

Review

Pulmonary Fibrosis in Response to Environmental Cues and Molecular Targets Involved in Its Pathogenesis

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Abstract: Chronic lung injury resulting from a variety of different causes is frequently associated with the development of pulmonary fibrosis in humans. Although the etiology of pulmonary fibrosis is generally unknown, several sources of evidence support the hypothesis that a number of environmental and occupational agents play an etiologic role in the pathogenesis of this disease. The agents discussed in this review include beryllium, nylon flock, textile printing aerosols, polyvinyl chloride and didecyldimethylammonium chloride. The authors also describe a variety of animal models, including genetically modified mice, in order to investigate the molecular mechanism of pulmonary fibrosis, focusing on chemokine receptors, regulatory T cells and transforming growth factor- β and bone morphogenetic protein signaling. Overall, we propose the concept of toxicological pulmonary fibrosis as a lung disease induced in response to environmental cues. (DOI: 10.1293/tox.24.9; *J Toxicol Pathol* 2011; 24: 9–24)

Key words: beryllium, nylon flock, DDAC, TGF- β , chemokine receptor, regulatory T cell

Introduction

Chronic lung injury resulting from a variety of different causes is frequently associated with the development of pulmonary fibrosis, which is characterized by replacement of the normal alveolar structure by a thickened extracellular matrix (ECM) with consequent reduction in the capacity for gas exchange¹. Interstitial lung diseases (ILDs), more accurately known as diffuse parenchymal lung diseases (DPLDs), are a diverse group of pulmonary disorders, which include idiopathic interstitial pneumonias (IIPs), drug-induced ILDs and occupational and environmental lung diseases². IIPs are categorized as heterogeneous clinicopathologic disorders, which include nonspecific interstitial pneumonia (NSIP), respiratory bronchiolitis (RB)/RB-associated ILD (RB-ILD), cryptogenic organizing pneumonia (COP)/organizing pneumonia (OP), desquamative interstitial pneumonia (DIP), lymphocytic interstitial pneumonia (LIP), acute interstitial pneumonia (AIP)/diffuse alveolar damage (DAD) and idiopathic pulmonary fibrosis (IPF)/usual interstitial pneumonia (UIP), which have been

defined by the American Thoracic Society/European Respiratory Society consensus classification^{3–5} (Table 1). IPF/UIP is the most common disorder among the IIPs, characterized by unrelenting progression of fibrosis; DIP and RB-ILD are related pathologically; and AIP, characterized by an acute onset of respiratory symptoms, is analogous to acute respiratory distress syndrome, differing only in that it is not preceded by an identifiable catastrophic event⁵.

Although the etiology of IPF is generally unknown, several sources of evidence support the hypothesis that a number of environmental and occupational agents such as cigarette smoke, agriculture/farming, livestock, wood dust, metal dust and stone/sand play an etiologic role in the pathogenesis of this disease⁶. With respect to occupational and environmental lung diseases, the industrial revolution of the 19th century resulted in widespread exposure to dust, which promoted the development of diseases such as silicosis, coal workers' pneumoconiosis and asbestosis; however, improvement in working conditions resulted in a decrease in the incidence of these lung diseases². In the past 20–30 years, an increase in industrial processes has led to the production and use of a large number of chemicals, some of which are currently reported as causes of ILDs (e.g., beryllium, nylon flock, textile printing aerosols, polyvinyl chloride (PVC) and organic vapors from flavorings). Sarcoidosis, a distinct entity of ILDs, is a granulomatous disease of unknown etiology, occurring most commonly in the lung; however, non-infectious environmental agents (e.g., beryllium, aluminum

Table 1. Histopathological Pattern of Interstitial lung Diseases (ILD)^{3–5}

Clinical diagnosis	Pathology	Key features
NSIP	NSIP	Cellular pattern: Mild to moderate interstitial chronic inflammation with hyperplastic pneumocytes. Fibrosing pattern: Diffuse thickening of alveolar walls by dense or loose connective tissues and interstitial chronic inflammation (basically lacking the UIP pattern and preserving alveolar architecture).
RB	RB-ILD	Bronchiolocentric accumulation of alveolar macrophages (laden iron) with mild bronchiolar fibrosis and chronic inflammation.
COP	OP	Patchy plugs of loose connective tissues within a bronchiole, the adjacent alveolar ducts and alveolar spaces and with mild interstitial chronic inflammation.
DIP	DIP	Marked alveolar accumulation of macrophages (laden iron) with mild interstitial thickening and chronic inflammation.
LIP	LIP	Diffuse thickening of alveolar walls by infiltration of T cells, plasma cells and macrophages and MALT hyperplasia.
AIP	DAD	Diffuse alveolar wall thickening by proliferation of connective tissues, inflammation and deposition of hyaline membranes.
IPF	UIP	Patch fibrosis with remodeling of the lung structures, fibroblast foci, dense collagenous scarring, overlying hyperplastic pneumocytes and cystic changes (honeycomb change lined by columnar respiratory epitheliums).

NSIP, nonspecific interstitial pneumonia; RB, respiratory bronchiolitis; RB-ILD, RB-associated with ILD; COP, cryptogenic organizing pneumonia; OP, organizing pneumonia; DIP, desquamative interstitial pneumonia; LIP, lymphocytic interstitial pneumonia; MALT, mucosa-associated lymphoid tissue; AIP, acute interstitial pneumonia; DAD, diffuse alveolar damage; IPF, idiopathic pulmonary fibrosis; UIP, usual interstitial pneumonia.

Table 2. Environmental and Occupational Agents and Pulmonary Fibrosis

Chemicals	Disease	Key factors/Mechanism	References
Beryllium	ABD/CBD ^ψ	Hypersensitivity, oxidative stress	10, 11, 16, 23, 24
Nylon flock	Flock Worker's Lung [#]	Hypersensitivity, iron-mediated	30–32
Textile paint	Ardystil syndrome [§]	Acramine F	37–41
PVC	Pulmonary fibrosis ^ψ	Residual additives, eosinophilia	43–45, 50–52
DDAC	†	Cytotoxicity, inflammation	63–65, 69

PVC, polyvinyl chloride; DDAC, didecyldimethylammonium chloride; ABD, acute beryllium disease; CBD, chronic beryllium disease.[#] Diagnosed as lymphocytic bronchiolitis and peribronchiolitis with lymphoid hyperplasia. [§] Diagnosed as organizing pneumonia. ^ψ With a granulomatous reaction. [†] Pulmonary fibrosis has not been reported, but asthma might occur based on epidemiological studies.

and zirconium) can produce pulmonary lesions similar to those of sarcoidosis². The agents discussed in this review include beryllium, nylon flock, textile printing aerosols, PVC and didecyldimethylammonium chloride (DDAC) (Table 2). Common ILD-causing drugs include amiodarone, antibiotics, nonsteroidal anti-inflammatory drugs, chemotherapeutic agents (e.g., bleomycin and methotrexate) and nitro drugs, all of which can produce virtually all the histopathologic patterns of interstitial pneumonia, including NSIP, OP, DIP and a pulmonary granulomatosis-like reaction⁷. However, this review does not include drug-induced ILDs, except for a bleomycin animal model, for discussing the mechanism of pulmonary fibrosis.

