Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis

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Previous reports have identified a circulating pool of CD45+ collagen I+ CXCR4+ (CD45+Col I+CXCR4+) cells, termed fibrocytes, that traffic to areas of fibrosis. No studies have demonstrated that these cells actually contribute to fibrosis, however. Pulmonary fibrosis was originally thought to be mediated solely by resident lung fibroblasts. Here we show that a population of human CD45+Col I+CXCR4+ circulating fibrocytes migrates in response to CXCL12 and traffics to the lungs in a murine model of bleomycin-induced pulmonary fibrosis. Next, we demonstrated that murine CD45+Col I+CXCR4+ fibrocytes also traffic to the lungs in response to a bleomycin challenge. Maximal intrapulmonary recruitment of CD45+Col I+CXCR4+ fibrocytes directly correlated with increased collagen deposition in the lungs. Treatment of bleomycin-exposed animals with specific neutralizing anti-CXCL12 Ab’s inhibited intrapulmonary recruitment of CD45+Col I+CXCR4+ circulating fibrocytes and attenuated lung fibrosis. Thus, our results demonstrate, we believe for the first time, that circulating fibrocytes contribute to the pathogenesis of pulmonary fibrosis.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and often fatal pulmonary disorder with a survival rate as low as 30% 5 years after diagnosis (1, 2). The prevalence of this disease increases with age — with an incidence as high as 250 cases per 100,000 in the elderly (2, 3). There also appears to be an increase in the incidence of the disease in many Western countries, including the United Kingdom, New Zealand, and Germany (3). Conventional approaches to treating IPF have focused on immunosuppressive therapy, predominantly steroid usage, which has yielded poor results. The pathogenesis of pulmonary fibrosis is characterized by dysregulated and inappropriate tissue remodeling and enhanced vascular remodeling, fibroproliferation, and deposition of ECM (4). Without resolution, continued fibrosis leads to loss of lung function and ultimately death.

Fibrocytes present in the peripheral circulation were first identified a decade ago (5). These cells comprised a minor component of the circulating pool of leukocytes (less than 1%) and express a characteristic pattern of markers, including collagen I (Col I) and CD45 (5). When cultured in vitro, these cells became adherent and developed a spindle-shaped morphology (5). Subsequent studies have revealed that these circulating fibrocytes express chemokine receptors such as CXCR4 and CCR7 (6). Furthermore, circulating fibrocytes have been shown to traffic to wounds (6). They express ECM proteins, such as pro-Col I and pro–Coll III, which may be involved in connective tissue formation (6–8). No study has actually demonstrated that fibrocytes contribute to deposition of ECM and the promotion of fibrosis in vivo, however.

Tissue fibroblasts have classically been held to be responsible for repair and remodeling within the lung, although this view has been challenged since those fibroblasts observed in the fibrotic lung show phenotypic differences to their counterparts in the lungs of healthy individuals (9, 10). Thus, it is possible that, in common with their perceived role in fibrosis and wound repair, circulating fibrocytes may also be recruited to injured lungs as an integral component of the pathogenesis of pulmonary fibrosis.

Results

Human fibrocytes express CD45, Col I, and CXCR4 and migrate in response to CXCL12. To dissect the role of fibrocytes in pulmonary fibrosis, we initially isolated and characterized a population of fibrocytes from human peripheral blood (Figure 1 and Table 1). Although these cells are a minor component of the PBMC pool, by using a technique of immunodepletion and culturing cells in vitro, we were able to purify a population of long, spindle-shaped cells that morphologically resemble fibrocytes (6). We began our characterization of these purified fibrocytes by using flow cytometry to assess changes in the cell surface phenotype of cultured fibrocytes similar to those described above (6, 11). By contrast, Col I expression remained elevated at all time points (28–35 days after cell isolation) there was an approximate threefold reduction in the expression of CD45 by fibrocytes (Table 1). Our results show that these newly purified fibrocytes expressed high levels of CD45, CD34, and Col I, and α-smooth muscle actin (α-SMA) as the cells matured in culture (Table 1).

Nonstandard abbreviations used: bronchoalveolar lavage fluid (BAL); collagen I (Col I); idiopathic pulmonary fibrosis (IPF); phycoerythrin (PE); α-smooth muscle actin (α-SMA).

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 114:438–446 (2004). doi:10.1172/JCI200420997.
cultured for 3 weeks (28–35 days after their initial isolation) were found to express intracellular Col I (Figure 1A), cell surface CXCR4 (Figure 1A), and α-SMA (Figure 1A).

