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A Profibrotic Function of IL-12p40 in Experimental Pulmonary Fibrosis[1]

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The p40 subunit of IL-12 (IL-12p40), but not the heterodimeric form IL-12p70, is secreted during the development of silica-induced lung fibrosis in C57BL/6 mice. To delineate the contribution of IL-12p40 to the lung inflammatory and fibrotic processes, we compared the pulmonary responses with silica particles of IL-12p35-deficient mice (IL-12p35$^{-/-}$, able to produce IL-12p40) and IL-12p40-deficient mice (IL-12p40$^{-/-}$). IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ animals developed strikingly contrasting responses to silica in comparison with wild-type C57BL/6 mice. Although the IL-12p40$^{-/-}$ mice exhibited limited inflammatory and fibrotic reactions, the IL-12p35$^{-/-}$ mice presented a robust and well-developed pulmonary inflammation and fibrosis. Furthermore, the silica-induced increase in lung IL-12p40 content was significantly higher in IL-12p35$^{-/-}$ mice than in wild-type controls, and was associated with extensive lung fibrosis and pulmonary macrophage infiltration. The contrasting responses observed between these two IL-12 subunit-deficient murine strains were not accompanied by a strict type 1 or type 2 polarization as estimated by the measurements of lung IFN-γ/IgG2a and IL-4/IgG1 content. In vitro proliferation, type I collagen expression, as well as myofibroblast differentiation of purified pulmonary fibroblasts were not affected by treatment with exogenous rIL-12p40. In vivo, supplementation with rIL-12p40 restored the impaired pulmonary fibrotic response and macrophage accumulation in silica-treated IL-12p40$^{-/-}$ mice, and also promoted fibrosis and macrophage influx in wild-type mice. Together, our data suggest that IL-12p40 plays an important role in silica-induced pulmonary inflammation and fibrosis, possibly by exacerbating macrophage recruitment. ***The Journal of Immunology,*** **2002, 169: 2653–2661.**

Pulmonary fibrosis is a progressive illness characterized by inflammation, with subsequent scarring characterized by abnormal and excessive deposition of extracellular matrix. The disease is caused by unknown or known factors and characterized by loss of normal alveolar structure and impaired lung function. The prognosis associated with this pulmonary disease remains poor due to the lack of effective therapy. Local persistence of activated immune cells appears to be important in the development of certain forms of pulmonary fibrosis. Although the exact role of granulocytes and lymphocytes is still a matter of debate in silica-induced lung injury and fibrosis, pulmonary macrophages seem to play a key role by secreting a wide range of mediators implicated in the process leading to fibrosis. Macrophage-derived molecules such as cytokines, growth factors, oxygen reactive species, destructive proteolytic enzymes, and eicosanoid metabolites participate in acute lung inflammation and injury, as well as in parenchymal and mesenchymal cell activation with exaggerated production and deposition of extracellular matrix proteins. However, the exact mechanism of lung macrophage accumulation during fibrosis has not been completely elucidated (1–3).

IL-12 (IL-12p70) is a heterodimer composed of two disulfide-linked subunits of 35 (p35) and 40 kDa (p40), which are encoded by two separate genes. Association of IL-12p35 and IL-12p40 subunits forms the bioactive heterodimer of 70–75 kDa (IL-12p70) (4, 5). Highly expressed by activated APCs, IL-12p70 is a key cytokine for induction of Th1 immune/inflammatory response and acts mainly on T and NK cells. IL-12p70 plays a major role in resistance to bacterial, viral, parasitic infections, and tumors, and in the pathogenesis of various autoimmune diseases (6). The biological activities of IL-12p70 require interaction of the IL-12p40 subunit with the β1-chain of the IL-12R, and the interaction of IL-12p35 subunit with the β2-chain of IL-12R (7).

In addition to dimerizing with IL-12p35 to form the heterodimeric molecule IL-12p70, the IL-12p40 subunit can also form homodimers. Both in vitro and in vivo studies have shown that IL-12p40 acts as an antagonist of IL-12p70 due to its ability to compete with IL-12p70 for binding to IL-12Rβ1. Moreover, on the basis of clearly different biological responses between IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice in models of cardiac allograft rejection (8) and infection (9–11), an agonist function for IL-12p40 has also been suggested. Thus, not only can IL-12p40 function both as IL-12 in its heterodimeric form with IL-12p35, or as an IL-12 inhibitor in its homodimeric form, it also can have additional activities separate from those associated with IL-12. Recently, a new cytokine named IL-23 has been identified, which is composed of the IL-12p40 subunit covalently bound to a newly

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discovered 19-kDa polypeptide (12). The biological activities and functions of IL-23 seem to be similar to those of IL-12p70.

In the present study, we compared pulmonary responses with silica particles between IL-12p35$^{-/-}$, IL-12p40$^{-/-}$, and wild-type mice to evaluate the role of endogenously produced IL-12p40 during the lung fibrosis process. The results show that IL-12p40 can act as a profibrotic and proinflammatory mediator independently of IL-12p70.

Materials and Methods

Mouse silicosis model

C57BL/6, IL-12p35-, and IL-12p40-deficient mice (13) were obtained from The Jackson Laboratory (Bar Harbor, ME). Female mice weighing between 18 and 22 g were purchased at 8 wk of age. Silica particles were heated for 2 h at 200°C before suspension in saline and intratracheal instillation. Wild-type and deficient mice were anesthetized with a mix of Ketalar (N.V. Warner-Lambert, Zaventem, Belgium) and Rompun (Bayer AG, Leverkusen, Germany) (1 and 0.2 mg, respectively/mouse, i.p.), and 50 μl of a suspension of silica particles (1, 2.5, or 5 mg) was injected into the lungs via the trachea upon visualization with a surgical incision.

Bronchoalveolar lavage and whole lung homogenates

At selected time points after silica instillation, the animals were sacrificed with sodium pentobarbital (20 mg/animal, i.p.). Bronchoalveolar lavage (BAL)[3] was performed by cannulating the trachea and lavaging the lungs six times with 1 ml sterile 0.9% NaCl. The BAL fluid (BALF) was centrifuged (1200 rpm, 10 min, 4°C), and the cell-free supernatant was used for biochemical measurements. BAL was then repeated twice with 1 ml sterile 0.9% saline. After pooling and centrifugation, cell pellets from all the lavage fractions were combined for each animal. Aliquots of the cell suspensions were used to determine total cell numbers and cell differentials. These were done on the cells pelleted onto glass slides by cytocentrifugation and subjected to DiffQuik staining (Dade, Brussels, Belgium). Polymorphonuclear and mononuclear cells were then counted by light microscopy at ×200 magnification (total of 300 cells counted). The remaining cells were used for flow cytometric analysis.

Separately, nonlavaged whole lungs were excised and placed into a Falcon tube chilled on ice, followed by addition of 2 ml cold 0.9% NaCl. After homogenization for 30 s using a Polytron PT1200 homogenizer (Kinematica, Littau, Lu, Switzerland), the tubes were centrifuged at 4°C (10,000 rpm, 15 min) and the supernatants were kept frozen at −80°C until use.

