treatment-refractory asthma (PwSA) showed enhanced migratory responses to stimulation with the C-C motif chemokine ligand 5 (CCL5), CCL11, and CCL24 in comparison with cells from patients with non-severe/treatment-responsive asthma (PwNSA). (**a**, **b**) Chemotaxis assay with the indicated concentrations of recombinant chemokines in the lower compartment of the chemotaxis chamber. The number of migrated cells under each experimental condition was expressed as percentage of input cells. Data are the means and s.d. (**a**) $^{**}P<0.01$ and $^{*}P<0.05$ compared with the spontaneous migration observed in the absence of chemokines by the paired Student's *t*-test ($n=4$). (**b**) $^{**}P<0.01$ and $^{*}P<0.05$ compared with the spontaneous migration and the chemokine-induced migration of fibrocytes from PwNSA by the two-way analysis of variance followed by the Bonferroni *post-hoc* test for multiple comparisons ($n=4$). (**c**) Checkerboard-like analysis of the chemotactic and chemokinetic effects of recombinant CCL5, CCL11, and CCL24 on circulating fibrocytes from PwSA. The number of migrated cells under each experimental condition was expressed as percentage of input cells. Data are the means and s.d. $^{**}P<0.01$ and $^{*}P<0.05$ compared with the spontaneous migration observed in the absence of chemokines or with the same concentration of each chemokine both in upper and in the lower compartment of the chemotaxis chamber by the analysis of variance followed by Dunnett's or the Tuckey's *post-hoc* test ($n=3$–4). Analysis of the data using the equivalent non-parametric tests provided similar results.

demonstrated for CCL5 and CCL11.[23,31,32] Patients' selection was based on previous observations that symptomatic patients with severe asthma show persistent airway eosinophilia[8,23,33,34] and higher levels of sputum CCL11 than patients with stable non-severe asthma,[23,32] although they receive a more intensive treatment with inhaled and oral corticosteroids. In one of these studies,[32] corticosteroid therapy was associated with reduced sputum concentrations of CCL11 in patients with stable asthma, but a similar association was not observed in patients with unstable disease, who had high amounts of CCL11 in their sputa irrespective of asthma severity and use of oral corticosteroids. The mean sputum contents of CCL11 were 289.0 pg ml^{-1} in patients with stable asthma not receiving corticosteroid therapy, 111.1 pg ml^{-1} in patients with stable

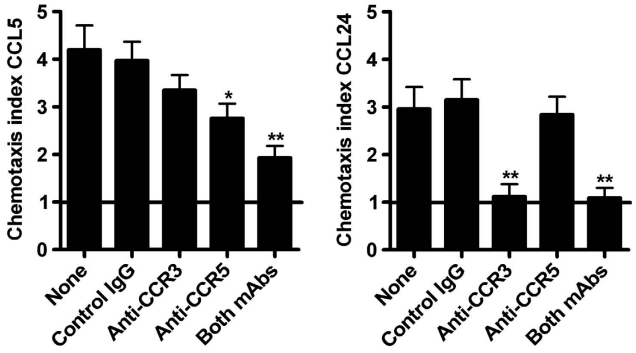

Figure 6 The chemotactic response of circulating fibrocytes from patients with severe/treatment-refractory asthma to the C-C motif chemokine ligand 5 (CCL5) and CCL24 was significantly attenuated or abrogated by inhibiting chemokine binding to the C-C motif chemokine receptor 5 (CCR5) and/or CCR3 on the cell surface with specific monoclonal antibodies (mAbs). The chemotactic index was calculated as the number of cells migrated in response to recombinant proteins divided by the number of cell migrated in response to assay medium alone (spontaneous migration). Data are the means and s.d. The horizontal lines indicate induced migratory responses identical to the spontaneous migration (chemotaxis index = 1). $^{**}P<0.01$ and $^{*}P<0.05$ compared with the chemotaxis index in the absence of blocking mAbs (none) or in the presence of the control immunoglobulin G (IgG) by the analysis of variance followed by Dunnett's or the Tuckey's *post-hoc* test ($n = 3$–4). Analysis of the data using the equivalent non-parametric tests provided similar results.

