Type 2 immune response associated with silicosis is not instrumental in the development of the disease

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Misson P, Brombacher F, Delos M, Lison D, Huaux F. Type 2 immune response associated with silicosis is not instrumental in the development of the disease. Am J Physiol Lung Cell Mol Physiol 292: L107–L113, 2007. First published September 22, 2006; doi:10.1152/ajplung.00503.2005.—It has been proposed that the development of lung fibrosis is associated with a T helper type 2 response, mainly characterized by IL-4 and IL-13 production. We investigated the potential role of type 2 immune polarization in the silicotic process and examined the pulmonary response to silica particles in mice genetically deficient for IL-4. We found that IL-4−/− mice were not protected against the development of silicosis, suggesting that IL-4 is not essential for the development of this fibrotic disease. By evaluating the intensity of silica-induced lung fibrosis in mice deficient for IL-4 receptor α (IL-4Rα), we showed that the establishment of pulmonary fibrosis was independent of both IL-4 and IL-13. Strong impairment of the type 2 immune response (IgG1) in the lungs of IL-4−/− and IL-4Rα−/− mice did not affect the development of the disease. Measurement of IL-13α2 receptor expression and IgG2a, IL-12p70, and IFN-γ levels in silica-treated IL-4−/− and IL-4Rα−/− animals showed that the development of silicosis was not related to an IL-13 signaling pathway or a switch to a type 1 response in deficient animals. Our data clearly indicate that the type 2 immune response associated with silicosis in mice is not required for the development of this inflammatory and fibrotic disease.

Inflammation; T helper type 2; lung fibrosis; cytokines

Fibrosis is a process characterized by an exaggerated accumulation of collagen and other extracellular matrix components, resulting in irreversible impairment of normal tissue architecture and function. Although the mechanisms leading to the development of fibrosis are still far from being fully understood, it is generally accepted that cytokines, and especially T helper type 2 (Th2) cytokines, are key mediators in the development of the fibrotic process. Several experimental and human studies have highlighted the profibrotic activities of Th2 cytokines, such as IL-4 and IL-13, as well as the association between the establishment of a fibrotic response and type 2 polarization of the immune response. This paradigm is supported by the findings of several reports on various organs, such as the lungs (37), skin (48), kidneys (50), and liver (53).

A number of experimental studies have implicated type 2 cytokines in the regulation of the fibrotic process in the lungs. Transgenic overexpression of IL-4 or IL-13 was shown to cause tissue fibrosis in mice (35, 38), suggesting strong profibrotic effects of these Th2 cytokines. Similar conclusions were drawn from in vitro experiments assessing cellular proliferation, collagen production, or myofibroblast differentiation in IL-4− or IL-13-treated pulmonary fibroblasts (9, 25, 27, 46, 55). In addition, targeting IL-4, IL-13, and/or their related receptors was accompanied by attenuation of bleomycin- and/or FITC-induced lung fibrosis in mice (16, 24, 29, 30, 36).

In experimental silicosis, both Th1 and Th2 polarization have been found to be associated with the development of the disease. We previously demonstrated that silica-induced fibrosis was correlated with markers of a Th2-like response. In mice, the development of silicosis was associated with upregulation of IL-4 levels in lung tissue and an increased IgG1/IgG2a ratio in bronchoalveolar lavage (BAL) fluid (1, 4, 23). IL-4 expression was also shown to be increased in the lungs of silica-treated rats (10). By contrast, overproduction of IFN-γ by lung and thoracic lymph node T lymphocytes was reported in mice and rats developing silicosis (13, 14, 17, 18, 22). A predominance of Th1 immune response in silicosis was also evidenced by enhanced levels of serum IgG2a (51) but reduced serum IgG1 concentrations in silica-treated mice (8). However, the role of Th1 response in the fibrotic process has not been conclusively defined, since contradictory findings on the functions of IFN-γ in experimental silicosis have been reported. Indeed, IFN-γ−/− C57BL/6 mice exposed to silica developed lower (12) or equivalent (15) levels of lung collagen accumulation compared with wild-type mice. Furthermore, administration of recombinant IFN-γ limited lung fibrosis through IL-4 and transforming growth factor-β (TGF-β) downregulation in a rat model (10). Together, these studies highlight the uncertainty that exists with regard to the respective roles of Th1 and Th2 immune responses in silicosis.