Flow cytometry

Erythrocytes in the BALF were lysed by incubation for 5 min in 0.15 M NH_4Cl. Fluorescent labeling of cells was undertaken upon resuspension in HBSS (Life Technologies-Invitrogen, Merelbeke, Belgium) with 3% decomplemented FBS (Cocalico Biologicals, Reamstown, PA) and 10 mM NaN_3. The following Abs were used: 1) FITC-conjugated anti-CD8 (clone 53-6.7; American Type Culture Collection (ATCC), Manassas, VA); 2) biotinylated anti-CD4 (clone GK1.5; ATCC), followed by PE-conjugated streptavidin (BD Biosciences, Bedford, MA); 3) PE-conjugated anti-pan-NK cells (clone DX5; BD Biosciences); and 4) FITC-conjugated anti-IgM (clone LOMM9; provided by H. Bazin, Catholic University of Louvain, Brussels, Belgium). After staining, cells were fixed in paraformaldehyde (1.25%) and 10^4 cells/sample were analyzed on a FACScan apparatus (BD Biosciences). Analysis of the lymphocyte population was undertaken with appropriate gating according to side and forward light scatter to exclude granulocytes, macrophages, and dead cells, as well as silica particles.

Biochemical and hydroxyproline assays

Lactate dehydrogenase (LDH) activity in BALF was assayed spectrophotometrically by monitoring the reduction of nicotinamide adenine dinucleotide at 340 nm in the presence of lactate. Total proteins in BALF were determined by the pyrogallol red staining method (Technicon RA system; Bayer Diagnostics, Domont, France).

Collagen deposition was estimated by measuring the hydroxyproline content of the whole lung. The lung was excised, homogenized, and hydrolyzed in 6 N HCl overnight at 110°C. Hydroxyproline was assessed by HPLC analysis (14), and data are expressed as micrograms of hydroxyproline per lung.

ELISA

Mouse IFN-γ, IL-4, IL-12p70, and p40 concentrations were measured in lung homogenates by ELISA kits obtained from R&D Systems (Minneapolis, MN), following the manufacturer's protocols. The detection limits of these ELISAs are 2 pg/ml for IFN-γ, IL-4; 5 pg/ml for IL-12p70; and 15.6 pg/ml for IL-12p40.

Fibronectin was measured in lung homogenates using a standardized sandwich ELISA. Nunc-immuno ELISA plates (MaxiSorp) were coated with rabbit anti-fibronectin capture polyclonal Ab (10 μg/ml; DAKO, Glostrup, Denmark) in a coating buffer (0.6 M NaCl, 0.26 M H_3BO_4, 0.08 M NaOH, pH 9.6) for 16 h at 4°C. The unbound capture Ab were washed away, and each plate was blocked with 2% BSA-PBS for 2 h at room temperature. Each ELISA plate was then washed with PBS/Tween 20 (0.05%; v/v), and samples were added and incubated for 2 h at room temperature. The ELISA plates were then thoroughly washed, and polyclonal rabbit anti-fibronectin Ab conjugated with peroxidase (0.22 μg/ml; DAKO) was added and incubated for 2 h at room temperature. After washing the plate, chromogen substrate (Life Technologies-Invitrogen) was added, and optical readings at 492 nm were obtained using an ELISA plate reader. Purified murine fibronectin (from fibroblast culture; Calbiochem, Darmstadt, Germany) was used to generate the standard curves for calculation of fibronectin concentration in each lung homogenate sample. The detection limit of this ELISA was consistently 40 ng/ml.

IgG subclass levels were measured in BALF using a sandwich ELISA. Polystyrene plates (Grenier, Nurtingen, Germany) were coated overnight with affinity-purified goat Ab for rabbit IgG, followed by rabbit Abs specific for these mouse IgG subclasses. After incubation for 2 h at 37°C with samples serially diluted in TBS (10 mM Tris, 10 mM merthiolate, and 130 mM NaCl, pH 7.4) supplemented with 5% FCS, biotinylated mAbs directed against IgG1 or IgG2a subclass were added for 2 h at 37°C. The plates were then visualized and quantitated using an avidin-peroxidase complex (15).

Type I collagen was measured in lung homogenates and in supernatants of fibroblast culture using a standardized direct ELISA. Samples and standards were diluted in PBS (Life Technologies-Invitrogen) and coated directly in Nunc-immuno ELISA plates (MaxiSorp) for overnight at 4°C. After blocking with BSA, polyclonal anti-mouse type I collagen Ab (1:200 times; Biodesign, Saco, ME) were then added and incubated 2 h at room temperature. Polyclonal HRP-conjugated goat anti-rabbit Ig Ab (1:1000; BD Biosciences) was used to measure the fixation of primary Abs. Purified mouse type I collagen obtained from Novotec (Saint Martin La Garenne, France) was used as standard to calibrate each assay. The detection limit of this ELISA is 40 ng/ml.

The same procedure was used to measure myofibroblast differentiation as defined by expression of α-smooth muscle actin (α-SMA). Sonicated lung fibroblast cultures were coated in ELISA plate, and Ab directed against α-SMA were added (1:1000, clone 1A4; Sigma-Aldrich, St. Louis, MO). After washing, specifically bound primary Ab was quantitated using a polyclonal HRP-conjugated goat anti-mouse Ig Ab (1:1000; BD Biosciences).

Isolation of pulmonary T lymphocytes

Lungs from mice were excised, washed in HBSS, cut into small pieces, agitated, and digested enzymatically for 80 min at 37°C. The digestion buffer was composed of collagenase type III (10 mg/lung) and DNase (250 μg/lung) (Worthington Biochemical, Lakewood, NJ) supplemented in HBSS (15 ml/lung) with 2% FBS and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone; Life Technologies-Invitrogen). The resulting cell suspension was filtered through 100- and 40-μm filters (BD Biosciences). After centrifugation (1200 rpm, 10 min), cells were washed with PBS, and lymphocytes and granulocytes were isolated by density centrifugation in 40% Percoll (16). Cells were washed twice, and T lymphocytes were isolated by positive selection with anti-CD90 (Thy-1.2), anti-CD4, and anti-CD8 magnetic beads using the MACS magnet system (Miltenyi Biotec, Auburn, CA). The resulting lymphocyte purity was 90% by microscopic examination of DiffQuik-stained cytocentrifuge preparations. Purified T cells were resuspended at 2×10^6/ml in complete RPMI medium supplemented with 10% FBS and antibiotics, then plated at 0.2 ml/well in 96-well plates precoated or not with anti-CD3 Ab for 2 h at 37°C (BD Biosciences). After 48 h of culture, supernatants were collected and analyzed by ELISA for IL-4 and IFN-γ secretion.

[3] Abbreviations used in this paper: BAL, bronchoalveolar lavage; BALF, BAL fluid; LDH, lactate dehydrogenase; PDGF, platelet-derived growth factor; SMA, smooth muscle actin; T.O., transoral.

