A Key Role for NOX4 in Epithelial Cell Death During Development of Lung Fibrosis

Stephanie Carnesecchi, Christine Deffert, Yves Donati, Olivier Basset, Boris Hinz, Olivier Preynat-Seauve, Cecile Guichard, Jack L. Arbiser, Botond Banfi, Jean-Claude Pache, Constance Barazzone-Argiroffo,* and Karl-Heinz Krause*.

Abstract

The pathogenesis of pulmonary fibrosis is linked to oxidative stress, possibly generated by the reactive oxygen species (ROS) generating NADPH oxidase NOX4. Epithelial cell death is a crucial early step in the development of the disease, followed only later by the fibrotic stage. We demonstrate that in lungs of patients with idiopathic lung fibrosis, there is strong expression of NOX4 in hyperplastic alveolar type II cells. 

Aim: To study a possible causative role of NOX4 in the death of alveolar cells, we have generated NOX4-deficient mice.

Results: Three weeks after administration of bleomycin, wild-type (WT) mice developed massive fibrosis, whereas NOX4-deficient mice displayed almost normal lung histology, and only little Smad2 phosphorylation and accumulation of myofibroblasts. However, the protective effects of NOX4 deficiency preceded the fibrotic stage. Indeed, at day 7 after bleomycin, lungs of WT mice showed massive increase in epithelial cell apoptosis and inflammation. In NOX4-deficient mice, no increase in apoptosis was observed, whereas inflammation was comparable to WT. In vitro, NOX4-deficient primary alveolar epithelial cells exposed to transforming growth factor-β1 did not generate ROS and were protected from apoptosis. Acute treatment with the NOX inhibitors also blunted transforming growth factor-β1-induced apoptosis. Conclusion: ROS generation by NOX4 is a key player in epithelial cell death leading to pulmonary fibrosis.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive, devastating lung disease in humans. Lung fibrosis can be observed in response to different types of damage to the alveoli (39, 50). However, in lung biopsies of IPF, the initial injury is found principally in epithelial cells (23). In reaction to epithelial destruction, there is an impaired re-epithelialization, myofibroblast formation, generation of fibroblastic foci, and ultimately fibrotic scars (22). Over time, pulmonary fibrosis also leads to pulmonary hypertension (33). While the sequence of pathophysiological changes in IPF is relatively well studied, the etiological causes are still unknown. Reactive oxygen species (ROS) have been involved in the disease, although an antioxidant treatment used in a multicenter patient trial showed modest beneficial effects (11, 12, 18).

ROS generation by NOX family NADPH oxidases might be implicated in the pathogenesis of IPF (2, 16, 27). Seven genes for...
NOX enzymes are known (3). NOX enzymes are involved in the development of several diseases (3). Of particular interest for lung fibrosis are NOX2, expressed in inflammatory cells, and NOX4, expressed in fibroblasts and in different types of epithelial cells. Two recent studies suggested increased expression of NOX4 in fibroblasts from IPF patients (2, 16).

Bleomycin-induced lung fibrosis is a widely used mouse model of IPF. This model is thought to be recapitulating key aspects of the human disease, in particular epithelial cell death, myofibroblast activation, and lung scarring (30, 31). There are three notable differences with human disease: (i) an important inflammatory phase is observed in the mouse model, but to a much lesser extent in human IPF; (ii) pulmonary hypertension can be observed in human IPF, but not in the mouse model; (iii) fibrotic foci are described only in humans. Similarly as seen for human IPF, there is an implication of ROS in the pathomechanism of bleomycin-induced fibrosis. ROS may contribute to the activation of fibrogenic molecules such as transforming growth factor (TGF)-β1 (21), which is a potent mediator of alveolar epithelial injury and myofibroblast activation (36). ROS have also been suggested to regulate TGF-β1 down-stream signaling (20, 21).

NOX2-deficient mice show a moderate protection from bleomycin-induced lung fibrosis (27); however, extrapolation to human IPF is difficult as inflammation might not be as prominent in human as compared to mice. NOX4-deficient mice have not been investigated in a model of lung fibrosis so far; however, instillation of siRNA directed against NOX4 showed beneficial effects in bleomycin-induced fibrosis (16). These effects were attributed to an impaired TGF-β1 signaling in NOX4-repressed fibroblasts, leading to decreased myofibroblast formation and collagen deposition.

Here we discovered a novel role for NOX4 in death of alveolar epithelial cells. We showed that NOX4 was highly expressed in hyperplastic alveolar type II cells in IPF patients. We demonstrated that NOX4-deficient mice are protected from bleomycin-induced pulmonary fibrosis through modulation of epithelial cell death in vivo. NOX4 deficiency as well

![FIG. 1. Immunohistological analysis of NOX4 expression in idiopathic pulmonary fibrosis (IPF) and in healthy lungs. (a) IPF lung tissue was analyzed for NOX4 by immunohistochemistry. Upper panels show a fibroblastic focus (recent lesion) and a fibrotic scar (older lesion; two different magnifications). Boxes represent magnified regions of fibrotic scars. Arrow points the regenerative NOX4-positive alveolar epithelium lying adjacent to a fibrotic focus (which itself is NOX4-negative). Asterisk indicates a NOX4-positive fibroblast in a fibrotic scar. Lower panels are stained with secondary antibody only (Ab). Scale bars, 50 μm. (b) Different structures from healthy lungs (panels on top line and third line) and from IPF patients (panels on second and fourth line) stained with NOX4 antibody (top line and second line) or with secondary antibody only (third and fourth lines). In both, healthy and in IPF lungs, the anti-NOX4 antibody stained pulmonary epithelial cells (arrows) and endothelial cells (arrowhead). Medial smooth muscle cells (asterisk) located in the pulmonary arteries were positive only in IPF samples. Note the presence of hemosiderin-laden macrophages in the alveoli of IPF lungs stained with the secondary antibody only. Scale bars, 50 μm. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).](image-url)
as NOX4 inhibitors prevent TGF-β1 induced cell death in vitro.
Some of these results have been previously reported in the form of abstract (7).