Next, we triple-stained newly purified fibrocytes with Col I, CD45, and CXCR4 and analyzed them by flow cytometry (Figure 1B). Initially, we gated CD45+ fibrocytes and then examined the resulting population for dual expression of CXCR4 and Col I. In this way, we identified a major population of CD45+Col I+CXCR4+ human fibrocytes and a smaller population of CD45+Col I+CXCR4− fibrocytes (Figure 1B). To determine whether the expression of CXCR4 on the surface of purified fibrocytes was functional, we performed chemotaxis assays to look for specific migration in response to CXCL12. We observed significant chemotaxis at both 30 and 100 ng/ml CXCL12 (Figure 1C), indicating that CD45+Col I+CXCR4+ fibrocytes undergo directed movement in the presence of a chemotactic gradient in vitro.

Having established that cultured human fibrocytes can migrate in response to CXCL12 in vitro, we wanted to examine whether these fibrocytes could migrate in vivo and, in particular, whether they could specifically migrate into lungs undergoing a fibrotic response. To test this possibility we used a murine model of bleomycin-induced pulmonary fibrosis. Initially, cohorts of mice were treated with either bleomycin, saline, or nothing (naive mouse, day 0) and then sacrificed at day 1, 2, 4, 8, 16, and 20 to look for evidence of fibrosis. Exuberant collagen deposition within the lung is a key marker of fibrosis (9); on this basis we examined changes in pro-Col I and pro-Col III gene expression using real-time quantitative RT-PCR (Figure 2, A and B). Our data revealed that transcription of both pro–Col III and pro-Col I was dramatically upregulated in mice exposed to bleomycin (up to 30-fold higher) as compared with mice treated with saline alone (Figure 2, A and B). To confirm that collagen gene expression was translated into protein, we next measured total collagen deposition in the lung by the Sircol assay (13). We observed that the level of total collagen deposition was significantly higher (as compared with mice treated with saline alone). These results suggest that a circulating pool of human fibrocytes exist that specifically migrate in response to CXCR4/ CXCL12 and traffic to fibrotic lungs.

A kinetic analysis of the onset of fibrosis reveals dramatic collagen deposition beginning 4 days after bleomycin exposure. To more fully elucidate the role of fibrocytes in the development of fibroproliferative disorders of the lung, we focused our studies on the murine model of bleomycin-induced pulmonary fibrosis (12). Initially, cohorts of mice were treated with either bleomycin, saline, or nothing (naive mouse, day 0) and then sacrificed at day 1, 2, 4, 8, 16, and 20 to look for evidence of fibrosis. Exuberant collagen deposition within the lung is a key marker of fibrosis (9); on this basis we examined changes in pro-Col I and pro-Col III gene expression using real-time quantitative RT-PCR (Figure 2, A and B). Our data revealed that transcription of both pro–Col III and pro-Col I was dramatically upregulated in mice exposed to bleomycin (up to 30-fold higher) as compared with mice treated with saline alone (Figure 2, A and B). To confirm that collagen gene expression was translated into protein, we next measured total collagen deposition in the lung by the Sircol assay (13). We observed that the level of total collagen deposition was significantly higher (as compared with mice treated with saline alone). These results suggest that a circulating pool of human fibrocytes exist that specifically migrate in response to CXCR4/ CXCL12 and traffic to fibrotic lungs.

Table 1

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collagen protein increased dramatically in bleomycin-treated mice, especially between days 4 and 20 (Figure 2C). The levels of collagen found in saline-treated mice increased slightly in the first 2 days and then decreased to the levels observed in the naive mouse (day 0; Figure 2C). Thus, this data reveals that 4 days after mice have been exposed to bleomycin there is a significant increase in both collagen gene expression and protein deposition, which is indicative of the onset of fibrosis.

**CD45**^+^**Col I**^+^**CXCR4**^+^ fibrocytes accumulate in the lungs of bleomycin-treated animals. To characterize the role of murine **CD45**^+^**Col I**^+^**CXCR4**^+^ fibrocytes in our mouse model of bleomycin-induced pulmonary fibrosis, we isolated cells from the lungs and blood buffy coats of mice that had been exposed to bleomycin or saline for 1, 2, 4, 8, 16, and 20 days, triple-stained for **CD45**, **Col I**, and **CXCR4**, and examined them by flow cytometry (Figure 3A). Our results reveal a dramatic increase in the number of infiltrating **CD45**^+^**Col I**^+^**CXCR4**^+^ fibrocytes found in the lungs of mice exposed to bleomycin. Increased expression of **CD45**^+^**Col I**^+^**CXCR4**^+^ cells began to appear 2 days after bleomycin treatment, became maximal at day 8, and remained elevated at days 16 and 20. Expression of **CD45**^+^**Col I**^+^**CXCR4**^+^ cells in saline-treated mice also increased initially before returning to the levels observed in the naive mouse (day 0) by days 16 and 20 (Figure 3A). The most likely explanation for this latter phenomenon is that intratracheal instillation of saline itself is not a normal event and could have promoted an inflammatory response. In contrast to the time-dependent accumulation of murine fibrocytes in the lungs of bleomycin-exposed animals, the steady-state levels of circulating **CD45**^+^**Col I**^+^**CXCR4**^+^ fibrocytes in the blood remained similar in both groups (Figure 3A).