Mouse lung fibroblast culture

To obtain mouse lung fibroblasts, lungs were cut and digested, as described above. After filtration, released cells were centrifuged, washed, and cultured in six wells in complete medium composed of DMEM (Life Technologies-Invitrogen) supplemented with 10% plasma-derived serum (Cocalico Biologicals), human recombinant platelet-derived growth factor (PDGF)-BB (5 ng/ml; R&D Systems), human recombinant epidermal growth factor (10 ng/ml; R&D Systems), insulin-transferrin-sodium selenite liquid medium supplement (1:100; Sigma-Aldrich), and antibiotics. Fibroblasts in this study were used after the first cell passage. The morphologic and synthetic characteristics of the cultured cells were consistent with those described before (17). Confluent cell monolayers were treated for 24 h with 10 ng/ml human rTGF-β or mouse rIL-12p40 homodimer (R&D Systems) diluted in medium supplemented with 0.5% of plasma. Fibroblast proliferation was estimated by [^{3}H]thymidine incorporation (17) in 96 wells. Type I collagen and α-SMA were measured by ELISA after sonication of the lung fibroblasts cultivated in 24-well plates.

IL-12p40 in vivo administration

When specified, silica-treated mice received at day 30 postinstillation either mouse rIL-12p40 reconstituted in PBS plus 0.1% of normal mouse serum or only PBS (0.1% serum) as control by transoral (T.O.) endotracheal instillation. rIL-12p40 homodimer was obtained from R&D Systems and was 97% pure, as determined by the silver staining of proteins separated by SDS-PAGE. Endotoxin level was <0.1 ng per 1 μg of the cytokine as determined by the *Limulus* amebocyte lysate method.

Histology

Animals were euthanized and perfused via the right ventricle with saline. Lungs were inflated with 1 ml 10% neutral buffered Formalin and fixed overnight. After dehydration in 70% ethanol, the lungs were then processed using standard procedures and embedded in paraffin. Sections were cut, mounted on slides, and stained with H&E or Masson's Trichrome.

Statistics

Treatment-related differences were evaluated using *t* tests or one-way ANOVA, followed by pairwise comparisons using the Student-Newman-Keuls test, as appropriate. For flow cytometry data, statistical analyses were performed by Mann-Whitney *U* test for unpaired values using Instat software (GraphPad Software, San Diego, CA). Statistical significance was considered at $p < 0.05$.

Results

Silica-induced lung injury, inflammation, and fibrosis

To assess the amplitude of lung injury and the inflammatory response to silica particles in the three strains studied, we compared LDH and protein levels as well as the number of cells in BALF 3 days after instillation of 1 and 5 mg silica particles per mouse. Although no significant difference in LDH and protein levels was observed between the three murine strains, IL-12p40$^{-/-}$ mice showed a reduction in alveolar macrophage number in comparison with wild-type and IL-12p35$^{-/-}$ mice (Table I). In contrast, a significantly greater increase in macrophage and neutrophil numbers was noted in the high dose silica-treated IL-12p35$^{-/-}$ mice relative to that in the wild-type animals (Table I). Fifteen and 60 days after silica administration, IL-12p40$^{-/-}$ mice were unable to mount an effective inflammatory response, as shown by the inability to recruit macrophages, lymphocytes, and neutrophils to the lung in response to silica instillation (Fig. 1). In striking contrast, IL-12p35$^{-/-}$ mice displayed an increased number of alveolar macrophages and lymphocytes in comparison with wild-type mice (Fig. 1). No significant difference in the number of neutrophils was observed between these two groups. The ratio and percentages of CD4$^+$, CD8$^+$, B, and NK cells were similar in all three strains, as determined by flow cytometry (data not shown).

The amplitude of the pulmonary fibrosis induced by 1, 2.5, and 5 mg silica particles at 15 and 60 days was determined by measuring lung hydroxyproline, type I collagen, and fibronectin contents, as well as by histology. IL-12p35$^{-/-}$ mice developed a more severe fibrosis than both the wild-type and IL-12p40$^{-/-}$ animals. After 15 and 60 days, this strain (IL-12p35$^{-/-}$) exhibited the highest levels of OH-proline after 1 and 5 mg silica (Fig. 2). Collagen I, fibronectin, and OH-proline levels were also significantly increased in IL-12p35$^{-/-}$ relative to C57BL/6 and IL-12p40$^{-/-}$ mice after instillation of 2.5 mg silica (Table II). In agreement with biochemical assays, histological examination revealed that pulmonary fibrosis in IL-12p35$^{-/-}$ mice was substantially more severe than in the two other strains. Indeed, lung fibrosis was more intense and more organized, affecting larger areas of the parenchyma than in wild-type and IL-12p40$^{-/-}$ mice (Fig. 3). Fibrotic lesions were not completely absent in IL-12p40$^{-/-}$ mice, but they were clearly fewer than in the lungs of treated IL-12p35$^{-/-}$ and wild-type mice. At day 60, all markers of fibrosis were significantly reduced after 2.5 and 5 mg silica (Fig. 2 and Table II) in treated IL-12p40$^{-/-}$ mice. Histological analysis also clearly demonstrated that the fibrotic response in IL-12p40$^{-/-}$ mice was limited to smaller focal areas and less well organized than in the other two strains (Fig. 3).

Collectively, these results show that silica-induced alveolitis and lung fibrosis are exacerbated in IL-12p35$^{-/-}$ mice and reduced in IL-12p40$^{-/-}$ mice relative to wild-type mice.

Lung IL-12p40 content

Although both IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice are deficient in IL-12p70, IL-12p35$^{-/-}$ animals retain the ability to express and secrete normal amounts of the p40 subunit (13). Because IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice showed opposite pulmonary responses to silica, we investigated the potential role of IL-12p40 in this contrasting response. First, we measured IL-12p70 and IL-12p40 contents by ELISA in lung homogenates of the three strains studied. IL-12 p70 levels were significantly and dose dependently

Table I. *Biochemical and cellular parameters in BALF 3 days after intratracheal instillation of silica in C57BL/6, IL-12p35$^{-/-}$, and IL-12p40$^{-/-}$ mice*

		LDH (U/L)	Protein (mg/L)	Macrophages ($\times 10^3$)	Neutrophils ($\times 10^3$)
C57BL/6	Saline	42.4 ± 4.3	49.3 ± 5.7	122.5 ± 18.7	0.3 ± 0.1
	1 mg	141.8 ± 6.5	459.3 ± 52.9	188.0 ± 40.6	208.9 ± 44.1
	5 mg	210.2 ± 18.7	1636.8 ± 202.9	327.9 ± 21.8	644.3 ± 93.1
IL-12p35$^{-/-}$	Saline	35.3 ± 1.0	48.0 ± 4.1	111.4 ± 19.6	0.1 ± 0.1
	1 mg	129.3 ± 24.4	517.0 ± 134.8	261.8 ± 44.4	148.0 ± 33.1
	5 mg	191.5 ± 32.7	1430 ± 206.7	454.6 ± 58.3*	1471.1 ± 309.6***
IL-12p40$^{-/-}$	Saline	34.0 ± 3.0	47.3 ± 5.0	129.6 ± 22.4	1.7 ± 1.0
	1 mg	153.3 ± 16.4	573.8 ± 121.3	172.9 ± 27.6	157.7 ± 25.1
	5 mg	188.2 ± 20.8	2040.6 ± 200.4	140.5 ± 18.8**	843.8 ± 68.3

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; denote significant differences in values measured in silica-treated IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice compared with silica-treated C57BL/6 mice.