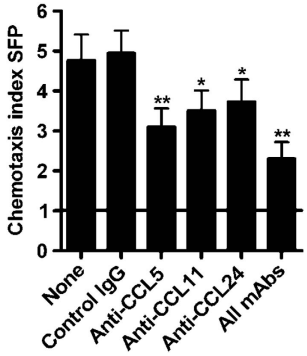

Figure 8 The fibrocytes chemotactic activity of the sputum fluid phase (SFP) from patients with severe/treatment-refractory asthma (PwSA) was significantly attenuated by neutralization of endogenous C-C motif chemokine ligand 5 (CCL5), CCL11, and CCL24 with specific neutralizing monoclonal antibodies (mAbs). The chemotactic index was calculated as the number of cells migrated in response to stimulation with 50% autologous SFP divided by the number of cell migrated in response to assay medium alone (spontaneous migration). Data are the means and s.d. The horizontal lines indicate induced migratory responses identical to the spontaneous migration (chemotaxis index = 1). $^{**}P<0.01$ and $^{*}P<0.05$ compared with the chemotaxis index in the absence of neutralizing mAbs (none) or in the presence of the control immunoglobulin G (IgG) by the analysis of variance followed by Dunnett's or the Tuckey's *post-hoc* test ($n = 5$–6). Analysis of these data using the equivalent non-parametric tests provided similar results.

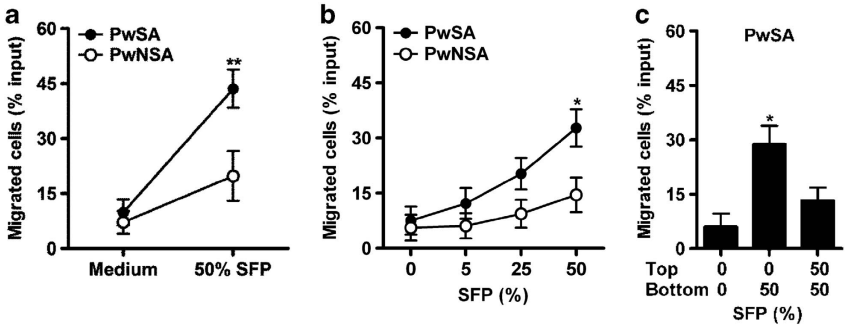

Figure 7 The sputum fluid phase (SFP) of patients with severe/treatment-refractory asthma (PwSA) showed potent chemotactic activity for autologous circulating fibrocytes. (**a**) Comparison of the migratory responses of circulating fibrocytes from PwSA and patients with non-severe/treatment-responsive asthma (PwNSA). The number of cells migrated in response to assay medium alone or medium supplemented with 50% autologous SFP was expressed as percentage of input cells. Data are the means and s.d. $^{**}P<0.01$ vs. medium alone by the paired Student's *t*-test (PwSA, $n = 12$; PwNSA, $n = 15$). (**b**) Migratory response of circulating fibrocytes from PwSA and PwNSA to increasing amounts of autologous SFP. Data are the means and s.d. $^{*}P<0.05$ compared with the spontaneous migration and the SFP-induced migration of fibrocytes from PwNSA by the two-way analysis of variance followed by the Bonferroni *post-hoc* test for multiple comparisons ($n = 4$). (**c**) Checkerboard-like analysis of the chemotactic and chemokinetic effects of autologous SFP on circulating fibrocytes from PwSA. Data are the means and s.d. $^{**}P<0.01$ and $^{*}P<0.05$ compared with the spontaneous migration observed in the absence of SFP or with the same amounts of SFP both in the upper and lower compartments of the chemotaxis chamber by the analysis of variance followed by Dunnett's or the Tuckey's *post-hoc* test ($n = 4$). Analysis of the data using the equivalent non-parametric tests provided similar results.

asthma receiving corticosteroid therapy, 404.7 pg ml^{-1} in patients with unstable asthma not treated with corticosteroids, and 529.9 pg ml^{-1} in patients with unstable asthma receiving corticosteroid therapy. In another study,[31] the sputum concentrations of CCL5 in symptomatic asthmatics receiving corticosteroids were, on the average, twofold higher than in asymptomatic patients not treated with corticosteroids. All these observations indicate that the airway release of CCL11 and related chemokines, like eosinophil accumulation,[8,23,33,34] is not responsive to corticosteroids in severe asthma but also suggest that the lack of asthma control may be a potential confounder when comparing the airway release of CCL11 and related chemokines in severe/treatment-refractory asthma and non-severe/treatment-responsive disease. Therefore, the PwNSA who were selected as controls in the present study were all asymptomatic patients with their disease well-controlled by current treatment.