The pathogenesis of pulmonary fibrosis is complex,

involving innate and adaptive immunoreactions against exogenous environmental cues and subsequent molecular and cellular responses, leading to an imbalance of profibrogenic and antifibrogenic effects in healing processes. This review also focuses on specific pathobiological mediators such as chemokines and chemokine receptors, regulatory T cells (T_{reg} s) and transforming growth factor- β (TGF- β) and bone morphogenetic protein (BMP) signaling.

Beryllium

Beryllium, which mostly occurs naturally as beryllium aluminum silicate, is the fourth lightest element, having a low density (1.85 g/cm³ at 20 °C), high melting point

(1287 °C) and high tensile strength, and these properties have led to its incorporation into many high-technology applications (e.g., electronics, aerospace, machinery and nuclear weapons manufacture)^{8,9}. Exposure to beryllium at workplaces continues to be a public health concern, with approximately one million individuals exposed and potentially at risk of developing acute beryllium disease (ABD) and chronic beryllium disease (CBD), both of which involve hypersensitivity reactions to beryllium^{10,11}. Genetic susceptibility to CBD has been linked to a major histocompatibility complex (MHC) class II isotype, human leukocyte antigen (HLA)-DP alleles, which contain a glutamic acid residue at amino acid position 69 in the β -chain^{12–14}. Among the mouse strains FVB/N, AKR, Balb/c, C3H/HeJ, C57/BL6, DBA/2 and SJL/J, the FVB/N strain has been reported to be the least responsive, while the SJL/J and C57/BL6 strains have been shown to be the highest responders¹⁵.

CBD is characterized by the presence of noncapsulating granulomatous inflammation in the lung and subsequent development of progressive fibrosis¹⁶. Accumulating evidence clearly suggests that CBD is recognized as an immune-mediated granulomatous reaction. Six to nine months after a single exposure of A/J and C3H/HeJ mice to beryllium for 90 min, lung responses comprised interstitial aggregates of lymphocytes—B cells centrally and T cells peripherally—and granulomatous pneumonia characterized by intra-alveolar foamy macrophages, giant cells and neutrophils, and multifocal interstitial granulomas¹⁷. Evidence suggests that recruitment of beryllium-specific CD4⁺ T cells to the lung significantly mediates the immunopathogenesis of CBD. These CD4⁺ T cells expressed an effector memory T cell phenotype^{10,13} and secreted the T helper 1 (Th1)-type cytokines interferon- γ (IFN- γ), interleukin-2 (IL-2) and tumor necrosis factor- α (TNF- α) upon beryllium exposure^{18–20}. Programmed death-1, which is a member of the CD28 family and a negative regulator of T cell function, was expressed at significantly high levels in bronchoalveolar lavage (BAL) CD4⁺ T cells from CBD patients, plausibly leading to induction of T cells and preventing activation-induced cell death²¹. Lately, ABD has also been recognized as a specific lymphocytic alveolitis rather than a nonspecific inflammatory process of an irritant¹¹. Beryllium-specific CD8⁺ T cells may play a minor role, if any, in the pathogenesis of this disease, as inferred from the results of analysis of BAL samples from CBD patients¹⁰.

Beryllium-induced oxidative stress plays a role in the pathogenesis of granulomatous inflammation in CBD. A decrease in intracellular thiols, glutathione and cysteine was observed in peripheral blood mononuclear cells from beryllium-sensitized and CBD patients, and beryllium-mediated T cell proliferation was inhibited by the thiol antioxidant *N*-acetylcysteine and the catalytic antioxidant manganese (III) *in vitro*²². Increased glutathione in lung epithelial lining fluid from CBD patients is correlated with glutathione peroxidase activity²³. Heme oxygenase-1 (HO-1) activity is also increased in induced sputum from CBD patients²⁴. To-

gether, the data imply that augmentation of glutathione- and HO-1-mediated antioxidant systems may be involved in the process of CBD.

Although cellular and molecular mechanisms underlying berylliosis have been investigated, the exact profibrogenic factors have not been identified thus far. Mast cells may contribute to the pathogenesis of CBD, serving as an important source of a 17.8-kDa isoform of basic fibroblast growth factor, which is a potent mitogenic and chemotactic factor, regulating fibroblast proliferation and ECM production²⁵. In contrast, beryllium at low concentrations inhibits the growth of normal human fibroblasts, as proved by G₀-G₁/pre-S phase arrest and senescence associated with elevation of the p53, p21 and p16 levels^{26,27}. At high concentrations, beryllium is somewhat carcinogenic to BALB/c-3T3 cells (mouse embryonic fibroblast cell line)²⁸. TGF- β signaling in CBD has not been fully investigated, although Jonth *et al.* (2007) have reported that the codons –509 C and 10T, implicated in the production of low levels of TGF- β 1 protein, are shared susceptibility factors associated with more severe granulomatous inflammation in CBD²⁹. Thus, investigations of profibrogenic/antifibrogenic signaling would provide a better understanding of the pathobiology of CBD and aid in exploring therapeutic targets.

Nylon Flock and Textile Paints

Flock is cut or pulverized fiber—synthetic or natural—of small diameter that produces a velvet-like coating when applied to an adhesive-coated fabric or other material. Flocked fabrics of nylon (polyamide), rayon (cellulose acetate), Dacron (ethylene glycol-terephthalic acid) and polyester fibers are becoming increasingly popular, especially as upholstery coverings and blankets. Kern *et al.* (2000) reported a chronic nongranulomatous ILD termed “Flock Worker’s Lung” in nylon flocking industry workers at a Rhode Island plant in the 1990s³⁰. The workers developed BAL eosinophilia, NSIP, OP and/or lymphocytic bronchiolitis and interstitial fibrosis. A small number of North American patients have also been reported to have nylon flock-related DIP. At a clinical pathology workshop hosted by the National Institute for Occupational Safety and Health, consulting pulmonary pathologists diagnosed “lymphocytic bronchiolitis and peribronchiolitis with lymphoid hyperplasia” in workers at 5 nylon flock facilities in 3 different U.S. states and a Canadian province, on the basis of clinical examination and histopathological findings³¹. This descriptive terminology was supported by the Centers for Disease Control (CDC) at a clinical-pathological workshop hosted by the CDC³².

Although nylon fibers may act as haptens and trigger an allergic reaction (e.g., occupational asthma)³³, *in vivo* experiments have failed to demonstrate pulmonary hypersensitivity reactions to nylon. Airborne dust (nylon flock) collected at a nylon flocking plant interacted with alveolar macrophages and induced mild-to-moderate, multifocal, suppu-

rative pneumonia around bronchioles after intratracheal instillation in rats (10 mg/kg body weight)³⁴. In rats exposed to aerosols of uncoated, finish-free nylon respirable-sized, fiber-shaped particulates at 6 hours per day and 5 days per week for 4 weeks, there were no significant increases in lung weight, indications of pulmonary inflammation or alveolar macrophage functional deficits, as compared with the control rats³⁵. Thus, since workers exposed to high concentrations of nylon fiber dust may be at an increased risk of ILDs, suitable animal models should be developed to clarify the role of nylon flock in immune-based pulmonary diseases.