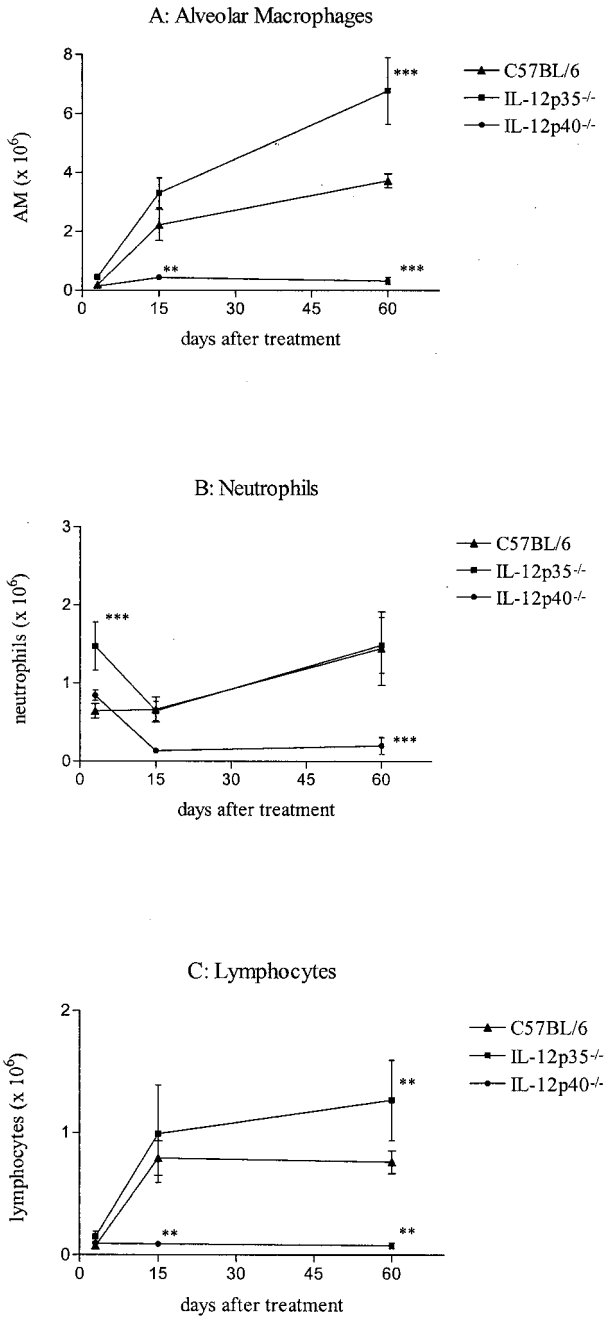

FIGURE 1. Time course of alveolar macrophage (*A*), neutrophil (*B*), and lymphocyte (*C*) counts in BAL samples obtained from silica-treated C57BL/6, IL-12p35$^{-/-}$, and IL-12p40$^{-/-}$ mice at days 3, 15, and 60 postinstillation of 5 mg silica particles. Symbols represent SEM of four to six animals. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; denote significant differences in values measured in silica-treated IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice compared with corresponding silica-treated C57BL/6 mice, as estimated by Student-Newman-Keuls multiple comparison test.

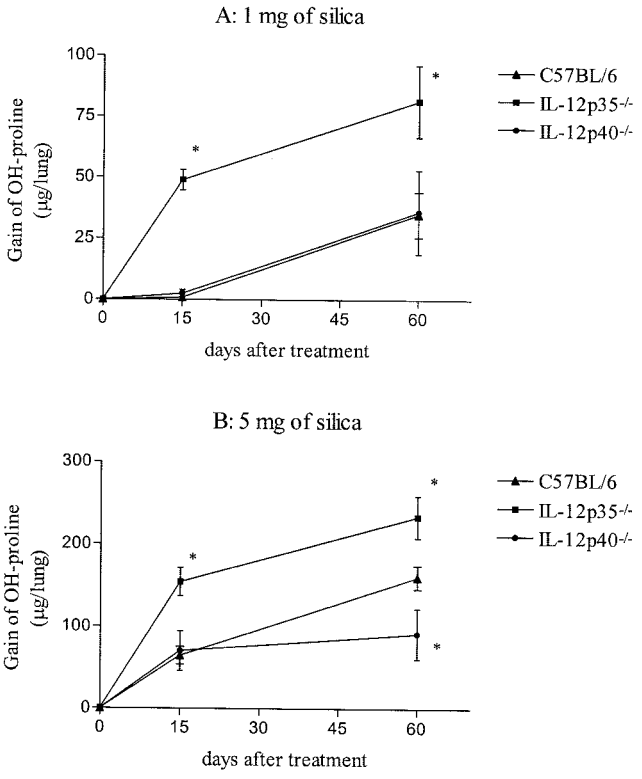

FIGURE 2. Time course of OH-proline gain in whole lung from C57BL/6, IL-12p35$^{-/-}$, and IL-12p40$^{-/-}$ mice treated with 1 (*A*) or 5 mg (*B*) silica at days 3, 15, and 60 postinstillation. Symbols represent SEM of four to six animals. *, $p < 0.05$; denotes significant differences in values measured in silica-treated IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice compared with corresponding silica-treated C57BL/6 mice, as estimated by Student-Newman-Keuls multiple comparison test.

decreased after silica administration in wild-type mice (Fig. 4). In striking contrast, IL-12p40 was clearly increased at the two doses used in wild-type mice. Interestingly, silica-exposed IL-12p35$^{-/-}$ mice expressed significantly higher levels of IL-12p40 than those observed in wild-type mice (Fig. 4). Thus, the levels of IL-12p40 correlated well with the amplitude of lung fibrosis in silica-treated wild-type and IL-12p35$^{-/-}$ mice. The value of IL-12p70 and IL-12p40 estimated by ELISA, respectively, in both IL-12-deficient mice and in IL-12p40$^{-/-}$ mice, was used to determine the detection threshold for these assays.

These results suggest a role for IL-12p40 in mediating pulmonary responses during fibrosis induced by silica particles, which is independent of IL-12p70. To further determine the biological activity of IL-12p40 in lung fibrosis, we assessed its potential activity on three different cell populations implicated in the pathogenesis of fibrosis: fibroblasts, T lymphocytes, and macrophages.