Sputum induction is a validated non-invasive method to sample soluble factors in asthmatic airways.[35] The procedure adopted in this study for sputum processing was specifically developed for measuring the concentrations of CCL11 and structurally related cytokines that cannot be accurately assayed

in sputa treated with thiol-reducing agents to obtain single-cell suspensions for cell counts.[22] The SFP recovered after precipitation of the mucous and cellular components by centrifugation has been successfully used to test the contribution of CCL11 to the eosinophils chemotactic activity of sputum from patients with moderate and severe asthma.[23] To mimic the *in vivo* condition and avoid the possible effects of prolonged cultivation on the migratory response of fibrocytes,[36] autologous circulating cells directly isolated from the peripheral blood, rather than fibrocytes derived from cultures of PBMCs or PBMC subpopulations,[7,8] were tested in a standard chemotaxis assay.

The novel findings of this study were the following: firstly, the SFP from PwSA, but not that from PwNSA, showed potent chemotactic activity for autologous circulating fibrocytes; secondly, >50% of the fibrocyte chemotactic activity of the SFP from PwSA was attributable to a combination of CCL5, CCL11, and CCL24; thirdly, circulating fibrocyte from PwSA expressed functional receptors for CCR3 and CCR5 ligands at high levels. Such observations may have clinical relevance because elevated numbers of $COL1^+\alpha\text{-}SMA^+$ fibrocytes have been detected in the bronchial subepithelial zone and smooth muscle bundle of PwSA.[8] It is worth noting that the circulating fibrocytes from PwSA expressed increased levels of COL3, a major component of the thickened *lamina reticularis* in asthmatic airways,[37–39] and the contractile protein α-SMA when cultured for 48 h in medium supplemented with 20% autologous SFP. Thus, this study also provides direct evidence that fibrocytes may contribute to the progression of subepithelial fibrosis and expansion of the population of $\alpha\text{-}SMA^+$ cells in severe asthma, as suggested by previous observations.[8] In consideration of the accumulating evidence that circulating fibrocytes function as a renewable source of non-resident bronchial $COL1^+\alpha\text{-}SMA^+$ cells in the asthmatic lung,[5,6,8] it would be more appropriate to use the term (myo)fibrocytes when referring to these cells.

CCL5, CCL11, and CCL24 are all chemokines involved in the airway recruitment of eosinophils in asthma.[10] The response of eosinophils and fibrocytes to these chemoattractants may be different, because the eosinophils express CCR3 but not CCR5.[11] Nonetheless, the findings of this investigation may explain why an increased number of airway fibrocytes is most frequently observed in association with airway eosinophilia in chronically severe/treatment-refractory asthma[8] and during an acute exacerbation of the disease triggered by allergen exposure in non-severe asthma.[9] In a previous study,[14] the early accumulation of eosinophils in the bronchial mucosa of patients with allergen-induced asthmatic responses occurred in concomitance with an increased expression of CCL11 in the airway epithelium. Notably, these patients were the same who also showed a progressive accumulation of fibrocytes in their airways during the same episode of allergen-induced asthma,[5] and the peak increase in CCL11 concentrations in the bronchoalveolar lavage of these patients at 4 h post-allergen inhalation[14] was associated with the first detectable increase in fibrocyte number in the bronchial mucosa.[5] In light of the findings of the present investigation, the existence of a relationship between CCR3 ligands and the concomitant accumulation of eosinophils and fibrocytes in allergen-exacerbated asthma is also indirectly supported by the combined results of previous experiments conducted with the same animal model of human disease in different laboratories. In one study,[5] a progressive increase in the density of $CD34^+COL1^+$ and $CD34^+\alpha\text{-}SMA^+$ fibrocytes was observed in the airway wall of mice subjected to repeated challenges with the allergen to which they had been sensitized. Fibrocyte infiltration of the subepithelial zone occurred in concomitance with airway eosinophilia and was associated with the development of subepithelial fibrosis. In another study,[40] the administration of a CCR3 antagonist in mice similarly subjected to chronic allergen challenge reduced eosinophils infiltration of the airway wall by only 50–60% but markedly attenuated the accumulation of myofibrocytes in the subepithelial zone and prevented the development of subepithelial fibrosis. The involvement of the CCR5 ligand CCL3 in the recruitment of circulating fibrocytes to injured lungs during fibrogenic tissue remodeling has been previously demonstrated in mice,[3] but there is no published information about the role of CCR5 ligands in the recruitment of fibrocytes in asthma or in animal models of human disease.