Synthetic fibers do not include metals. Synthetic fibers present an insoluble solid-liquid interface to the surrounding host tissue, and the fiber surface includes functional groups having the ability to mobilize metal from endogenous sources, introducing a metal-catalyzed oxidative stress in the lower respiratory tract. Ghio *et al.* (2006) reported that a ferruginous body, with its accumulated iron, functions as an indicator of both metal accumulation and as a potential oxidative stressor³⁶. The clinical presentations of 2 patients employed in a textile mill were consistent with a chronic fibrotic disease of the lung, in which there were areas of traction bronchiectasis with chronic inflammation and bronchial metaplasia and bronchiolitis with mixed lymphocytic and eosinophilic infiltration. Staining of resected tissue for iron revealed structures having the appearance of ferruginous bodies. Energy dispersive X-ray analysis and scanning electron microscopy revealed that the fibers isolated from the lung were carbonaceous and originated from the mill. Thus, metal, particularly iron, may play a role in the pathogenesis of Flock Worker's Lung.

Besides synthetic fibers, textile paints may affect human health in these industries. An outbreak of severe respiratory disease called "Ardystil syndrome" occurred in the Community of Valencia, Spain, among factory workers who worked where textiles were air-sprayed with dyes by using the Acramin F paint system. Twenty-two cases of OP were identified, and of the 22 patients, 6 died within a few months³⁷. Besides OP, interstitial fibrosis and DAD were evident³⁸. In another study, after a 1 year follow-up of 27 patients heavily exposed to textile sprays, symptoms were observed to persist in 15 cases³⁹. The main polycationic components of textile sprays are Acramin FWR (a polyurea), Acramin FWN (a polyamide-amine) and Acrafix FHN (a polyamine), and the non-polycationic component is Acramoll W. Acramin FWR, Acramin FWN and Acrafix FHN exhibited considerable toxicity in rat and human type II pneumocytes and alveolar macrophages, while Acramoll W was almost nontoxic⁴⁰. Consistent with the results of *in vitro* experiments, lung damage was caused by Acramin FWR, Acramin FWN, Acrafix FHN or their mixture after intratracheal instillation in hamsters⁴¹. Protein concentration, lactate dehydrogenase (LDH) activity, inflammatory cell number and percentage of polymorphonuclear neutrophils were increased in BAL fluid during the first week, and lung weight remained high for at least a month after

intratracheal instillation. Inflammatory cell infiltration and subsequent fibrosis with collagen deposition were detected in lung histology, which was confirmed by increased hydroxyproline content in dried lung tissue. Acramoll W did not show toxic effects even *in vivo*. Interestingly, multiple positive charges might play an important role in the toxicity mechanism of the polycations with no irritant properties, since the cytotoxicities decreased in the presence of polyanions⁴². Overall, combined exposures to textile dyes and nylon flock could be involved in development of the diseases, and cross-talk between them should be further investigated.

PVC

PVC is the second most commonly used plastic material worldwide, and it is used in numerous products, e.g., credit cards, computer keyboards, flooring material, food containers, medical cannulae, telephones, highway sound barriers and toys. At the end of the synthesis process, the material may exist as respirable dust, the inhalation of which may result in lung function impairment. Epidemiological surveys have identified PVC dust as an etiologic agent in dyspnea with decreased pulmonary function and appearance of opacities in chest radiograph⁴³, in a particular type of pulmonary fibrosis with a granulomatous reaction⁴⁴ and in interstitial fibrosing pneumonia⁴⁵. Electron microscopy revealed a nonhomogenous material in the cytoplasm of giant multinucleated cells and/or alveolar macrophages, which was identified to be PVC particles^{44,46,47}. Human alveolar macrophages that engulfed PVC powder showed a similar ultrastructural appearance⁴⁶. It is important to note that early PVC pneumoconiosis may regress in human cases⁴⁸.

Several studies on PVC exposure to animals have been conducted, although there is no evidence of fibroproliferation in the lung. Rats, guinea pigs and monkeys were exposed by inhalation (6 hours per day, 5 days per week) for 22 months; autopsies of rats and guinea pigs and of monkeys were performed after 12 and 22 months of exposure, respectively. PVC particles were found in aggregated alveolar macrophages, but there were no other effects either on lung histology or pulmonary function⁴⁹. Hoet and his colleagues have conducted detailed investigations of the effects of PVC exposure *in vitro* and *in vivo*. Emulsion of PVC particles with a mean diameter of 2 μm exhibited moderate toxicity in cultures of different pulmonary cells, i.e., rat and human alveolar macrophages, rat type II pneumocytes, A549 cells (type II cell type) and THP-1 cells (macrophage-like). It was found that the cytotoxicity and inflammatory potential of some PVC particles might be mostly due to their residual additives, since removal of the residual additives considerably reduced their toxic effects⁵⁰. However, *in vivo* experiments did not confirm the conclusion from the *in vitro* toxicity tests. Male Wistar rats received a single intratracheal instillation of the washed "additive-free" PVC particles. As a result, they exhibited pulmonary inflammation and damage similar to that in the silica-exposed rats at 2 days; how-

ever, at 90 days, most parameters returned to the control level, except for minor histopathological lesions. Interestingly, additive-free PVC particles caused less neutrophil but more eosinophil influx than the original PVC particles containing residual detergents. The washed additive-free PVC particles might be more hydrophobic than the original PVC particles, but pulmonary eosinophilia was induced via an unknown mechanism⁵¹. Repeated intratracheal instillation of PVC particles for 3 weeks increased the number of CD4⁺ and CD8⁺ T cells in BAL fluid, and compared with the original PVC particles, the additive-free PVC particles tended to induce more changes in many endpoints, including BAL eosinophilia⁵², as observed in the single intratracheal instillation study⁵¹. Experimental BAL eosinophilia is apparently linked to asthma, as reported in epidemiological studies on PVC^{53,54}. Together, deposition of PVC particles with or without residual detergents in alveolar macrophages and subsequent immunoreactions may contribute to the development of PVC-induced pneumoconiosis.