Previous studies have reported that fibrocytes can express **CCR7** in addition to **CXCR4** (6), and we have now identified a second fibrocyte population that is **CD45**^+^**Col I**^+^**CCR7** (Figure 3B and data not shown). These **CCR7**^+^ fibrocytes also traffic to the lungs of bleomycin-treated mice (Figure 3B); however, the absolute number of **CCR7**^+^ fibrocytes found in the fibrotic lung is twofold to threefold lower than the number of **CXCR4**^+^ fibrocytes present under similar conditions (i.e., compare A and B in Figure 3). Thus, these data suggest that although two chemotactic chemokine receptors are present on the surface of fibrocytes, **CXCR4** appears predominant for the recruitment of fibrocytes to fibrotic lungs.

We have already shown that human **CD45**^+^**Col I**^+^**CXCR4**^+^ fibrocytes traffic through the bloodstream and are recruited into the lungs of bleomycin-treated animals (Figure 1D), and this suggests that murine **CD45**^+^**Col I**^+^**CXCR4**^+^ fibrocytes may be recruited in a similar manner. There appears to be insufficient peripheral blood fibrocytes present to drive the dramatic intrapulmonary recruitment of progenitor cells (Figure 3A), however. One explanation to account for this disparity is that the circulating pool of fibrocytes is continually replenished from the bone marrow. In support of this notion, recent work has suggested that mesenchymal stem cells originating from the bone marrow traffic to organizing alveolitis in pulmonary irradiated bone marrow chimeric mice (14). To formally test this hypothesis, therefore, bone marrow was recovered from mice treated with bleomycin or saline for 8 days, triple-stained for **CD45**, **Col I**, and **CXCR4**, and analyzed by flow cytometry (Figure 3C). Our results clearly indicate a dramatic increase in the number of **CD45**^+^**Col I**^+^**CXCR4**^+^ fibrocytes found in the bone marrow of bleomycin-challenged animals in comparison with the normal saline control mice. These data therefore suggest that bone marrow is at least one source of **CD45**^+^**Col I**^+^**CXCR4**^+^ circulating fibrocytes and that fibrocyte numbers in the bone marrow appear to be higher in mice treated with bleomycin.

For **CD45**^+^**Col I**^+^**CXCR4**^+^ fibrocytes to enter the lungs from the peripheral circulation, the cells must be actively recruited. One likely mechanism for achieving this is the chemokine receptor **CXCR4** and its cognate ligand **CXCL12**, which has been shown to regulate cellular migration in other systems (6, 15, 16). To establish whether indeed a **CXCL12** gradient exists between the lungs and the peripheral blood, lung homogenates were prepared and the levels of **CXCL12** protein determined by ELISA (Figure 3D). Whereas plasma steady-state levels of **CXCL12** protein in lungs of mice exposed to intratracheal bleomycin compared with saline control as determined by real-time quantitative PCR. **n** = 6 lungs in each group. Data represent the mean ± SEM. *P < 0.05, significant differences between bleomycin and saline groups. (C) Kinetics of total collagen protein deposition in lungs of mice exposed to either intratracheal bleomycin, saline, or naive (time 0) control as determined by the Sircol assay. **n** = 6 lungs in each group. Data represent the mean ± SEM. *P < 0.05, significant differences between bleomycin and saline groups.
they decreased by day 20, again suggesting that the initial instillation of saline promoted an inflammatory response. Thus, these data support the notion that a CXCL12 gradient exists between the lungs and plasma of bleomycin-treated mice, which could promote the recruitment of CD45+ fibrocytes and correlates with collagen deposition in the lungs of bleomycin-exposed mice. (A and B) Single-cell suspensions were isolated from bleomycin- or saline-challenged lungs and blood buffy coats at the times indicated, triple-stained for CD45, Col I, and CXCR4 (A), or CD45, Col I, and CCR7 (lungs only) (B), and then examined by FACS analysis. n = 6 samples per group. BC, buffy coat. Data represent the mean ± SEM. *P < 0.05, significant differences between bleomycin and saline groups. **P < 0.05, significant differences between saline-exposed mice and the naive mice. (C) Mice were treated with either intratracheal bleomycin or saline for 8 days. Bone marrow was removed, triple-stained for CD45, Col I, and CXCR4, and then examined by FACS analysis. n = 3 samples per group. Data represent the mean ± SEM. *P < 0.05. (D) Kinetics of CXCL12 protein expression in lung tissue and plasma of mice exposed to either intratracheal bleomycin, saline, or naive control (day 0) as determined by ELISA. n = 6 samples in each group. Data represents the mean ± SEM. *P < 0.05, significant differences between bleomycin and saline groups. **P < 0.05, significant differences between saline-exposed mice and the naive mice.