In conclusion, this study provides the first evidence that an excessive release of biologically active CCL5, CCL11, and CCL24 may promote the airway recruitment of fibrocytes in severe/treatment-refractory asthma. It also demonstrates that human circulating fibrocytes express functional receptors for CCR3 and CCR5 ligands, and this finding may be relevant to other human disorders associated with the infiltration of fibrocytes at tissue sites.

METHODS

Sputum induction and processing. Sputum induction by hypertonic saline was performed according to a standard procedure.[21] Whole expectorated samples were processed to collect the SFP according to a method specifically developed to assay CCL11.[22,23] Each sputum sample was mixed in the ratio of 1:3 with phosphate-buffered saline containing 22.5 μl per ml of a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO), vortexed for 15 s, and placed on a rotor mixer for 30 min on ice. The sample was then filtered and centrifuged at 400 *g* for 10 min. The supernatant was decanted and subjected to sonication and centrifugation at 12,000 *g* for 2 min. Aliquots of the SFP obtained in this way were used for measurement of the concentrations of soluble factors or evaluation of the SFP biological activity in the assays described below.

Quantification of soluble factors in the SFP. ELISA kits from R&D Systems (Minneapolis, MN) were used for the quantification of sputum concentrations of CCL5 and CCL11, while an ELISA kit from Abcam (Cambridge, UK) was used for the quantification of sputum concentrations of CCL24. In preliminary experiments, all standard proteins were tested for recovery of known amounts after spiking into the SFP from five asthmatic individuals. The chemokine recovery was always in the recommended acceptable range,[35] varying from 86% to 102%. The results of the assays were corrected for the recovery rates and original sample dilution. Measurements of the chemokine concentration in the SFP from six healthy individuals provided values in keeping with the data reported in previous studies where sputum

processing was performed in a similar way.[22,31,32] The median contents (interquartile range) of CCL5, CCL11, and CCL24 were 67.8 (97.6) pg ml^{-1}, 51.2 (91.3) pg ml^{-1}, and 41.8 (44.7) pg ml^{-1}, respectively.

Isolation of circulating fibrocytes and phenotypic analysis. The methodology used for the isolation and enumeration of circulating fibrocytes has been described in detail elsewhere[24] and is summarized below. Venous blood samples were collected into heparinized blood collection tubes, and a pure population of CD34$^+$ cells was initially separated from PBMCs by using the Dynal CD34 Progenitor Cell Selection System (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. After detachment of the antibody-coupled magnetic particles, the released CD34$^+$ cells were checked for purity with an allophycocyanin-conjugated CD34 mAb (StemCell Technologies, Vancouver, British Columbia, Canada). Cells were then repeatedly washed and triple-stained with the following mAbs: Pacific Blue-labeled anti-CD45RO (clone UCHL1, BioLegend, San Diego, CA), phycoerythrin-labeled anti-CD11b (clone MEM-174, Abcam), and peridinin chlorophyll protein-eFluor 710-labeled anti-CD13 (clone WM-15, eBioscience, San Diego, CA). The CD34$^+$cells that coexpressed CD45RO, CD11b, and CD13 were sorted on a BD FACSAria II (BD Biosciences) as previously described.[24] Samples of the sorted cell population were used to evaluate the expression of intracellular COL1 and the surface expression of CCR3 and CCR5 by flow cytometry. For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm kit (BD Biosciences) and either sequentially incubated with rat anti-COL1A1 (clone M58, Chemicon International, Temecula, CA) and a fluorescein isothiocyanate (FITC)-labeled goat anti-rat secondary Ab (Invitrogen) or directly stained with a mouse anti-COL1 (LS-C79516, LifeSpan BioSciences, Seattle, WA) after mAb labeling with EasyLink FITC Conjugation Kit (Abcam) or LNK031APC (AbD Serotec/Morphosys AbD, Düsseldorf, Germany). The mAbs used to evaluate the expression of CCR3 and CCR5 on the cell surface were a FITC-labeled anti-CCR3 (clone 444-11) and a FITC-labeled anti-CCR5 (clone T227) from MBL International (Woburn, MA). Analysis of the cells stained with these Abs or the appropriate controls was performed with a FACSCalibur flow cytometry system (BD Biosciences) as previously described.[9,24,41]