DDAC

DDAC [C₁₀H₂₁N(CH₃)₂C₁₀H₂₁·Cl], a representative dialkyl quaternary ammonium compound (QAC), is used as a detergent in wood preservatives, as a disinfectant against pathogens and in other applications^{55–58}. DDAC formulations are directly added to water in swimming pools, spas and humidifiers and used for treatment of surfaces in institutional, commercial, industrial and residential settings by fogging, flooding, immersion, wiping, mopping, aerosol spray treatment and low- and high-pressure spray treatments with final spray concentrations ranging from 0.5 to 26320 ppm⁵⁹. The action of DDAC on the cell membrane causes leakage of intracellular molecules⁶⁰, together with autolysis and subsequent death of *Escherichia coli* and *Staphylococcus aureus*⁶¹. Occupational exposure to QACs, including DDAC, therefore, is known to cause contact dermatitis, conjunctivitis⁶² and asthma^{63–65} among professionals working in healthcare and cleaning industries. Although DDAC does not seem to contaminate the indoor hospital atmosphere during the disinfection process, it can contaminate working atmospheres if it is put in suspension by aerosolization⁶⁶. The same health concerns are expected to exist for veterinary professionals, since DDAC is a useful disinfectant for preventing the entry and spread of infectious disease agents, including enveloped and non-enveloped viruses, in domestic animals^{67,68}.

Recently, we reported that DDAC induces pulmonary injury and fibrosis in mice⁶⁹. To our knowledge, this was the first report on pulmonary fibroproliferative response to topically applied DDAC. In mice with intratracheally instilled DDAC, pulmonary cytotoxicity (increase in protein concentration and LDH activity in BAL fluid) was evident in association with inflammation, which was confirmed by the expression of monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1 α , MIP-1 β

and regulated upon activation, normal T cell expressed and secreted (RANTES) in BAL fluid. These changes were accompanied by altered gene expression of the chemokine (C–C motif) receptors Ccr1, Ccr2, Ccr3 and Ccr5 in the lung. Expression of chemokines might principally be derived from alveolar macrophages, as *in vitro* cytotoxicity was higher in the macrophages (J774.1) than in epithelial cells (A549) and isolated mouse lung fibroblast (MLF)-like cells (Ohnuma *et al.*, unpublished data). The cytotoxic and inflammatory phases were accompanied or followed by pulmonary remodeling, i.e., fibrosis, which was evident by an increase in interstitial connective tissue and fibroblasts/myofibroblasts, as demonstrated by Masson's trichrome stain; immunohistochemistry for α -smooth muscle actin (α -SMA); and mRNA expression of type I procollagen⁶⁹. Overall, pulmonary injury peaked in the first week after a single instillation of DDAC in the lung; fibroproliferation gradually became profound in the first week and peaked in the second week, subsequently reducing in the third week (Fig 1). The role of TGF- β and BMP in fibrogenesis is currently being investigated in our laboratories.

Limited toxicology information is currently available for DDAC^{59,70}. Similar to DDAC, benzalkonium chloride (BAC) is a mixture of alkylbenzyltrimethylammonium chlorides belonging to the QAC group and is widely used as a disinfectant and in preservatives and stabilizers. Rats that aspirated BAC following oral administration showed AIP⁷¹. Furthermore, BAC inhalation caused strong inflammation via an irritant activity, as demonstrated by an increase in lung weight and high levels of total protein, LDH activity, matrix metalloproteinase-9 (MMP-9), TNF- α and MIP-2 in BAL fluid; however, no histopathological examinations were carried out⁷². The mode of action of DDAC and BAC hypothetically poses human health concerns, particularly if people are unknowingly exposed to these QACs or experience prolonged workplace exposure. In our current protocol, a similar sequence of pulmonary changes as those occurring in bleomycin- or fluorescein isothiocyanate (FITC)-induced lung injury^{73–77} was observed on DDAC administration; it seemed that the fibrotic response to DDAC administration was more rapid, but weaker, than that to bleomycin or FITC treatment. This unique property of pulmonary responses would be helpful in better understanding pulmonary fibrotic diseases particularly at promoting and regenerating phases.

Molecular Targets Involved in the Pathogenesis of Pulmonary Fibrosis

Chemokines and CCRs

Inflammatory reactions, involving an influx of particular cell types expressing cytokines, chemokines and cell surface molecules, precede and accompany pulmonary fibrosis, whereas nonspecific inflammatory processes end with nonfibrous tissue repair⁷⁸. Several cytokines and chemokines and their receptors have been investigated with regard to their contribution to the pathogenesis of pulmonary