Figure 3
Intrapulmonary recruitment of CD45+ Col I+ CXCR4+ fibrocytes is greater than CD45+Col I+ CCR7+ fibrocytes and correlates with collagen deposition in the lungs of bleomycin-exposed mice. (A and B) Single-cell suspensions were isolated from bleomycin- or saline-challenged lungs and blood buffy coats at the times indicated, triple-stained for CD45, Col I, and CXCR4 (A), or CD45, Col I, and CCR7 (lungs only) (B), and then examined by FACS analysis. n = 6 samples per group. BC, buffy coat. Data represent the mean ± SEM. *P < 0.05, significant differences between bleomycin and saline groups. **P < 0.05, significant differences between saline-exposed mice and the naive mice. (C) Mice were treated with either intratracheal bleomycin or saline for 8 days. Bone marrow was removed, triple-stained for CD45, Col I, and CXCR4, and then examined by FACS analysis. n = 3 samples per group. Data represent the mean ± SEM. *P < 0.05. (D) Kinetics of CXCL12 protein expression in lung tissue and plasma of mice exposed to either intratracheal bleomycin, saline, or naive control (day 0) as determined by ELISA. n = 6 samples in each group. Data represents the mean ± SEM. *P < 0.05, significant differences between bleomycin and saline groups. **P < 0.05, significant differences between saline-exposed mice and the naive mice.

The Journal of Clinical Investigation http://www.jci.org Volume 114 Number 3 August 2004

with those mice receiving bleomycin and control Ab’s (Figure 4B). The addition of neutralizing CXCL12 Ab’s to bleomycin-treated lungs did not, however, completely attenuate collagen deposition to the level of the saline control (Figure 4B). Taken together, therefore, these data indicate that inhibition of the CXCR4/CXCL12 chemotactic axis reduces intrapulmonary recruitment of CD45+ Col I+ CXCR4+ fibrocytes and significantly abrogates lung fibrosis in bleomycin-exposed mice.

To ascertain whether the reduction in collagen deposition was truly a reflection of fewer infiltrating CD45+ Col I+ CXCR4+ fibrocytes, rather than as a result of generalized migratory inhibition of other circulating CXCR4+PBMCs, we also examined changes in the recruitment of a panel of CXCR4+ cells to bleomycin-exposed lungs treated with anti-CXCL12 Ab’s (Figure 4C). Thus, mice were treated with bleomycin and given daily injections of either anti-CXCL12 or control Ab’s for 8 days. Thereafter, cells were isolated from bleomycin-exposed lungs treated with either anti-CXCL12 or control Ab’s and stained with a panel of markers (CD3, CD4, CD8, NK1.1, Ly6, and Mac) prior to analysis by flow cytometry. Our results show that there was no statistical difference in the numbers of CD4+ (CD4) and CD8+ (CD8) T cells, NK cells (NK1.1),

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Figure 4
Neutralizing anti-CXCL12 Ab's inhibit intrapulmonary recruitment of CD45^+Col I^+CXCR4^+ fibrocytes and significantly attenuate lung fibrosis in bleomycin-treated mice. (A) Single-cell suspensions were isolated from the lungs of naive or saline- or bleomycin-exposed mice, where the bleomycin-treated mice also received daily injections of either anti-CXCL12 Ab or control Ab for 16 days. These cells were then triple-stained for CD45, Col I, and CXCR4 and analyzed by flow cytometry. n = 5 samples per group. Data represent the mean ± SEM. *P < 0.05. (B) Total collagen present in lung tissue of naive, saline-, or bleomycin-exposed mice, where the bleomycin-treated mice also received daily injections of either anti-CXCL12 Ab or control Ab for 16 days. Total collagen levels were determined by the Sircol assay. n = 4 lungs in each group. Data represent the mean ± SEM. *P < 0.05. (C) Single-cell suspensions were isolated from the lungs of bleomycin-exposed mice treated with either daily injections of anti-CXCL12 or control Ab's for 8 days. These cells were then individually stained for CD3, CD4, CD8, NK1.1, Ly6, and Mac519 (Mac) and analyzed by flow cytometry. n = 3 lungs per group.

Figure 5C) Thus, by blocking the recruitment of CD45^+Col I^+CXCR4^+ fibrocytes to bleomycin-exposed lungs, expression of α-SMA was also reduced. This suggests that CD45^+Col I^+CXCR4^+ fibrocytes recruited from the peripheral circulation may ultimately develop an α-SMA^+ phenotype in fibrotic lungs, which is compatible with our in vitro findings for human fibrocytes (Figure 1A and Table 1).

Discussion
This study provides, to our knowledge, the first evidence to show that circulating CD45^+Col I^+CXCR4^+ fibrocytes contribute to the pathogenesis of pulmonary fibrosis. Initially, we identified circulating pools of both human and murine CD45^+Col I^+CXCR4^+ fibrocytes, which traffic to the lungs during pulmonary fibrosis. In the murine model of bleomycin-induced pulmonary fibrosis, exuberant collagen deposition, a key component of IPF, is observed between 4 and 20 days after bleomycin exposure. Day 8 also represents the peak period of CD45^+Col I^+CXCR4^+ fibrocyte infiltration. A CXCL12 gradient exists between the lungs and plasma of bleomycin-treated mice, which we inhibited by the addition of specific neutralizing anti-CXCL12 Ab's. Under these conditions, the trafficking of CD45^+Col I^+CXCR4^+ fibrocytes into the lungs was reduced and pulmonary fibrosis attenuated.