Effects of IL-12p40 on pulmonary fibroblasts in vitro

Fibroblast activation and myofibroblast differentiation are hallmarks of fibrotic disease and collagen deposition. Therefore, we first compared the in vitro activity of mouse rIL-12p40 with that of TGF-β on purified lung fibroblasts from saline- or silica-treated C57BL/6, IL-12p35$^{-/-}$, and IL-12p40$^{-/-}$ mice. Although TGF-β significantly increased thymidine incorporation, myofibroblast differentiation (α-SMA expression), and type I collagen production in comparison with medium alone, rIL-12p40 was unable to activate fibroblasts obtained from saline-treated wild-type mice using the same endpoints (Table III). Similar results were obtained with the three strains studied and with fibroblasts purified from silica-treated mice at day 60 (data not shown). These results demonstrated that IL-12p40 did not directly activate lung fibroblasts.

Type 1 vs type 2 immune response in silica-treated mice

Recently, it has been proposed that Th differentiation could be implicated in the pathogenesis of lung fibrosis (18). IL-12 p70 promotes Th1 differentiation through IFN-γ production and a type

Table II. *Levels of OH-proline, fibronectin, and type I collagen in whole lung of silica-treated C57BL/6, IL-12p35$^{-/-}$, and IL-12p40$^{-/-}$ mice (2 mo, 2.5 mg silica)*

		OH-Proline (μg/lung)	Gain of OH-Proline (μg/lung)	Collagen I (mg/lung)	Gain of Collagen I (mg/lung)	Fibronectin (μg/lung)
C57BL/6	Saline	155.6 ± 12.4		4.8 ± 0.06		4.8 ± 0.8
	Silica	245.4 ± 17.0	89.8 ± 17.0	8.0 ± 0.6	3.2 ± 0.6	91.4 ± 5.6
IL-12p35$^{-/-}$	Saline	155.8 ± 10.6		6.4 ± 0.8		5.2 ± 0.8
	Silica	288.4 ± 13.8*	132.6 ± 12.0*	11.4 ± 0.3***	5.0 ± 0.3***	119.0 ± 4.8***
IL-12p40$^{-/-}$	Saline	181.8 ± 3.2		6.8 ± 0.04		3.4 ± 0.2
	Silica	224.2 ± 5.6*	42.4 ± 5.6**	8.6 ± 0.4	1.8 ± 0.4*	53.6 ± 3.6***

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; denote significant differences in values measured in silica-treated IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice compared with silica-treated C57BL/6 mice.

1 cellular immune response. Therefore, we assessed the balance between type 1 and type 2 immune responses by measuring IFN-γ and IL-4 in pulmonary T cell cultures as well as IgG2a and IgG1 levels in BALF. Samples were obtained 60 days postinstillation of saline or silica, and purified T cells from the lung were stimulated or not with Con A in vitro to measure IFN-γ and IL-4 expression.

Purified T cells from all silica-treated mice produced spontaneously detectable amounts of IFN-γ, but no IL-4 (Table IV), although the levels of IFN-γ were significantly reduced in IL-12p40$^{-/-}$ cell cultures relative to the other strains. After in vitro stimulation with Con A, T cells from silica-treated animals from the three groups showed an increased IFN-γ response, but a decreased IL-4 response in comparison with the saline situation. IFN-γ and IL-4 levels were significantly lower in IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ cells than in wild-type cell cultures (Table IV).

In saline groups, IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ had less IgG1 in BALF than wild-type mice (C57BL/6 = 2266 ± 477.7; IL-12p35$^{-/-}$ = 449.8 ± 147.1; and IL-12p40$^{-/-}$ = 438.3 ± 37.9 ng/ml), but similar IgG2a levels (C57BL/6 = 892 ± 117.6; IL-12p35$^{-/-}$ = 608.5 ± 197.8; and IL-12p40$^{-/-}$ = 705.3 ± 146.6 ng/ml). Silica significantly increased both IgG2a and IgG1 subtypes in BALF in all strains. IL-12p35$^{-/-}$ mice, which developed the most pronounced lung fibrosis, presented the highest levels of IgG1 in comparison with C57BL/6 and IL-12p40$^{-/-}$ mice (Fig. 5). No significant difference in terms of IgG2a levels was noted between silica-exposed mice from the three groups.

Collectively, these data showed that the intensity of lung fibrosis was not intimately related with a specific type 1 or type 2 pattern, which could not account for the difference in response to silica between the strains studied.

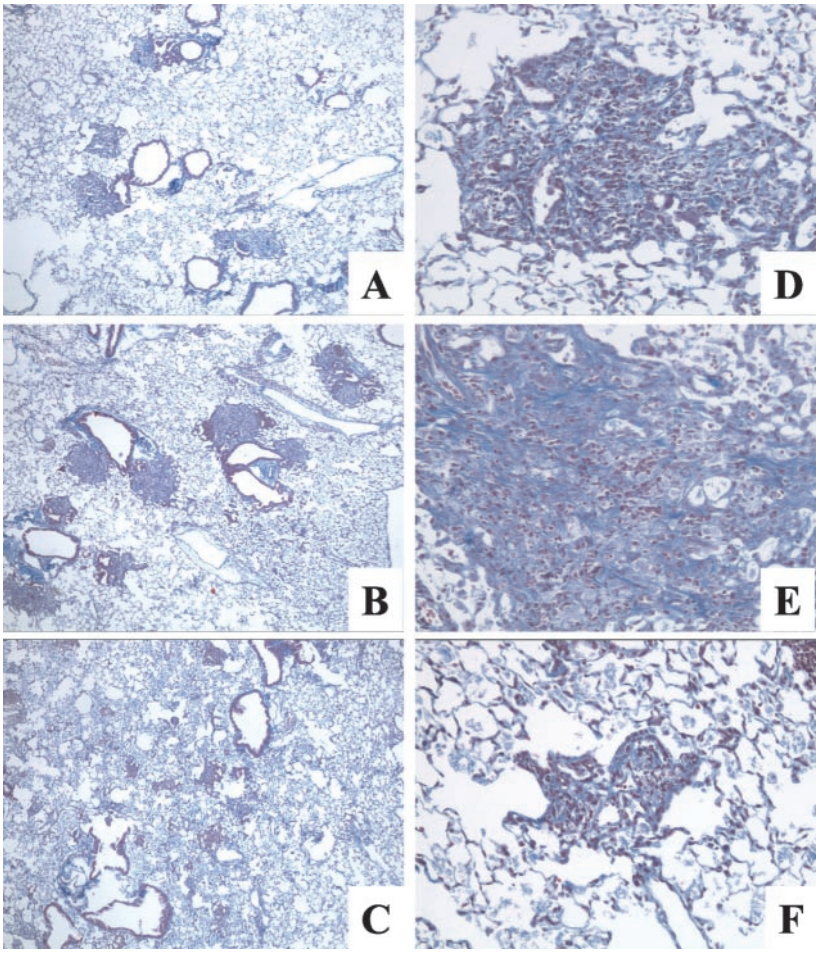

FIGURE 3. Representative of Blue Masson Trichrome-stained lung sections from C57BL/6 (*A* and *D*), IL-12p35$^{-/-}$ (*B* and *E*), and IL-12p40$^{-/-}$ (*C* and *F*) mice treated with 2.5 mg silica at days 60 postinstillation. Pulmonary fibrosis was substantially more intense in IL-12p35$^{-/-}$ and less severe in IL-12p40$^{-/-}$ mice than that observed in C57BL/6. *Left panel*, Original magnification was ×100. *Right panel*, Original magnification was ×200.