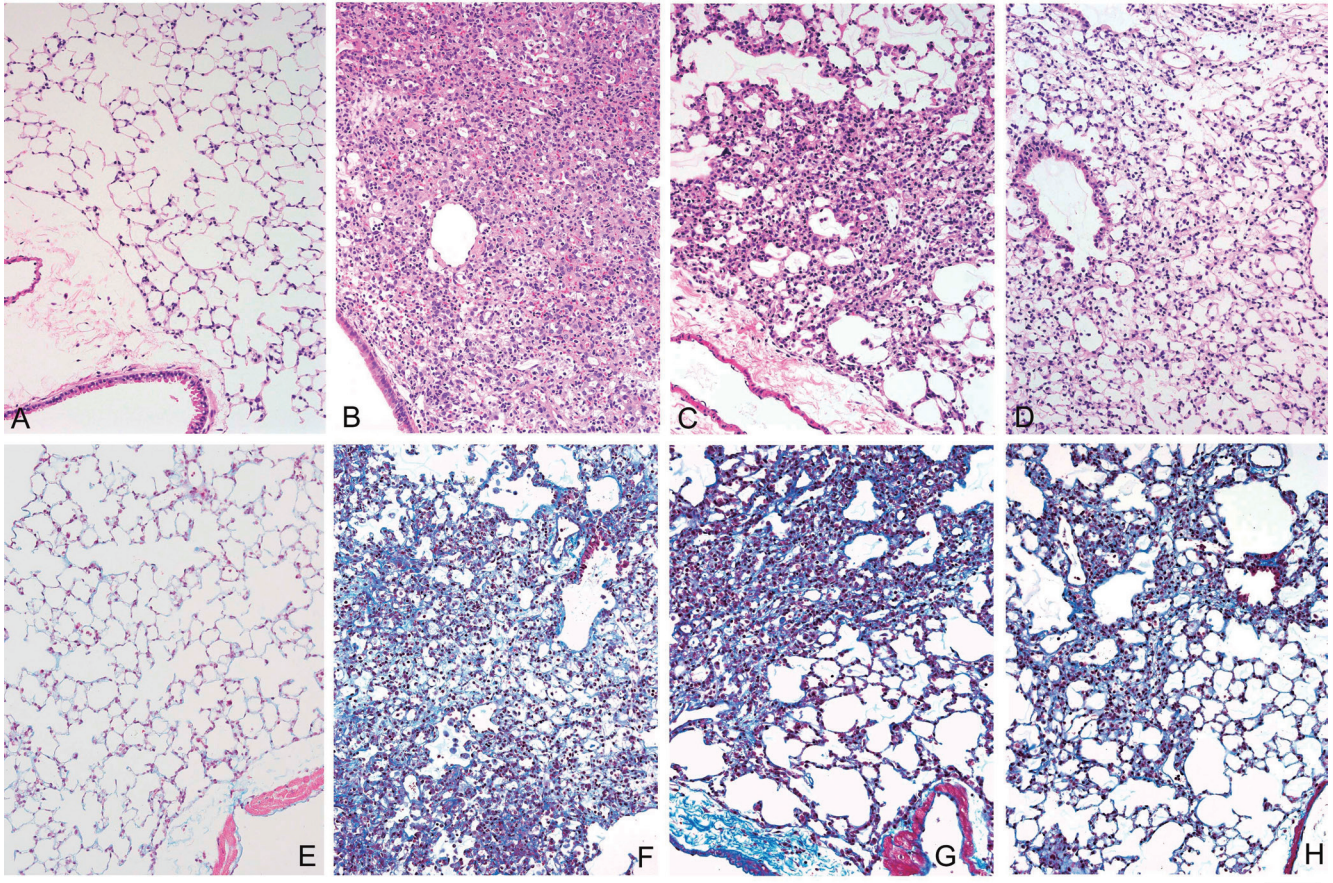


Fig. 1. DDAC induces inflammation and fibroproliferation in the lungs. Mice were intratracheally instilled with 0.01% of DDAC or the vehicle control and were sacrificed on day 7, 13, or 20 after the treatment⁶⁹. Lung tissue samples were subjected to histopathological examination. Representative images of the lung tissues stained with hematoxylin and eosin (A–D) and Masson trichrome (E–H) from the vehicle control (A, E) and DDAC-treated mice on day 7 (B,F), 13 (C,G) or 20 (D,H). Scale bar: 50 μ m.

fibrosis in human and animal models^{75,79–82}.

Murine MCP-1, also known as JE or chemokine (C–C motif) ligand 2 (CCL2), is considered homologous to human CCL2, which is produced by a variety of cells, including endothelial cells, fibroblasts, monocytes, and smooth muscle cells⁸³. MCP-1 and its receptor CCR2 are involved in fibrosis via regulation of profibrotic cytokine generation and ECM formation^{84–86}. This is supported by the fact that CCR2-knockout mice with FITC- or bleomycin-induced pulmonary fibrosis showed improvement in fibrosis^{74,87}. The protective effect was associated with suppressed macrophage infiltration and macrophage-derived MMP production⁸⁸ and enhanced alveolar epithelial cell inhibition of fibroblast proliferation⁸⁹. CCR2 also mediates neutrophil recruitment through effects on intravascular adherence and subsequent transmigration⁹⁰. Surprisingly, MCP-1 binds not only to alveolar macrophages but also to bone marrow-derived fibrocytes, both of which express CCR2⁹¹. Interestingly, transplant of CCR2^{+/+} bone marrow cells into CCR2^{-/-} recipients restored recruitment of lung fibrocytes and susceptibility to FITC-induced fibrosis. The study showed that fibrocytes

may contribute to fibrogenesis in several ways: (1) fibrocytes may directly contribute to fibrosis by secreting collagen, since the amount of collagen secreted by CCR2^{+/+} fibrocytes exposed to MCP-1 was greater than that secreted by CCR2^{-/-} fibrocytes; (2) fibrocytes secrete TGF- β 1, and thus, CCR2-mediated recruitment of fibrocytes to the lung may serve to activate resident fibroblasts via the secretion of TGF- β 1; and (3) fibrocytes may differentiate into effector fibroblasts. Murine MCP-5/CCL12, which is homologous to human CCL2, may have a higher affinity for activated macrophages than MCP-1⁹². Moore *et al.* (2006) proposed that MCP-5 rather than MCP-1 is more likely to drive fibroproliferation in mice via its interaction with CCR2⁹³. Inconsistent with this, Tsou *et al.* (2007) demonstrated that MCP-1 and MCP-3, rather than MCP-2 and MCP-5, are the CCR2 agonists that are most critical for the maintenance of monocytes⁹⁴. Thus, although the contribution of CCR2 agonists remains controversial, the MCP/CCR2 system may represent the link between the macrophage-dominant phase and the subsequent remodeling phase.

CCR1 is constitutively expressed on monocytes, neu-

trophils, lymphocytes and eosinophils and interacts with MIP-1 α , RANTES, MCP-2 and MCP-3^{83,95}. After bleomycin challenge in mice, CCR1 mRNA levels and CCR1-positive cells increased and peaked on day 7, which paralleled the expression of MIP-1 α and RANTES⁷³. Anti-CCR1 antibody inhibited inflammatory cell accumulation and collagen deposition, resulting in dramatic improvement in survival. In contrast, bleomycin-induced an increase in intrapulmonary macrophage and fibrocyte numbers, and collagen deposition was attenuated in MIP-1 α ^{-/-} mice but not in CCR1^{-/-} mice⁷⁷. CCR1 was observed to play a limited role in pulmonary granuloma formation, since the granuloma size remained unchanged in CCR1^{-/-} mice, as compared with CCR1^{+/+} mice, challenged with Sepharose beads coupled to the purified protein derivative of *Mycobacterium bovis* or soluble antigens derived from *Schistosoma mansoni* eggs⁹⁶. However, natural killer (NK) cell recruitment and Th1 response (expression of IL-2 and IFN- γ) were reduced, while Th2 response (expression of IL-5 and IL-13) was enhanced in CCR1^{-/-} mice in comparison with CCR1^{+/+} mice. Thus, CCR1 seemed to be critical in immune-mediated fibrotic disease, and this was supported by the fact that airway remodeling, e.g., goblet cell production and subepithelial fibrosis, was absent in CCR1^{-/-} mice during chronic fungal allergic airway disease⁹⁷. Rather than CCR1, CCR5, another receptor of MIP-1 α , seemed to be critical in regulating fibrosis, since CCR5^{-/-} mice showed attenuation of collagen deposition and macrophage infiltration induced by bleomycin⁷⁷.

CCR3 is highly expressed on neutrophils and eosinophils, and its ligand is eotaxin-1/CCL11^{83,95}. Administration of bleomycin induces a marked pulmonary expression of eotaxin-1 and CCR3⁷⁶. Eotaxin-1^{-/-} mice showed reduced pulmonary fibrosis associated with a decrease in TGF- β 1 mRNA expression, whereas increased expression of eotaxin-1 by using eotaxin-1-expressing adenovirus enhanced bleomycin-induced lung fibrosis. Similar to eotaxin-1^{-/-} mice, those treated with neutralizing CCR3 antibodies showed reduced pulmonary fibrosis, eosinophilia, neutrophilia and TGF- β 1 mRNA expression.