CXCR4 is one of about 20 chemokine receptors that have been cloned so far (17, 20–22), while more than 40 chemokines have also been described (17, 20–22). CXCL12 is the only chemokine known to bind to CXCR4, and the interplay between this receptor/ligand pair enables cellular migration to be exquisitely regulated. Although earlier studies provided conflicting results regarding the ability of CXCR4^+ fibrocytes to migrate in response to CXCL12 in vitro, this may be explained by the fact that different methodologies were performed (6). In our assay, we counted fibrocytes that had migrated across the membrane and were adhered to the underside, while in the study of Abe and colleagues (6), only those cells that had fallen through and collected in the lower chamber were deemed to have migrated. Furthermore, we performed our chemotaxis assays in media containing 10% FCS, rather than 1% BSA, and possibly the most important factor is that we incubated our cells for 6 hours in the presence of CXCL12 rather than the 3 hours used by Abe et al. (6). In addition, recent evidence has indicated that in vitro bone marrow–derived lung CXCR4^+ cells can migrate in response to CXCL12 (23). Moreover, our in vivo studies strongly support the notion that the systemic addition of specific neutralizing anti-CXCL12 Ab's reduce intrapulmonary recruitment of CD45^+Col I^+CXCR4^+ fibrocytes and significantly attenuate lung fibrosis.

Lung fibrosis is not completely abolished by treatment with anti-CXCL12 Ab's, however. Thus, it is possible that other mechanisms could be involved in the intrapulmonary recruitment of fibrocytes and the associated development of lung fibrosis. In this respect, CCR7 is another chemokine receptor that has been shown to be expressed on fibrocytes and the associated development of lung fibrosis. In this respect, CCR7 is another chemokine receptor that has been shown to be expressed on fibrocytes and the associated development of lung fibrosis. In this respect, CCR7 is another chemokine receptor that has been shown to be expressed on fibrocytes and the associated development of lung fibrosis. In this respect, CCR7 is another chemokine receptor that has been shown to be expressed on fibrocytes and the associated development of lung fibrosis.
shown to be important in the regulated movement of fibrocytes (6). Indeed, flow-cytometric analysis of our purified human fibrocytes indicated that at least two phenotypically distinct fibrocyte populations (CD45+Col1CXCR4+ and CD45–Col1CXCR4–) were present. Thus it is possible that CD45+Col1CXCR4+ fibrocytes actually express CCR7 and respond to its cognate ligands CCL19 and CCL21. To that end, we have identified a second population of fibrocytes in our mouse model that expresses CD45, Col1, and CCR7, and that also traffics to the lungs of bleomycin-exposed animals. Furthermore, it is possible that our neutralizing CXCL12 Ab was not completely effective in perturbing the putative CXCL12 chemotactic gradient that we believe develops between the lungs and blood plasma during bleomycin exposure and that recruits CD45+Col1CXCR4+ fibrocytes to the lungs during the onset of pulmonary fibrosis. Thus, in future studies we hope to design appropriate strategies to more fully attenuate CXCR4 and CCR7 biology and subsequently to determine whether this has an additive effect on abrogating the fibrictic response in our mouse model of bleomycin-induced pulmonary fibrosis.

In addition, several different fibroblast phenotypes, including Thy-1+, Thy-1+, cyclooxygenase1β, and telomerase+, have been shown to be present at the site of evolving pulmonary fibrosis (10, 24–30). Although it would appear that these fibroblast phenotypes may contribute to the deposition of ECM components during lung fibrosis, it is not clear whether the origin of Thy-1+, Thy-1+, cyclooxygenase1β, and telomerase+ fibroblasts is from an intrapulmonary or extrapulmonary cellular source. If these fibroblast phenotypes originate from circulating CXCR4+ fibrocytes, it would be expected that neutralizing CXCL12 Ab’s would block both the recruitment of Thy-1+, Thy-1+, cyclooxygenase1β, and telomerase+ fibroblasts and attenuate their ability to secrete ECM components. Alternatively, if Thy-1+, Thy-1+, cyclooxygenase1β, and telomerase+ fibroblasts originate from a cellular source other than circulating CXCR4+ fibrocytes, then their role in mediating pulmonary fibrosis would not be impaired under conditions of blocking the CXCL12/CXCR4 biological axis. Thus, collagen deposition mediated by these additional fibroblast phenotypes could further explain why the reduction in collagen expression observed in bleomycin-exposed mice treated with anti-CXCL12 Ab’s failed to reach the levels of the saline or naïve control. Other cell types, including pericytes, have also been postulated to play a role in the onset of fibrosis in the liver and dermis (31–33), although there is currently no evidence to suggest that these cells are an important source of collagen deposition in pulmonary fibrosis.