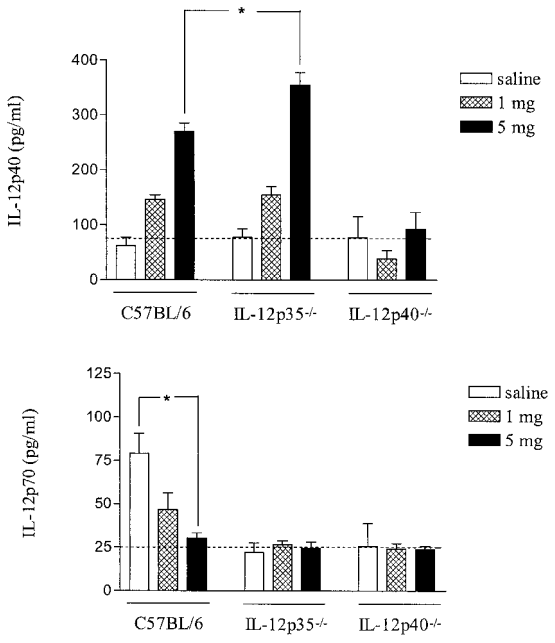

FIGURE 4. IL-12p40 and IL-12p70 levels in lung homogenates from saline- or silica (1 and 5 mg)-treated C57BL/6, IL-12p35$^{-/-}$, and IL-12p40$^{-/-}$ mice at day 60 postinstillation. Bars represent SEM of four to six animals. *, $p < 0.05$; denotes significant differences in values measured in silica-treated IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice compared with corresponding silica-treated C57BL/6 mice, as estimated by Student-Newman-Keuls multiple comparison test. The lines drawn over the figure represent the threshold of detection for IL-12p40 and IL-12p70 estimated by ELISA.

Activity of IL-12p40 in pulmonary macrophage accumulation

Pulmonary fibrosis and in particular silicosis are driven by activated macrophages. Consequently, we addressed whether the potential profibrotic role of IL-12p40 is mediated by an exacerbation of macrophage accumulation. Silica-treated wild-type mice were treated on day 30 (after silica instillation) with 0.25 or 0.5 μg mouse rIL-12p40 by T.O. endotracheal instillation. After different times of incubation (24 until 72 h), mice were sacrificed and lavaged. Cells collected in BALF were used for total and differential count. T.O. instillation of rIL-12p40 markedly increased the numbers of alveolar macrophages in a dose-dependent manner at 48 h compared with silica-treated mice injected with PBS (Fig. 6). The number of macrophages returned to normal values after 72 h. There was no statistical difference in the number of neutrophils and lymphocytes after administration of rIL-12p40 (Fig. 6). This pulmonary macrophage accumulation was also observed in silica-treated IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice, 24 h after T.O. administration of rIL-12p40 (Fig. 7). Together, these results suggest a role for IL-12p40 in mediating selective macrophage recruitment during lung fibrosis induced by silica particles, which is independent of IL-12p70.

Effects of in vivo supplementation with rIL-12p40

To determine whether this observation could be relevant in the pulmonary fibrosis reaction, we administrated rIL-12p40 (0.5 μg three times per week during 4 wk by T.O.) to wild-type and IL-12p40$^{-/-}$ mice treated with silica 30 days before. After an additional 30 days, as estimated by measuring OH-proline, type I collagen, as well as fibronectin in lung homogenates, increased pulmonary fibrosis in rIL-12p40-supplemented wild-type and IL-12p40$^{-/-}$ animals was observed relative to silicotic mice treated with PBS (Table V). These data demonstrated that exogenously administrated IL-12p40 is able to restore at least in part the defective fibrotic reaction observed in IL-12p40$^{-/-}$ mice, suggesting a profibrotic role for IL-12p40.

Table III. *In vitro [^{3}H]thymidine incorporation, α-SMA, and type I collagen expression in lung mouse fibroblasts purified from saline-treated C57BL/6 mice*

	[^{3}H]Thymidine (cpm × 1000)	SMA (OD)	Collagen I (μg/ml)
Medium	4.4 ± 0.2	0.6 ± 0.07	27.5 ± 1.2
TGF-β (10 ng/ml)	19.4 ± 0.8***	2.1 ± 0.08***	34.6 ± 0.4***
IL-12p40 (10 ng/ml)	3.4 ± 0.1	0.5 ± 0.04	23.0 ± 1.6

***, $p < 0.001$; denotes significant differences in values measured in cytokine-treated wells compared with wells without cytokines.

Discussion

Pulmonary macrophages play an important role in the pathogenesis of lung fibrosis by promoting inflammation and fibrotic responses (19, 20). IL-12p70, composed of p35 and p40 subunits, is a major Th1-driving cytokine establishing cell-mediated immunity (6) and preferentially expressed by APC cells such as pulmonary macrophages (21, 22). We have therefore studied the relative contribution of this cytokine and its subunits in the development of a mouse model of pulmonary fibrosis induced by silica particles.

Results from our laboratory (23) and others (24–26) have demonstrated that the IL-12p40 subunit is overproduced during the establishment of an experimental fibrotic process, but not the IL-12p70 heterodimer. IL-12p40 has been found to be produced largely by activated pulmonary macrophages during the fibrotic reaction (23). In this study, we report in the C57BL/6 strain that experimental silicosis is also related with increased IL-12p40 levels, but, in contrast, with a parallel decrease of IL-12p70 content (Fig. 4). The key importance of IL-12p40 in fibrotic process was demonstrated by using IL-12p40$^{-/-}$ mice. These animals were relatively resistant to the toxicity of silica, showing a reduced pulmonary inflammation and fibrosis in comparison with wild-type animals. Silica-treated IL-12p35$^{-/-}$ mice, which expressed the highest concentrations of IL-12p40, developed the most severe lung fibrosis and the alveolar macrophage accumulation compared with the wild-type and IL-12p40$^{-/-}$ mice, emphasizing that the development of the fibrotic reaction is dependent on IL-12p40 expression, but independent of IL-12p70 expression. Moreover, the striking differences observed between both IL-12 subunit-deficient strains in their pulmonary responses to silica and their synthesis of IL-12p40 suggest a profibrotic role for IL-12p40. In vivo supplementation of IL-12p40$^{-/-}$ mice with rIL-12p40 restored in part the amplitude of the attenuated lung fibrotic process, confirming its profibrotic activity.

IL-12p40 monomeric and homodimeric forms have been first identified to exert an antagonistic activity in numerous studies both in vitro and in vivo by inhibiting the biological activity of IL-12p70 (27, 28). For example, administration of rIL-12p40 is able to protect mice from septic shock and to reduce in vivo IFN-γ responses in IL-12-dependent models (29, 30). Reports studying IL-12p40 transgenic mice have confirmed that overproduction of IL-12p40 suppressed IFN-γ production and Th1 responses in models of hepatic infection (31) and lupus-like autoimmune disease (32). It has been proposed that sustained production of IL-12p40, often associated with IL-12p70 synthesis, tempers the exaggerated effect of IL-12p70-mediated immune responses by behaving as an antagonist in competing with IL-12p70 for the binding to the IL-12Rβ1-chain of the heterodimeric IL-12R (33).