CCR4 is a selective marker for Th2 lymphocytes and is expressed in dendritic cells, NK cells and monocytes; thymus- and activation-regulated chemokine (TARC)/CCL17 and macrophage-derived chemokine (MDC)/CCL22 are both high-affinity ligands and high-potency agonists for CCR4^{83,95,98}. The mRNA and protein expressions of TARC and MDC were elevated in lung tissues after bleomycin treatment, which was related to alveolar expression of CCR4⁹⁹. Neutralization of TARC, but not MDC, led to a reduction in collagen deposition and BAL leukocytes, including CD4⁺ T cells, CD8⁺ T cells, NK cells, macrophages and neutrophils. TARC was expressed in alveolar epithelial cells in the lung tissues of bleomycin-treated mice and human patients with IPF. These data support the results of an experiment conducted using CCR4^{-/-} mice, which protected from bleomycin-induced lethal inflammatory and fibrotic

responses, with evidence of a reduction in the TARC and MDC levels observed in lung homogenates¹⁰⁰. A major contribution to this protective effect might be the regulatory role of CCR4 in determining the phenotype of macrophages during the acute inflammatory phase by switching classically activated or M1 macrophages (type-1 polarization, induced by Th1 signals, i.e., IFN- γ expression) to alternatively activated or M2 macrophages (type-2 polarization, induced by Th2 signals, i.e., IL-4 and IL-13 expression). In the presence of CCR4, TARC and MDC promoted tissue injury through induction of the M1 macrophage phenotype with increased nitric oxide synthase 2 (NOS2) and decreased arginase 1 expression; in the absence of CCR4, M2 macrophage activation occurred through suppression of NOS2 and increased expression of arginase 1 and found in inflammatory zone 1 (FIZZ1), favoring upregulation of a decoy receptor, D6, which attenuates inflammation and tissue injury by internalizing and degrading chemokines. Thus, it appears that alterations in M1/M2 marker expression are confined to the early inflammatory stage and that the establishment of a fibrotic process is not necessarily associated with M2 polarization¹⁰¹. Furthermore, reduction in arginase 1 in lung epithelial cells enhanced the levels of NO metabolites and S-nitrosylated proteins and resulted in increased TNF- α - or lipopolysaccharide (LPS)-stimulated nuclear factor- κ B (NF- κ B) DNA binding¹⁰². Coculture of FIZZ1-expressing type II alveolar epithelial cells with fibroblasts stimulates α -SMA and type I collagen expression independent of TGF- β ¹⁰³ and dependent on Notch/Jagged 1¹⁰⁴. The imbalance of arginase 1 and NOS2 in association with the induction of FIZZ1 may contribute to fibrogenesis, depending on the stage of inflammation and specific cell types.

CCR7 is expressed by T and B cells and plays an important role in T cell and dendritic cell trafficking in association with its ligands, Epstein-Barr-induced 1 (EBI1)-ligand chemokine (ELC)/CCL19 and secondary lymphoid-tissue chemokine (SLC)/CCL21^{83,98}. CCR7 expression is raised in a severe form of IIP, UIP, and localized to focal areas consisting of activated resident fibroblasts, rather than myofibroblasts and bone marrow-derived fibrocytes¹⁰⁵. This was supported by Pierce *et al.* (2007), who showed that CCR7 is expressed by primary IPF/UIP fibroblasts but not by normal fibroblasts. IPF/UIP fibroblasts showed migration and proliferation in response to CCR7 activation by SLC, but not by ELC, and SLC-mediated CCR7 activation seemed to be limited to the extracellular signal-regulated kinase (ERK)1/2 and 90-kDa ribosomal S6 kinase (p90RSK) pathways, since the p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways were not activated by SLC¹⁰⁶. The investigators adopted an unusual technique to clarify the role of SLC/CCR7 axis elegantly. IPF/UIP fibroblasts, NSIP fibroblasts and normal fibroblasts were intravenously injected into severe combined immunodeficiency (SCID) mice lacking adaptive and innate immune features. Interestingly, the mice with IPF/UIP fibroblasts showed patchy interstitial fibrosis and those with NSIP fi-

broblasts showed a more diffuse interstitial fibrosis, with an increased hydroxyproline content in both cases. The injected normal fibroblasts did not induce interstitial remodeling. Neutralization of SLC or CCR7 attenuated pulmonary fibrosis in mice that received either type of fibroblasts. However, delayed onset of pulmonary remodeling, which was noted at 35 days after intravenous injection, remains to be clarified. Similarly, Trujillo *et al.* (2010) showed that CCR7^{-/-} mice were protected from bleomycin-induced pulmonary fibrosis; the effect was related to an early increase in lung CD4⁺ T cells and late increase in CD4⁺CD25⁺FoxP3⁺ T_{regs}, which was consistent with increased IL-2 expression¹⁰⁷, indicating that CCR7-independent T_{regs} expansion is a novel therapeutic target for pulmonary fibrosis.

T_{regs}

T cells are present in the lungs of patients with pulmonary fibrosis because a variety of causes play a pivotal role in the fibrotic process. CD4⁺ T cells are abundant in the lung in granulomatous lung diseases such as IPF, sarcoidosis and berylliosis^{10,13,108}. In fact, CD4⁺ T cells from IPF patients have characteristics typical of cell-mediated pathologic responses, including augmented effector functions (production of TGF-β1, IL-10 and TNF-α), provision of facultative help for immunoglobulin G (IgG) autoantibody production, oligoclonal expansions and proliferations driven by an antigen present in diseased tissues¹⁰⁹. Most previous studies investigated whole T cells, while the emerging novel subpopulations of naturally occurring and adaptive T_{regs} take the center stage as the crucial immunoregulatory cells that are capable of suppressing Th1- and Th2-mediated adaptive immune responses in a cell contact-dependent fashion directly or by acting on antigen-presenting cells¹¹⁰. Since the transcription factor Foxp3 has been shown to be selectively expressed in T_{regs} in humans and mice, Foxp3 mRNA represents a more specific marker than cell surface molecules such as CD25, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and glucocorticoid-induced TNF receptor (GITR)^{111–114}.

In CBD and beryllium-sensitized patients, a small population of BAL CD4⁺ T cells retained CD27 expression, and these CD4⁺CD27⁺ T cells contained the FoxP3-expressing naturally occurring T_{reg} subset, increasing T cell expansion, while the vast majority of CD4⁺ T cells had lost surface CD27 expression, which inversely correlated with lung inflammation in the patients¹¹⁵. The number of CD4⁺CD25⁺FOXP3⁺ T_{regs} and their activities (demonstrated by Th1- and Th2-cytokine expression) were reduced in both BAL and peripheral blood of patients with IPF as compared with those of healthy volunteers and patients without IPF; further, the defective function of BAL T_{regs} highly correlated with the parameters of disease severity, as determined by pulmonary function tests¹¹⁶. Furthermore, downregulation of CD28, which induces FoxP3 and generates CD4⁺CD25⁺ T_{regs}, was evident in IPF patients, and these patients demonstrated infiltrations of CD4⁺CD28^{null} T cells in lung tissues and a positive cor-

relation between the presence of these cells and indication of lung transplantation or death in the next year¹¹⁷. Thus, the data suggested that T_{regs} play a pivotal role in pulmonary fibrosis; however, additional studies extensively addressing T_{reg} interactions with cellular and soluble factors in animal models and human patients are required, since it has been reported that T_{reg} depletion accelerates *in vitro* granuloma growth in mononuclear cell cultures of healthy controls but not in those of patients with active sarcoidosis¹¹⁸.