A key marker of IPF is the dramatic rise in both collagen gene expression and deposition of collagen protein within the lungs. In our murine model of bleomycin-induced pulmonary fibrosis, increased expression of both pro-Col I and pro–Col III was observed beginning 4 days after instillation of bleomycin. In addition, collagen deposition was clearly detectable in lungs within 4 days of bleomycin exposure. Morphometric analysis of picrosirius red staining of lung sections at day 16 after bleomycin exposure further verified the presence of pulmonary collagen deposition. This collagen deposition was strongly attenuated in bleomycin-exposed mice treated with daily injections of neutralizing anti-CXCL12 Ab’s. The magnitude of collagen reduction we observed here was similar to that observed in several other cytokine neutralization studies (34–39).

We measured total collagen deposition using the Sircol assay, which we felt was actually more reproducible and reliable than the hydroxyproline assay (our unpublished observations). The kinetics of collagen deposition we report here are in keeping with results found in other published work, where significant collagen deposition was detectable within 3 days of bleomycin treatment using the hydroxyproline assay (34). In addition, the concept that collagen is detectable very early in the inflammatory process is consistent with several studies that have shown markedly elevated levels of collagen during acute lung injury in patients (40–42). Indeed, increased collagen levels in the bronchoalveolar lavage (BAL) fluid of patients suffering from acute respiratory distress syndrome were detectable 3 days after the onset of the disease (40). Moreover, early detection of collagen in BAL fluid in these patients was strongly associated with an increased risk of mortality (40). This does not mean, however, that we will ultimately see fibrosis in these patients, especially in light of the magnitude of counterbalancing measures such as proteinases present at the same time, which would lead to collagen degradation, remodeling of collagen deposition, and resolution. This concept would be analogous to the saline control–treated lungs. In contrast, under conditions of bleomycin-induced pulmonary fibrosis, we speculate that the previously published find-
ings of elevated TGF-β in this model contributes to tipping the balance in favor of increased collagen production and at the same time decreased collagen degradation, which would result in more fibrosis. Therefore, although elevated numbers of fibrocytes may be present under conditions of saline exposure, as compared with naïve mice there is no marked accumulation of collagen.

We observed significant recruitment of CD45+ Col I+CXCR4+ fibrocytes into the fibrotic lung as early as 2 days after bleomycin exposure and 2 days before significant collagen deposition was detected, further suggesting that CD45+ Col I+CXCR4+ fibrocytes may be involved in the onset of fibrosis. Maximal recruitment of CD45+ Col I+CXCR4+ fibrocytes was observed 8 days after the instillation of bleomycin, and by days 16 and 20 the number of infiltrating CD45+ Col I+CXCR4+ fibrocytes appeared to decrease. The fate of these cells remains to be determined, however. Indeed, the apparent loss of CD45+ Col I+CXCR4+ fibrocytes from fibrotic lungs, may, in fact, reflect a change in the phenotype of these cells. Myofibroblasts (CD45+ Col I+α-SMA+), which have been found in pulmonary fibrotic lesions but not in normal lungs (9), is one potential differentiation phenotype for fibrocytes. Although it has been suggested that myofibroblasts arise from tissue fibroblasts within the lung itself (30, 43), the true origin of these cells has yet to be definitively established (9). In addition, myofibroblasts are known to be a major source of pro-Col I expression in pulmonary fibrotic lesions (9, 44), and we have clearly demonstrated that in the presence of anti-CXCL12 Ab’s collagen deposition is significantly reduced in bleomycin-exposed mice. Further support for the notion that circulating fibrocytes may ultimately differentiate into myofibroblasts within the lungs stems from our observations and other recent reports (6, 11) that cultured human fibrocytes eventually lose expression of CD34 and CD45 and upregulate α-SMA, which is consistent with the known myofibroblast phenotype (CD34−CD45+ Col I+α-SMA+) (9).

Another factor that may determine the fate of CD45+ Col I+CXCR4+ fibrocytes during pulmonary fibrosis is apoptosis. The inflammatory environment associated with the onset of fibrosis typically contributes extensively to the programmed cell death of injured cells, including those of the respiratory epithelium and infiltrating white blood cells. Thus, it is important for cells migrating into the lungs to have in place a mechanism to survive the apoptotic landscape. In this respect, CXCR4+ fibrocytes may be ideally situated to avoid programmed cell death since ligation of CXCR4 by CXCL12 has been shown to regulate the cell death machinery through downstream activation of the PI3K, AKT, and MAP kinase pathways (45–49).

In conclusion, we have shown, we believe for the first time, that CD45+ Col I+CXCR4+ circulating fibrocytes contribute to the pathogenesis of pulmonary fibrosis. These cells, therefore, offer a novel therapeutic target for developing more effective treatments against this devastating disease.