To further confirm the nature of the cells isolated from the peripheral blood of PwSA and PwNSA, their ability to express α-SMA and COL3 was also evaluated. Aliquots of the freshly isolated cells were resuspended in culture medium (Dulbecco's Modified Eagle's Medium containing 2 mM L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, 100 U ml^{-1} penicillin, and 0.1 mg ml^{-1} streptomycin) supplemented with either 20% autologous serum or 20% autologous SFP. The cells were seeded into the wells of standard 96-well culture plates (2×10^4 cells per well) and incubated for 12–72 h in a humidified atmosphere (5% CO_2 in air). The expression of α-SMA and COL3 was then examined by western blot analysis of cell lysates as previously described.[9,42] The mAb against α-SMA and β-tubulin, the loading control, were purchased from Sigma-Aldrich and Abcam, respectively.[9] The polyclonal Ab against COL3A1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).[42] Densitometric analysis was performed with the ImageQuanta TL (Amersham Biosciences, Piscataway, NJ), using the loading control for signal normalization and quantification of the relative densitometric units.

Chemotaxis assay. The chemotactic activity of the SFP or recombinant CCL5, CCL11, and CCL24 (R&D Systems) was evaluated in a fluorescence-based, modified Boyden chamber assay, using an uncoated 8-μm pore-size insert membrane (QCM Chemotaxis 96-Well Cell Migration Assay, Chemicon International). Assay medium alone (Hank's balanced salt solution containing 25 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 120 mM NaCl, and 0.5% bovine serum albumin) and medium supplemented with 5–50% SFP or recombinant proteins were separately placed into the bottom compartments of the chemotaxis chamber (150 μl per well) and preincubated for 30 min at 37 °C. The fibrocytes were then placed into the upper compartment of the chamber (3×10^4 cells in 100 μl assay medium per well) and allowed to migrate for 3 h at 37 °C in a humidified incubator (5% CO_2 in air). The migratory cells on the bottom of the insert membrane were dissociated from the membrane, lysed, and stained with a fluorescent dye. Fluorescence was read with a fluorescence plate reader and the number of migrated cells was estimated on the basis of the standard curve obtained in preliminary experiments by using stained lysates of known numbers of fibrocytes. Dose–response curves and checkerboard-like analyses were conducted according to standard methods.[42]

CCR3 and CCR5 blockage. In blocking experiments, the fibrocytes were preincubated for 30 min with medium containing 10 μg per ml of the anti-CCR3 mAb (clone 444-11, MBL International) or the anti-CCR5 mAb (Pharmingen clone 2D7, BD Biosciences) used in a previous study[43] or with the equivalent concentration of an irrelevant mAb (Clone 20102, R&D Systems). The cell suspension was subjected to gentle rotation on a rocker platform to prevent fibrocyte attachment. The chemotaxis assay was then performed as described above, in the continuing presence of each mAb in the upper compartment of the chemotaxis chamber.

Neutralization of endogenous chemokines. To evaluate the contribution of endogenous CCL5, CCL11, and CCL24 to the chemotactic activity of the SFP, 8–20 μg per ml of specific neutralizing mAbs (all mouse IgG_1, clones 16411, 43911, and 61016 from R&D Systems) were added singly or in combination to separate aliquots of each SFP 60 min before the chemotaxis assay. An irrelevant mouse IgG_1 (R&D Systems) was used at equivalent concentrations as control. The chemotaxis assay was then repeated as described above.

Statistical analysis. Statistical analysis was performed with GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA).

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DISCLOSURE

Sabrina Mattoli is a founding shareholder and board member of AVAIL GmbH. She is named as inventor in patent applications concerning improvements of asthma treatment. The other authors declared no conflict of interest.

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