TGF-β and BMP signaling

TGF-β1 has the ability to induce the expression of ECM proteins in mesenchymal cells¹¹⁹. Cellular TGF-β1 mRNA levels were higher than cellular TGF-β2 and TGF-β3 mRNA levels in IPF patients¹²⁰ and bleomycin-treated mice¹²¹. However, TGF-β2 and TGF-β3 have the potential for *in vitro* fibrogenic activity that is the same as or higher than the *in vitro* fibrogenic activity of TGF-β1¹²². Signaling by TGF-β family members occurs via TGF-β type I and type II receptors (TGF-βRI and TGF-βRII, respectively)^{119,123,124}. Upon phosphorylation by activated TGF-βRI/activin receptor-like kinase-5 (ALK-5), TGF-β-activated Smad2/Smad3 forms a heteromeric complex with Smad4, which is translocated to the nucleus where it functions as a transcription factor. Smad3, in particular, is of critical importance in the development of fibrosis^{124,125}, since loss of Smad3 greatly attenuated histopathological fibrotic responses to bleomycin¹²⁶ or active TGF-β1-expressing adenovirus¹²⁷ in the mouse lung. Active TGF-β1 is a major cytokine that stimulates transcription of the *Collα2* gene, as determined by the binding of Smad3-Smad4 complex to the *cis*-element TGF-β-responsive element (TbRE) in the proximal promoter region¹²⁸.

It is important to note that repression of collagen gene expression is critical for pulmonary disease therapy, and lately, two complex underlying mechanisms have been proposed by Xu *et al.* (2006, 2007): methylation-mediated repression may be mediated by interaction of the regulatory factor for X-box 1 (RFX1) and histone deacetylase 1 (HDAC1) at the TATA box, or IFN-γ-mediated repression may be mediated by the interaction of RFX5, class II transactivator, HDAC2, and peroxisome proliferator-activated receptor γ at the CCAAT and TATTA boxes^{129,130}. Lin *et al.* (2006) identified protein phosphatase 1A (PP1A)/PP2Cα as a Smad2/Smad3 SXS motif-specific phosphatase that inhibits Smad2/Smad3 phosphorylation¹³¹. Interestingly, phosphatase and tensin homolog (PTEN) deleted on chromosome 10 plays a negative role in the TGF-β pathway by forming a complex with PP1A/PP2Cα¹³². A lipid mediator, sphingosine 1-phosphate (S1P), mimics TGF-β-induced cell responses through cross-activation of Smad signaling¹³³ or Rho signaling¹³⁴ cascades, while dihydrosphingosine 1-phosphate (dS1P) plays an opposite role in the regulation of TGF-β signaling by stimulating phosphorylation of PTEN and its subsequent translocation to the nucleus¹³². Thus, repression of collagen gene expression and negative

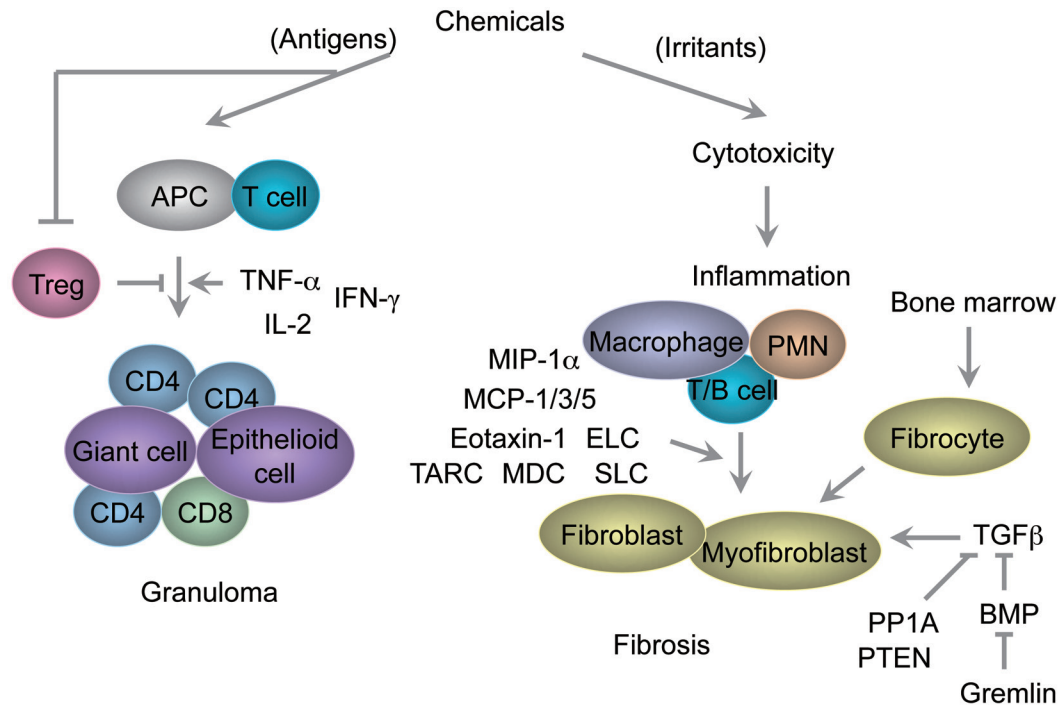


Fig. 2. Proposed concept of toxicological pulmonary granuloma and fibrosis as lung diseases induced in response to environmental cues. Chemicals have the potential to function as either antigens or irritants or both for alveolar macrophages, dendritic cells and/or epithelial cells. Antigens are presented to T cells by APCs, inducing Th1-type inflammation (expressing TNF- α , IFN- γ and IL-2) and recruiting many CD4⁺ and CD8⁺ T cells, which may contribute to the formation of giant and epithelioid cells. Only a few T_{regs} are found in affected humans and animals as compared with the controls, subsequently disturbing the Th1/Th2 balance. On the other hand, cytotoxicity by irritants induces inflammation in association with the expression of chemokines (MIP-1 α , MCP-1, MCP-3, MCP-5, eotaxin-1, TARC, MDC, ELC and SLC) and recruitment of macrophages, PMN and lymphocytes (T cells/B cells), followed by the proliferation of fibroblasts and myofibroblasts. Circulating fibrocytes derived from the bone marrow contribute to fibrotic disorders in the lung. Profibrotic and antifibrotic signaling may be regulated by TGF- β /BMP/gremlin and PPIA/PTEN signaling. APCs, antigen-presenting cells; Th1, T helper 1; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ ; IL-2, interleukin-2; T_{regs}, regulatory T cells; Th2, T helper 2; MIP, macrophage inflammatory protein; MCP-1, monocyte chemoattractant protein-1; TARC, thymus- and activation-regulated chemokine; MDC, macrophage-derived chemokine; ELC, Epstein-Barr-induced 1 (EBI1)-ligand chemokine; SLC, secondary lymphoid-tissue chemokine; PMN, polymorphonuclear neutrophils; TGF- β , transforming growth factor- β ; BMP, bone morphogenetic protein; PPIA, protein phosphatase 1A; PTEN, phosphatase and tensin homolog.

regulation of TGF- β /Smad signaling through formation of a PPIA/PP2C α -PTEN complex, probably mediated by dSIP, may open the possibilities to control the progression of pulmonary fibrosis.