Methods
Animal model of pulmonary fibrosis. Female C57Bl/6 mice (6–8 weeks old) purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) were anesthetized with ketamine and given intratracheal injections of either 0.025 U of bleomycin (0.15 U/kg Blenoxane; Sigma-Aldrich, St. Louis, Missouri, USA) or sterile saline. Mice were sacrificed for analysis 1, 2, 4, 8, 16, and 20 days after exposure to bleomycin. In separate experiments, mice treated with bleomycin were given daily injections (500 μl) of 10 mg/ml F(ab’2); fragments of either neutralizing goat anti-CXCL12 Ab or control Ab’s for 8 or 16 days prior to sacrifice. Animal use for these studies was approved by the Department of Laboratory Animal Medicine, David Geffen School of Medicine at UCLA.

Lung tissue preparation for protein assays. Bleomycin- or saline control–treated lungs were homogenized and sonicated in complete buffer (Roche Diagnostics Corp., Indianapolis, Indiana, USA) as previously described (41). Homogenates were centrifuged at 900 g for 15 minutes, filtered through a 1.2 μm Sterile Acrodiscs (Gelman Sciences Inc., Ann Arbor, Michigan, USA), and frozen at −70°C until required.

CXCL12 ELISA. Lung homogenate and plasma were assayed for CXCL12 levels as previously described (50).

Sircol assay. The Sircol collagen assay (Biocolor Ltd., Belfast, United Kingdom) was performed following the manufacturer’s instructions (13). Briefly, Sirius red reagent (50 μl) was added to each lung homogentate (50 μl) and mixed for 30 minutes. The collagen–dye complex was precipitated by centrifugation at 16,000 g for 5 minutes, washed with ethanol, and dissolved in 0.5 M NaOH. Finally, the samples were introduced into a microplate reader and the absorbance determined at 540 nm.

Total RNA isolation and real-time quantitative RT-PCR. Total RNA was prepared using TRIzol and by following the manufacturer’s instructions. Real-time quantitative RT-PCR was performed using specific TaqMan primers and probes, the ABI Prism 7700 sequence detector, and SDS analysis software (Applied Biosystems, Foster City, California, USA), as previously described (50).

Purification of circulating human fibrocytes. Human fibrocytes were isolated according to previously published methods (5, 6, 11). Briefly, PBMCs were isolated from leukopheresis packs (HemaCare Inc., Woodward Hills, California, USA) using Ficoll density centrifugation. Cells isolated in this way were then cultured in DMEM supplemented with 20% FCS, penicillin, streptomycin, and l-glutamine for 7–14 days. Typically, 2 × 10⁶ PBMCs were plated into each T150 tissue culture flask. After 7–14 days, the media was aspirated from the PBMC culture and the remaining adherent cells washed with sterile PBS. Subsequently, the adherent cells were harvested using Accutase (Innovative Cell Technologies Inc., San Diego, California, USA), which we have found to be more efficient than the standard trypsin protocol for removing adherent cells from plastic. Previous data (6) have indicated that the main contaminating cell types in these cultures are B lymphocytes (about 3%), T lymphocytes (about 13%), and monocytes/macrophages (up to 20% of the PBMC culture). Thus, we used immunomagnetic selection to deplete B lymphocytes (Dynabeads M-450 anti-CD19; Dynal Inc., Lake Success, New York, USA), T lymphocytes (Dynabeads M-450 anti-CD2), and monocytes/macrophages (Dynabeads M-450 anti-CD14) from our crude fibrocyte preparation. Purified fibrocytes prepared in this way were returned to culture (DMEM supplemented with 20% FCS, penicillin, streptomycin, and l-glutamine) for an additional 5 days before analysis. Fibrocytes were routinely greater than 90% pure as determined by staining with CD45, Col I, and CXCR4. Typically, our isolated fibrocytes represented about 0.5% of the overall pool of peripheral white blood cells.

FACS analysis. Cells isolated from the bone marrow, lungs, and peripheral circulation of mice were initially stained with FITC-labeled CXCR4 or FITC-labeled CCR7 and peridinin chlorophyll-a protein–labeled (PerCP-labeled) CD45. Subsequently, the cells were permeabilized using cytofix/cytoperm (BD Biosciences — Pharmingen, San Diego, California, USA) to facilitate intracellular staining of Col I. Col I was stained initially with anti-human or anti-mouse Col I (Chemicon International, Temecula, California, USA) and then phycoerythrin-conjugated (PE-conjugated) goat anti-rabbit Ig (Molecular Probes Inc., Eugene, Oregon, USA). Three-color analysis of the stained cells was performed on a FACScan flow cytometer (BD Biosciences — Pharmingen) using Cellquest 3.2.1f software. In separate experiments cells isolated from the lungs were individually stained with PE-conjugated
Purified human fibrocytes cultured for either 1, 2, or 3 weeks after their initial isolation were taken and stained with FITC-labeled CXCXR4, APC-labeled CD34, and PerCP-labeled CD45. Next, the cells were permeabilized using cytofix/cytoperm to facilitate intracellular staining of Col I and α-SMA. Col I and α-SMA were then stained with unconjugated rabbit anti-human Col I and mouse anti-human α-SMA Ab’s followed by anti-rabbit FITC and anti-mouse FITC, respectively. Finally, single-color analysis of the stained cells was performed on a FACScan flow cytometer using Cellquest 3.2.1f software.