Table IV. In vitro *IFN-γ and IL-4 production of lung T cells purified from saline or silica-treated C57BL/6, IL-12p35$^{-/-}$, and IL-12p40$^{-/-}$ mice (2 mo, 2.5 mg silica)*

		IFN-γ			IL-4		
		C57BL/6	IL-12p35$^{-/-}$	IL-12p40$^{-/-}$	C57BL/6	IL-12p35$^{-/-}$	IL-12p40$^{-/-}$
T cells							
without Con A	Saline	N.D.[a]	N.D.	N.D.	N.D.	N.D.	N.D.
	Silica	37.4 ± 11.7	39.0 ± 12.5	10.3 ± 5.0*	N.D.	N.D.	N.D.
with Con A	Saline	280.4 ± 63.3	434.0 ± 24.6	440.3 ± 28.0	137.2 ± 11.0	164.1 ± 6.9	131.9 ± 12.2
	Silica	847.8 ± 12.5	757.3 ± 10.5**	789.0 ± 19.8*	52.0 ± 8.7	20.6 ± 2.7***	17.1 ± 3.4***

[a] N.D., Not detected.

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; denote significant differences in values measured in silica-treated IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice compared with silica-treated C57BL/6 mice.

However, recent publications have suggested the additional possibility that IL-12p40 under certain conditions can function as an IL-12p70 agonist in its own right. Using both mice deficient in IL-12p70 and the administration of Abs blocking IL-12p40 or rIL-12p40, these authors have elegantly demonstrated that, as IL-12p70, IL-12p40 contributed to Th1 induction responses and production of IFN-γ in experimental models of cardiac allograft rejection (8), as well as bacterial and viral infections (9–11). In addition, transgenic mice overproducing IL-12p40 in basal keratinocytes spontaneously developed inflammatory skin disease, which is also obtained by injection of IL-12p70 (34). Recently, a p19 protein has been identified that combines with IL-12p40 to form a novel cytokine named IL-23 (12). This new cytokine has similar activity as IL-12p70, which could explain at least in part the observed agonist activity of IL-12p40.

Of particular relevance to the results of this study, specific biological activity of IL-12p40 itself, separate from its IL-12p70-like activity, has not been excluded. In absence of IL-12p70, the physiological effect of rIL-12p40 in a mouse model of mycobacterial infection (10) and in our experimental model of lung fibrosis (Figs. 6 and 7, Table V) argues for such a novel specific activity. It is not clear for the moment how IL-12p40 could function as an independent mediator in terms of receptor and signaling mechanisms. The fact that IL-12p40 binds IL-12Rβ1, which is associated with a different Janus kinase (Tyk2) than IL-12Rβ2 (Jak2) used by the

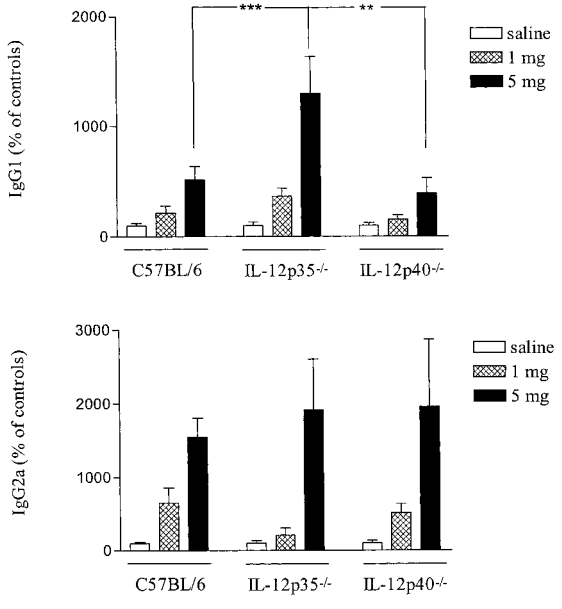

FIGURE 5. IgG1 and IgG2a levels in BALF from saline- or silica (1 and 5 mg)-treated C57BL/6, IL-12p35$^{-/-}$, and IL-12p40$^{-/-}$ mice at day 60 postinstillation. Bars represent SEM of four to six animals. **, $p < 0.01$; ***, $p < 0.001$; denote significant differences in values measured in silica-treated C57BL/6 and IL-12p40$^{-/-}$ mice compared with silica-treated IL-12p35$^{-/-}$ mice, as estimated by Student-Newman-Keuls multiple comparison test.

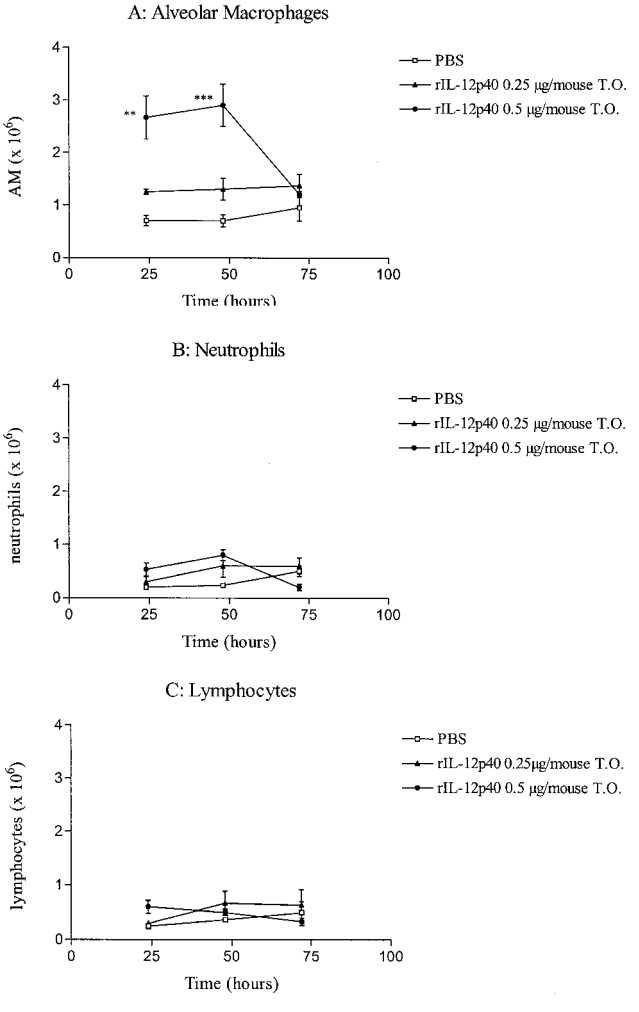

FIGURE 6. Alveolar macrophage (*A*), neutrophil (*B*), and lymphocyte (*C*) counts in BAL samples obtained from silica (2.5 mg)-treated C57BL/6 mice at various times after T.O. administration of different concentrations of mouse rIL-12p40. Symbols represent SEM of four to six animals. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; denote significant differences in values measured in rIL-12p40-administrated mice compared with corresponding PBS-administrated mice, as estimated by Student-Newman-Keuls multiple comparison test.