In this context, the present study was initiated to examine the role of type 2 immune polarization in the silicotic process by investigating the silica-induced pulmonary response in mice genetically deficient for IL-4, the prototypic Th2 cytokine.

MATERIALS AND METHODS

Animals. C57BL/6, IL-4−/−C57BL/6 (43), and IL-4 receptor α (IL-4Rα)-deficient (41) mice, backcrossed to C57BL/6 for nine generations, were provided by Frank Brombacher (University of Cape Town, South Africa) and bred in our local animal facility. The mice were genotyped by PCR of tail biopsies as previously reported (31, 41). Eight- to 10-wk-old female mice were used for the study. The animals were housed in positive-pressure air-conditioned units (25°C, 50% relative humidity) on a 12-h light/dark cycles. The animal use committee at the Université Catholique de Louvain approved all experimental procedures involving mice.

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Instillation method. For sterilization and inactivation of any traces of endotoxin, crystalline silica particles [DQ12, d50 (mean particle size) = 2.2 μm, kindly donated by Dr. L. Armbruster, Essen, Germany] were heated at 200°C for 2 h immediately before suspension and administration. The mice were anesthetized with a combination of Ketalar (N. V. Warner-Lambert, Zaventem, Belgium) and Rompun (Bayer, Leverkusen, Germany) (1 and 0.2 mg/mouse ip, respectively). A suspension of particles in sterile 0.9% saline was injected into the lungs via the trachea by transoral instillation (2.5 mg; 60 μl/mouse).

BAL and lung homogenates. We previously demonstrated that silica-induced inflammation in mice is acute in the first days following intratracheal instillation (3–5 days). Silica-induced lung fibrosis is progressive up to 120 days and detectable after 60 days (1, 23, 34, 39). On the basis of these data, we selected days 3 and 60 as the most relevant time points to assess lung inflammation and fibrosis, respectively. The animals were euthanized with pentobarbital sodium (20 mg/animal ip), and BAL was performed by cannulating the trachea and rising the lungs with 1 ml of sterile 0.9% NaCl. The BAL fluid was centrifuged (1,500 rpm, 10 min, 4°C), and the cell-free supernatant was used for biochemical measurements. The cell pellet was resuspended in 1 ml of sterile saline and used for total BAL cell number determination. Cell differentiation was performed on cytocentrifuge preparations fixed in methanol and stained with Diff-Quik (Baxter, Lessines, Belgium). After BAL, whole lungs were perfused with 5 ml of sterile 0.9% NaCl and then excised and transferred to 3 ml of cold PBS for hydroxyproline and soluble collagen measurements. The contents of each tube were subsequently homogenized for 30 s using an Ultra-Turax T25 homogenizer (Janke and Kunkel, Brussels, Belgium) and stored at −80°C.

Western blot analyses. Western blotting was used to detect IL-13Ra2 protein expression. Twenty micrograms of protein from lung homogenates was loaded onto precast Novex (Carlsbad, CA) SDS-polyacrylamide gels (8%) and transferred electrophoretically to nitrocellulose membranes (Hybond C; Amersham Biosciences, Piscataway, NJ). The membranes were then blocked in 5% nonfat dry milk, washed, and probed with rat antibodies specific for mouse IL-13Ra (R&D Systems, Oxon, UK). Blots were reprobed with anti-actin antibodies (Sigma, St. Louis, MO) as controls. A SuperSignal West Pico detection kit (Pierce, Rockford, IL) was used for detection.

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

Collagen assays. Collagen deposition was evaluated by measuring the hydroxyproline and soluble collagen contents of lung homogenates. Hydroxyproline was assessed by HPLC analysis (6), and data were expressed as micrograms of hydroxyproline per lung. Soluble collagen levels were calculated by the Sirocl collagen assay according to the manufacturer’s protocol (Biocolor, Westbury, NY).

Histology. Animals were euthanized and perfused via the right ventricle with saline. Their lungs were inflated with 1 ml of 10% neutral buffered formalin and fixed overnight. After dehydration in 70% ethanol, the lungs were processed using standard procedures and embedded in paraffin. Sections were cut, mounted on slides, and stained with hematoxylin-eosin or Masson’s trichrome.

Enzyme-linked immunosorbent assays. Mouse IgG1, IgG2a, (Bethyl Laboratories, Montgomery, TX), IFN-γ, and IL-12p70 (R&D Systems) concentrations in BAL fluid and lung homogenates were measured using ELISA kits following the manufacturer’s instructions. The detection limits of these ELISAs were 3.9, 3.9, 5, and 8 pg/ml, respectively.