Further, TGF- β terminates the induction of its own target genes by induction of Smad7, which competes with Smad2 and Smad3 for binding to TGF- β RI²⁵; inhibits receptor-activated Smad2 phosphorylation¹³⁵; recruits the E3 ubiquitin ligases Smurf1, Smurf2, Nedd4-2 and WW domain-containing protein 1 (WWP1)/Tiul1 to TGF- β RI and Smads, causing their degradation¹³⁶; or specifically binds to the Smad-responsive element via its Mad homology 2 (MH2) domain and disrupts formation of a TGF- β -induced functional Smad-DNA complex¹³⁷. As expected,

a decrease in the expression of Smad7 was observed after bleomycin instillation in rats, in contrast to a persistent increase in the phosphorylation and nuclear localization of Smad2/Smad3¹³⁸. The same groups compared the levels of phosphorylated Smad3 and Smad7 in isolated fibroblasts from the lungs of control and bleomycin-treated rats under normal conditions and after TGF- β treatment¹³⁹. The basal levels of phosphorylated Smad3 were higher in the normal lung fibroblasts than in bleomycin-treated lung fibroblasts, but the basal levels of Smad7 were comparable. After TGF- β treatment, the levels of phosphorylated Smad3 and Smad7 mRNA were increased in both treated and normal cells, while the protein level of Smad7 was increased in the normal lung fibroblasts as compared with the bleomycin-

treated lung fibroblasts. The investigators concluded that inadequate response to TGF- β and consequent imbalance between the levels of Smad7 and phosphorylated Smad3 might contribute to the fibrotic phenotype characteristic of activated fibroblasts. In fact, bleomycin-induced collagen deposition and phosphorylation of Smad3 were prevented by an intratracheal injection of a recombinant adenovirus carrying mouse Smad7 cDNA¹⁴⁰. Intriguingly, inhibition of TGF- β 2 release by IFN- γ ¹⁴¹ and myofibroblast differentiation by hepatocyte growth factor (HGF)¹⁴² has been reported to be dependent on Smad7. Overall, overexpression of Smad7 may be a therapeutic target for IIPs.

BMPs member, one of the TGF- β family, have been investigated in pulmonary vascular disorders, especially pulmonary hypertension, which has been linked to mutations in the BMP receptor II gene¹²³ and expression of BMP-2 and BMP-4. BMP-2 expression is increased by proinflammatory stimuli and induces endothelial dysfunction, oxidative stress, and endothelial activation, including increased adhesion of a monocyte cell line¹⁴³. BMP-4 promotes migration of vascular smooth muscle cells, possibly contributing to vascular remodeling, i.e., medial wall thickening and muscularization of vessel walls¹⁴⁴. Lately, the role of BMPs, particularly BMP-7 and BMP-4, and that of gremlin in pulmonary fibrosis has been investigated. BMP-7 is known to act as an antagonist of TGF- β 1-dependent fibrogenic activity in mouse pulmonary myofibroblasts by inducing inhibitor of differentiation 2¹⁴⁵, which is an important myoepithelial cell marker during epithelial-mesenchymal transition^{146,147}. However, the role of BMP-7 in fibrosis remains controversial, since BMP-7 treatment significantly reduced the hydroxyproline content in asbestos-treated mice¹⁴⁸ but not in bleomycin-treated mice¹⁴⁹. No alterations in BMP-4 levels have been reported in lung samples of IPF patients, but IPF fibroblasts are less responsive to exogenous BMP-4, which inhibits the growth of and induces apoptosis in myofibroblasts¹²⁰. However, BMP-4 and BMP-7 accurately regulate fibrogenesis in different ways: BMP-4, but not BMP-7, reduces TGF- β 1-induced ECM production, while BMP-7, but not BMP-4, inhibits TGF- β 1-induced myofibroblast transformation in normal human lung fibroblasts¹⁵⁰. On the other hand, TGF- β 1 induces the expression of a BMP inhibitor, gremlin, in A549 lung epithelial cells, and IPF fibroblasts and their lung samples show high levels of gremlin mRNA and protein^{120,148}. Pulmonary gremlin mRNA levels were also upregulated in asbestos-treated mice¹⁴⁸. Increased gremlin level may decrease BMP-4-mediated myofibroblast apoptosis and increase TGF- β signaling through suppression of BMP signaling. This concept was supported by evidence of latent TGF- β -binding protein-4^{-/-} fibroblasts, which exhibited reduced activation of TGF- β due to increased expression of BMP-4 and decreased expression of gremlin¹⁵¹. Thus, profibrotic and antifibrotic signaling regulated by TGF- β and BMPs and the role of gremlin in disrupting the balance between both the mediators provides the complex feedback loop coordinating fibrogenesis¹⁵².

Conclusion

The mechanism by which cytotoxicity and inflammation cause fibrosis in the lung in response to environmental cues is not fully understood. In most pulmonary fibrotic conditions, cytotoxicity, inflammation and repair could be dysregulated by imbalance in the production of chemokines, cytokines and growth factors, and pulmonary wound repair is an extremely dynamic process intersecting immunology, structural biology and airway physiology¹⁵³. When a cytotoxic effect is minimal or self-limiting, repair would proceed to restore the normal structure, but when an injury is more extensive, tissue repair might result in scarring and/or fibrosis¹⁵⁴. The significance of new discoveries in understanding the pathogenesis of diseases and the potential molecular targets involved in advancing or interfering with fibrogenesis continues to be evaluated using *in vivo* analyses of animal models substituting for patients with ILDs/DPLDs¹⁵⁵. Important advances have been made in identifying molecules that are able to inhibit or induce lung granulomas and fibrosis, including TGF- β and BMP signaling and chemokines and their receptors in relation to reduction in T_{regs} (Fig. 2). Evidence by toxicological approaches would be expected to explore new aspects of the pulmonary diseases. Exposure to beryllium causes a Th1 cytokine (TNF- α , IFN- γ and IL-2)-dominant granulomatous process, which might share the features of sarcoidosis, a more frequent disorder with similar pathological features^{156,157}. Discovering the role of T_{regs} in CBD provides new insights in the mechanism of development of granulomatous lesions. On the other hand, nongranulomatous fibrosis has been found in nylon flock-exposed individuals, with or without textile paint exposure, although experimental data are quite limited as of 2010. PVC is known to be an etiological agent of pneumoconiosis, including granulomatous or nongranulomatous fibrosis, and of lung cancer, hepatic angiosarcoma and leukemia/lymphoma¹⁵⁸. QACs, including DDAC, may cause pulmonary diseases such as asthma or fibrosis, as shown by etiological research^{63–65} and our experimental study⁶⁹. Because there are several types of ILDs/DPLDs (Table 1), there should be a note of caution regarding the pathological characteristics in human and animal cases.

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