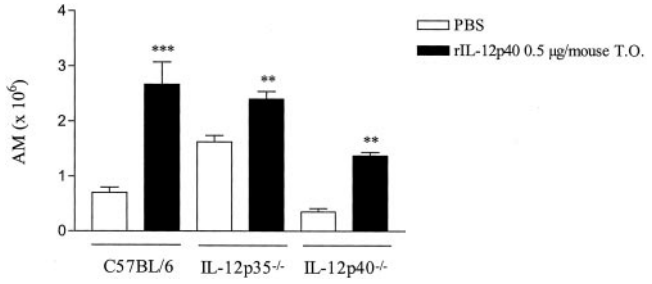

FIGURE 7. Alveolar macrophage counts in BAL samples obtained from silica (2.5 mg)-treated C57BL/6, IL-12p35$^{-/-}$, and IL-12p40$^{-/-}$ mice at 24 h after T.O. administration of mouse rIL-12p40 (0.5 μg/mouse). Bars represent SEM of four to six animals. **, $p < 0.01$; ***, $p < 0.001$; denote significant differences in values measured in rIL-12p40-administrated mice compared with corresponding PBS-administrated mice, as estimated by Student-Newman-Keuls multiple comparison test.

IL-12p35 subunit, suggests distinct signaling pathways, resulting in a novel separate biological activity of IL-12p40 (35). Interestingly, it has been shown that a new component is associated with the IL-12Rβ1 subunit, which could possibly impart greater selectivity in terms of ligand binding and consequent signaling via this receptor (36).

Considering in our study that 1) pulmonary IL-12p40 content is clearly related with the intensity of fibrosis, 2) clear different fibrotic responses were observed between silica-treated IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice, and 3) rIL-12p40 exacerbated lung fibrosis, it is likely that IL-12p40 has a direct and independent (i.e., separate from activity as IL-12p70) activity in the promotion of pulmonary fibrosis in our silicosis model.

To further define the role of IL-12p40 in regulating lung fibrotic process and the cellular mechanisms underlying its profibrotic activities, we subsequently analyzed the response of pulmonary fibroblasts, T cells, and macrophages to IL-12p40.

During the fibrotic process, a complex interplay between cytokines is responsible for the exaggerated activation of fibroblast functions such as proliferation (PDGF, epidermal growth factor, fibroblast growth factor, TNF-α), myofibroblast differentiation (TGF-β), and collagen synthesis (TGF-β, IL-4, IL-13) (37). By studying these cellular parameters, we have demonstrated in vitro that rIL-12p40 was not acting directly on fibroblasts (Table III). We concluded that its profibrotic activity was not due to a direct activation of the pulmonary fibroblast.

With respect to an agonistic role of IL-12p40 in mediating Th1 immune responses and its potential contribution to the Th1 and Th2 balance, we did not find marked correlation between selected Th markers (IFN-γ, IgG2a for Th1 response and IL-4, IgG1 for Th2 response) and fibrosis intensity in the three strains used. Our data suggest that IL-12p40 plays a minimal role in the polarization of Th-mediated immune response during lung fibrosis. Only lung IgG1 content was associated with the intensity of fibrosis, emphasizing a possible relationship between Th2 responses and fibrosis. T cells purified from silica-exposed animals preferentially produced in vitro IFN-γ over IL-4. This observation is in accordance with data published by Davis et al. (38), showing that purified T cells from silicotic mice expressed IFN-γ, but not IL-4. However, in our study, lung IFN-γ expression did not correlate with the amplitude of lung fibrosis and thus could not account for the striking difference in fibrosis between IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice.

In contrast, the level of lung IL-12p40 expression showed good correlation with the extent of macrophage accumulation in silica-treated lungs. Our data obtained with in vivo administration of rIL-12p40 confirmed a major role for IL-12p40 in the accumulation of pulmonary macrophages during experimental silicosis. These observations are reminiscent of previous studies showing that IL-12p40 is chemotactic for macrophages both in vitro and in vivo (39, 40). It has been well demonstrated in both experimental and human studies that lung fibrosis, and silicosis in particular, is characterized by an alveolar macrophage-dominant alveolitis. The numerous and activated macrophages present in fibrotic lesions release exaggerated amounts of mediators capable of injuring lung parenchymal cells (e.g., superoxide anion and hydrogen peroxide) (41), recruiting neutrophils (TNF-α, IL-1, and IL-8) (42, 43), or promoting the growth and/or collagen production of mesenchymal cells (fibronectin, TGF-β, TNF-α, PDGF, and insulin-like growth factor-1) (43–46). On the basis of this recognized key role of macrophages, we suggest that excessive IL-12p40 found in silica-treated mice can induce selective macrophage accumulation in tissue and airspace compartments, and thus sustain its detrimental activity in the lung fibrotic process. However, we cannot exclude that IL-12p40 may have additional mechanisms to influence lung inflammation and fibrosis. Further investigations will have to determine whether macrophage-derived IL-12p40 synthesis is induced by a direct effect of silica particles or by intermediate molecules produced during the lung fibrotic process and identified as specific inducers of IL-12p40, such as IL-4 and PGE_2 (47, 48). Further support of the concept that IL-12p40 is an important profibrotic factor is provided by a recent study showing that IL-12p40-neutralizing Abs block the extension of bleomycin-induced pulmonary fibrosis in the apparent absence of IL-12p70 synthesis (26). The functional role of IL-12p40 proposed in this study could also reconcile apparently contradictory experiments in which, in the same model, rIL-12p70 administration also attenuates bleomycin-induced pulmonary fibrosis (49).

In summary, we describe in this study induction of pulmonary IL-12p40 expression in response to silica particles and demonstrate that overproduction of IL-12p40 contributes to the extension of lung fibrosis through pulmonary macrophage accumulation.

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Table V. *Levels of OH-proline, fibronectin, and collagen type I after repeated T.O. administration of mouse rIL-12p40 in silica-treated C57BL/6 or IL-12p40$^{-/-}$ mice (2 mo, 2.5 mg silica)*

	C57BL/6 + PBS	C57BL/6 + rIL-12p40	IL-12p40$^{-/-}$ + PBS	IL-12p40$^{-/-}$ + rIL-12p40
OH-proline (μg/lung)	214.7 ± 15.0	251.4 ± 3.9	176.2 ± 23.8	233.1 ± 10.4*
Collagen I (mg/lung)	10.7 ± 0.7	12.6 ± 0.6	9.6 ± 0.2	12.5 ± 0.5*
Fibronectin (μg/lung)	79.1 ± 5.8	87.6 ± 2.5	59.4 ± 8.9	85.0 ± 7.3*

*, $p < 0.05$; denotes significant differences in values measured in rIL-12p40-administrated mice compared with corresponding PBS-administrated mice.

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