Preparation of F(ab’2) fragments of goat anti-human CXCXR12 whole Ig, F(ab’2) fragments of goat anti-human CXCXR12 (16) were prepared using the ImmunoPure F(ab’2) kit (Pierce Chemical Co., Rockford, Illinois, USA). Briefly, immobilized pepsin was used to digest whole Ig into Fc and F(ab’2) fractions. These fractions were then prepared by protein A-affinity chromatography such that the Fc portion bound to the column, while the F(ab’2) portion was eluted, collected, and further purified by dialyzing against PBS, pH 7.4, overnight.

Morphometric analysis and picrosirius red staining. Paraaffin-embedded sections (3 μm thick) of bleomycin-exposed lungs treated with either a control Ab or neutralizing anti-CXCXR12 Ab were exposed to xylene (twice, 1 minute each) and then washed initially in 100% ethanol (twice, 1 minute), followed by 50% ethanol (twice, 1 minute), and finally rinsed in distilled water for 10 minutes. Subsequently, the sections were treated with 0.2% phosphomolybdic acid (2 minutes), left to dry overnight. After drying overnight, Permount was added, together with a coverslip, and the sections were then viewed by microscopy.

Statistical analysis. The animal studies involved 3–6 mice for each treatment group. Data were analyzed on a Dell PC (Dell, Round Rock, Texas, USA) computer using the Statview 5.0 statistical package (Abacus Concepts Inc., Berkeley, California, USA). Comparisons were evaluated by Student’s unpaired t test. Results were statistically significant if P values were 0.05 or less.

Acknowledgments
Funding was provided by NIH grants HL-66027 (to R.M. Strieter), P50HL-67665 (to M.P. Keane and R.M. Strieter), and HL-03906 (to M.P. Keane).

Received for publication January 7, 2004, and accepted in revised form June 15, 2004.

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CD3, CD4, CD8, NK-1, LY-6, and Mac519 (Sertec Inc., Raleigh, North Carolina, USA). Except where indicated, all mAb’s were purchased from BD Biosciences — Pharmingen. To obtain sufficient cells from the blood for analysis by FACS, two buffy coats (500 μl per buffy coat) were combined from each treatment condition. Thus data from fibrocytes isolated from the peripheral circulation are presented as cells per milliliter of blood.

Immunostaining. Histopathologic sections of lung tissue were prepared from mice who had been exposed to bleomycin for 16 days in the presence of either control or neutralizing anti-CXCXR12 Ab’s and assessed by immunostaining of α-SMA (R&D Systems Inc., Minneapolis, Minnesota, USA). Purified fibrocytes (104 cells per well) were cultured on TissueTech (BD Biosciences — Pharmingen) for the times indicated and then immunostained. Briefly, the cells were fixed in 4% paraformaldehyde and then incubated with a 1:1 mixture of 3% hydrogen peroxide in methanol. Subsequently, the cells were exposed to Power Block (BioGenex Laboratories, San Ramon, California, USA) for 30 minutes and, after washing in PBS, stained with a primary Ab (human and murine Col I from Chemicon International; CXCXR4 from R&D Systems Inc; α-SMA; or an isotype control) for a further 30 minutes at room temperature. After incubation with the primary Ab, the cells were washed with PBS and then exposed to a biotinylated anti-mouse secondary Ab for 30 minutes. Subsequently, the cells were washed in PBS and avidin-binding complex (Vector Laboratories, Burlingame, California, USA). Reagent was added, and the cells incubated again for 30 minutes. After an additional PBS wash, the cells were exposed to the chromogen 3,3’-diaminobenzidine tetrahydrochloride (Vector Laboratories), which turns the positively staining cells brown. Finally, the cells were counterstained with hematoxylin, neutralized in 10% ammonia, and left to dry overnight. After drying overnight, Permount was added, together with a coverslip, and the sections were then viewed by microscopy.

Histology. Lungs were perfused with saline and then infiltrated with 1 ml of 4% paraformaldehyde at a constant pressure of 30 cm H2O. Lungs were ligated at the trachea, removed en bloc, and immersed in 4% paraformaldehyde for 24 hours, at which time they were changed to 70% alcohol before paraffin embedding, followed by sectioning and H&E staining.

Fibrocyte chemotaxis. Chemotaxis was performed using fibronectin-coated filters (5 μm pore) as previously described (16).

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