Statistics. Treatment-related differences were evaluated using t-tests or one-way analysis of variance, followed by pairwise comparisons with the Student-Newman-Keuls test, as appropriate. P < 0.05 was considered statistically significant.

RESULTS

IL-4 deficiency does not affect the intensity of silica-induced lung inflammation. Three days after intratracheal instillation, LDH activity, TP levels, and neutrophil number were assessed in the BAL fluid of IL-4+/+ and IL-4−/− animals to evaluate the influence of IL-4 deficiency on the extent of the silica-induced inflammatory reaction. Instillation of silica particles in IL-4+/+ mice led to marked pulmonary inflammation, characterized by a significant increase in LDH and protein levels (Fig. 1, A and B), as well as extensive recruitment of polymorphonuclear neutrophils (Fig. 1C), macrophages, and lymphocytes (data not shown) in the lungs. No statistically significant difference was observed for any of these inflammatory markers between IL-4+/+ and IL-4−/− mice. Similar observations were made during the chronic inflammatory stage of the disease (2 mo; data not shown). We concluded that IL-4 is not essential for silica-induced inflammation.

IL-4 deficiency does not affect the intensity of silica-induced lung fibrosis. To determine whether IL-4 deficiency could affect the intensity of silica-induced lung fibrosis, we measured hydroxyproline and soluble collagen levels in the lung homogenates of IL-4+/+ and IL-4−/− mice 2 mo after instillation. As shown in Fig. 4, both markers were significantly upregulated in silica-treated animals, clearly indicating the development of lung fibrosis at similar intensity in IL-4+/+ and IL-4−/− mice. The histological appearance of the lungs after silica treatment was also similar in both strains, and no difference was observed in the organization of the fibrotic tissue (parenchymal densification, micronodules including monocytes, polymor-

Fig. 1. IL-4 deficiency does not affect silica-induced lung inflammation. Three days after instillation, lactate dehydrogenase (LDH) (A), total proteins (B), and neutrophil numbers (C) were assessed in the bronchoalveolar lavage (BAL) fluid of saline- or silica-treated C57BL/6 IL-4+/+ and IL-4−/− mice. Bars represent means ± SE (n = 6). Significant differences (P < 0.001, not indicated in the graph) were noted between silica- and saline-treated mice, as assessed by the Student-Newman-Keuls multiple comparison test. No significant difference was observed for any of the markers between silica-treated IL-4+/+ and IL-4−/− mice. WT, wild-type.
phonuclear neutrophils, or lymphocyte foci; Fig. 2). These results were supported by additional data obtained from Sv/129 IL-4/IL-4/H11001 and IL-4/IL-4/H11002 mice (data not shown). We concluded that IL-4 is not a crucial mediator in the development of silica-induced fibrosis in mice.

IL-4R/H9251/H11002 mice are not protected against the development of silicosis. IL-4 and IL-13 are both type 2 cytokines that exhibit many overlapping functions because they share a common receptor chain, IL-4R/H9251, for signal transduction. To determine whether IL-13 could compensate for the absence of IL-4, we evaluated the development of silica-induced fibrosis in IL-4R/H9251/H11002 mice. Wild-type and IL-4/IL-4/H11002 mice were also treated concomitantly to allow adequate comparison and interpretation of the results.

To monitor the development of silica-induced type 2 immune response, we measured IgG1 concentrations in the BAL fluid of these animals 2 mo after instillation. As shown in Fig. 3, total IgG1 levels were significantly increased in silica-treated wild-type mice. This upregulation was drastically reduced in IL-4−/− animals, however, and even appeared to be completely absent in IL-4R−/− mice, indicating considerable impairment of silica-induced type 2 immune response in both knockout strains.

We then measured hydroxyproline and soluble collagen levels in the lung homogenates of the same mice to evaluate the development of fibrosis in the absence of IL-4Rα-mediated signal transduction. The results for both markers (Fig. 4) showed that, 2 mo after administration of silica particles, all three strains developed fibrosis of similar intensity. Histological analysis confirmed these observations (data not shown).

It has recently been recognized that IL-13Rα2, formerly thought to function only as a decoy receptor, is essential for...
IL-13 signaling, TGF-β production, and bleomycin-induced fibrosis (16). We therefore investigated whether IL-13Ra2 expression was upregulated in the lungs of silica-treated mice and whether IL-13Ra2 could compensate for the absence of IL-4Ra. Whereas IL-13Ra2 was strongly induced by silica in wild-type mice, IL-4Ra deficiency was found to completely abrogate IL-13Ra2 expression (Fig. 5). These data demonstrate that IL-13 did not compensate for IL-4 during the establishment of fibrosis in IL-4−/− mice.

Silicosis in IL-4−/− and IL-4Ra−/− mice is not associated with type 1 immune polarization. Although development of Th2 inflammation is the most widely evoked mechanism in the pathogenesis of silica-induced fibrosis, some authors have reported results disrupting this paradigm (12, 14).

To investigate whether establishment of silica-induced fibrosis in IL-4−/− and IL-4Ra−/− mice may be associated with a type 1 immune response, we measured concentrations of IgG2a, IFN-γ, and IL-12p70 in the BAL fluid and lung homogenates of wild-type, IL-4−/−, and IL-4Ra−/− animals treated with silica. The results revealed that expression of these markers was not modified in any of the three strains examined in BAL fluid (Fig. 6) nor lung homogenates (data not shown). We concluded that silicosis development is not related to a switch to a type 1 response in the absence of IL-4- or IL-13-mediated signal transduction.

DISCUSSION

This study examined the role of type 2 immune polarization in the development of silica-induced lung fibrosis. The main finding of this report was that the Th2-like response associated with the development of silicosis is not essential for the establishment of the fibrotic process.

It is commonly proposed that the pathogenesis of fibroproliferative diseases is closely regulated by type 2 immune responses (37, 56). The importance of Th2 cytokines in the development of fibrotic lung diseases can be partially explained by in vitro studies, demonstrating a potent effect of IL-4 and IL-13 on the biology of key cell types involved in the pathogenesis of fibrosis. A number of studies have shown both IL-4 and IL-13 to stimulate proliferation of lung fibroblasts (24, 26) and induce synthesis of collagen (44, 46, 49) and growth factors, such as IGF-1 (55). Furthermore, recent in vitro data suggest that strong induction of FIZZ1 in alveolar type II epithelial cells by IL-4 and IL-13 constitutes another potential mechanism by which IL-4/IL-13 may play a role in the pathogenesis of lung fibrosis (36).

This hypothesis is supported by the results of several studies conducted in different animal models. Following bleomycin administration, Huaux et al. (24) reported that IL-4−/− mice developed significantly less lung fibrosis than IL-4+/+ animals. Similarly, the development of tuberculosis-associated fibrosis was reduced in infected IL-4−/− mice compared with IL-4+/+ controls (21). In line with these data, overexpression of IL-4 in the lungs of C57BL/6 mice was recently shown to be associated with fibrotic lesions, demonstrating that IL-4 exhibits strong profibrotic activity in the lungs (38).

However, conflicting observations have also been reported. Indeed, bleomycin-induced lung fibrosis was similar in both IL-4−/− and IL-4+/+ mice, and mice overexpressing IL-4 appeared to be protected from bleomycin-induced lung lesions.
(28). Moreover, neutralizing antibodies directed against IL-4 were unable to reduce bleomycin-induced lung fibrosis, unlike anti-IL-13 antibodies (5). Finally, FITC-induced lung fibrosis in BALB/c mice was not modified by IL-4 deficiency (30). These observations suggest that cellular and molecular events, as well as genetic factors, may contribute to the differential fibrotic effects of IL-4, as suggested by Ma et al. (38).

The role of IL-13 has also been extensively studied in experimental models of lung fibrosis. In murine models of FITC- or bleomycin-induced lung fibrosis, IL-13-/- and IL-13Rα2-/- mice were shown to be significantly protected from fibrosis development (16, 30). Neutralizing antibodies against IL-13 or pulmonary administration of chimeric IL-13 immunotoxin molecule (IL-13-PE) had marked therapeutic effects on bleomycin-induced lung fibrosis (5, 29). Using a transgenic mouse model, Lee and colleagues (35) demonstrated that overexpression of IL-13 in the lungs led to the development of subepithelial and adventitial airway fibrosis, probably mediated by TGF-β1 stimulation and activation. In murine schistosomiasis, egg-induced lung granuloma and fibrosis were found to be dependent on IL-13 responsiveness, whereas IL-4/IL-13-stimulated alternative macrophage activation was not essential, as recently demonstrated in a macrophage/neutrophil-specific IL-4Rα-deficient mouse model (20).

The Th2 paradigm is also supported by the results of several human studies, showing preferential Th2 polarization in fibrotic lung diseases. Increased levels of IL-4 and IL-13, but not IFN-γ, were detected in BAL cells recovered from patients with pulmonary fibrosis, compared with healthy subjects (2, 19, 54). Flow cytometry analysis of intracellular cytokines of BAL cells also revealed a predominant Th2 profile in patients with idiopathic pulmonary fibrosis (IPF) (45). Finally, a pathogenic role has recently been suggested for IL-4 gene promoter polymorphisms in the etiology and pathogenesis of IPF (52).

Clearly, immune polarization status during the development of silica-induced lung fibrosis is still a matter for debate. Both Th1 (8, 13, 14, 17, 18, 22, 51) and Th2 responses (1, 4, 10, 22) have been found to be associated with the development of silicosis. Thus, in our study, we examined the potential role of type 2 immune polarization by investigating the functions of IL-4 and IL-13 in the fibrotic process in response to silica particles in C57BL/6 wild-type, IL-4-/-, and IL-4Rα-/- mice. Upregulation of IgG1, but not IgG2a, in the BAL fluid of wild-type animals treated with silica indicated that the fibrotic process was associated with a Th2-like immune response. Two months after silica instillation, measurement of IgG1 levels in the BAL fluid of IL-4-/- and IL-4Rα-/- mice showed this type 2 immune response to be strongly impaired in both strains of knockout mice. This is consistent with the results of several studies in IL-4-/- or IL-4Rα-/- mice that have reported IL-4 to be the promoting factor of Th2 differentiation (31, 32, 41). IL-4-independent mechanisms have also been identified in in vivo Th2 development, however (7, 11, 40).

Evaluation of the different parameters of the silicotic response in IL-4-/- and IL-4Rα-/- mice demonstrated that impairment of the silica-induced type 2 immune response did not influence the inflammatory response or intensity, or the histological characteristics of lung fibrosis in either mouse strain. Analysis of IgG2a, IL-12p70, and IFN-γ levels in the BAL fluid of knockout animals proved that the development of pulmonary fibrosis in IL-4-/- and IL-4Rα-/- mice could not be explained by a switch to a type 1 immune response in the absence of IL-4- or IL-13-mediated signal transduction. Other studies investigating lung fibrosis using Schistosoma mansoni eggs or gamma-herpesvirus showed that treated IL-4/IL-10- or IL-13/IL-10-deficient mice developed Th1-biased immune response (IFN-γ overproduction), tissue damage, and limited fibrosis (47), whereas Th2-polarized mice (IL-12/IL-10- or IFN-γR-deficient mice) developed massive lung collagen deposition and upregulated Th2 cytokine expression (42, 47). These studies clearly demonstrate a shift between the two responses and their specific pathophysiological roles. However, these mutually exclusive responses do not occur in silica-induced lung fibrosis (Fig. 6). We propose that, whereas either a type 1 or type 2 immune response might well be induced during the different stages of silicosis, neither plays a critical role in the evolution of lung fibrosis.

During the last few years, a number of reports have been published on the involvement of CD4+ lymphocytes in the pathogenesis of silicosis (3, 12, 33). They demonstrated that treatment with anti-CD4 antibodies reduced the intensity of silica-induced lung fibrosis in mice, suggesting that this T cell population actually contributes to the establishment and/or evolution of fibrotic lesions. Our present data indicate that significant impairment of the Th2-like response does not influence the establishment of silica-induced fibrosis, which suggests that other CD4+ lymphocyte populations could be closely associated with the development of the disease. These observations are consistent with our previously published data on silicosis development, showing no clear association with alternative polarization of alveolar macrophages (M2) (39). Our data thus demonstrate, contrary to other models of lung fibrosis, that the Th2-like immune response associated with the

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**Fig. 6.** Silicosis in IL-4−/− and IL-4Rα−/− mice is not associated with type 1 immune polarization. Two months after instillation, IgG2a (A), IFN-γ (B), and IL-12p70 (C) levels were assessed in the BAL fluid of saline- or silica-treated C57BL/6 IL-4−/−, IL-4−/−, and IL-4Rα−/− mice. Bars represent means ± SE (n = 6). No significant difference was observed for any of the markers between either saline- or silica-treated IL-4−/−, IL-4−/−, or IL-4Rα−/− mice.
development of silicosis is not required for the establishment of the fibrotic process.

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