Results
NOX4 is highly expressed in alveolar epithelial cells of IPF lungs
To study expression of NOX4, we have performed immuno-histochemistry with a NOX4 antibody in sections of IPF and of healthy lungs (Fig. 1a, b). Negative controls were obtained by staining lung sections with secondary antibody only (Fig. 1a, b). Fibroblastic foci, which are relatively early lesions in the fibrosis stage, did not contain NOX4-expressing fibroblasts, even if the adjacent alveolar epithelium was clearly NOX4 positive (Fig. 1a). Within fibroblastic scars, which are later stage fibrotic lesions, some, but not all, of the fibroblasts expressed NOX4 (Fig. 1a). Thus, NOX4 expression in typical IPF lesions is variable and appears to be a late—rather than early—event.

We next analyzed other lung structures in healthy and IPF lungs (Fig. 1b). In healthy lungs, NOX4 was found in alveolar epithelial cells and in endothelial cells. In IPF lungs, the most striking finding was a strong staining of the hyperplastic alveolar type II cells. Strong NOX4 staining was also found in the thickened media of IPF lung arteries (33). Thus, NOX4 is expressed in alveolar epithelium and pulmonary vascular endothelium under physiological conditions, and in addition in the vascular media of IPF lungs. In addition, there is variable expression of NOX4 in late fibrotic lesions.

NOX4 deficiency decreases bleomycin-induced lung fibrosis
Both epithelial cell death and development of lung fibrosis are reproduced in the mouse model of bleomycin-induced lung fibrosis. To test the causal involvement of NOX4 in these pathophysiological mechanisms, we have generated NOX4-deficient mice by homologous recombination (Fig. 2a–c) and speed back-crossing into a pure C57Bl/6 genetic background (25). These mice, as expected, NOX4 mRNA was not expressed in any tissue; examples shown are spleen, kidney, and lung (Fig. 2d). Similarly, there was no NOX4 protein detected in lungs from NOX4-deficient mice, whereas NOX4 expression was conserved in NOX1- and NOX2-deficient mice (Fig. 2e). In addition, bleomycin-induced ROS production was blunt in NOX4-deficient mice as measured by dihydroethidium (DHE) staining (Fig. 2f). Further, these mice were healthy and did not display any spontaneous phenotype, besides a moderate tendency for accelerated increase in weight (data not shown).

We have compared the impact of bleomycin on the development of pulmonary fibrosis in wild-type (WT) and NOX4-deficient mice. WT mice showed massive weight loss in response to bleomycin, whereas NOX4-deficient mice rapidly returned to normal after a minor initial decrease in weight (Fig. 3a). Histochemical analysis (Masson’s Trichrome staining) demonstrated that WT mice developed severe pulmonary fibrosis in response to bleomycin, in contrast to few fibrotic lesions that were observed in NOX4-deficient mice (Fig. 3b). These results were quantitatively confirmed by analysis of total pulmonary collagen content (Fig. 3c) and procollagen IIIa mRNA levels (Fig. 3d). Thus, NOX4-deficient mice developed decreased pulmonary fibrosis in response to bleomycin.

NOX4 does not modulate inflammation induced by bleomycin
Inflammation is observed during the initial phase of bleomycin-induced pulmonary fibrosis and thought to contribute to fibrosis development (49). To investigate whether the protection provided by NOX4 deficiency is linked to inflammation, we investigated bronchoalveolar lavage fluid (BALF) for cell content (Fig. 4a–d) and levels of selected cytokines (Fig. 4e, f). None of these parameters was different between WT and knock-out animals. Note also that no significant changes were observed in the following inflammatory markers: MIP1α, RANTES, MIP-2, TNF, IL-10, and IFN (data not shown). Taken together, this suggests that NOX4 does not contribute to the pathogenesis of pulmonary fibrosis through modulation of inflammation.

Accumulation of myofibroblasts and phosphorylation of Smad2 induced by bleomycin depends on NOX4
Accumulation of myofibroblasts, to a large extent driven by TGF-β1 signaling (13, 36), is a hallmark of advanced and progressing lung fibrosis in response to bleomycin. Activation of transcription factors of the Smad family, in particular of Smad2 and Smad3, is one of the major TGF-β1-dependent signals (28, 36, 38). This activation occurs through phosphorylation by the TGF-β1 receptor, which is a serine/threonine kinase (32). Marked increase in Smad2 phosphorylation was observed in lung homogenates of WT mice exposed for 21 days to bleomycin. In contrast, the levels of phospho-Smad2 in NOX4-deficient mice did not increase and were similar to saline controls (Fig. 5a). To investigate whether NOX4 is important for myofibroblast accumulation, we performed immunohistochemistry using an antibody against z-smooth muscle actin (z-SMA), the hallmark of myofibroblasts (17, 44). In saline-treated mice (both WT and NOX4-deficient), predominantly smooth muscle cells of blood vessels were stained by the antibody. In bleomycin-treated WT mice, fibroblasts present in pulmonary parenchyma were strongly labeled (Fig. 5b), indicating the massive accumulation of myofibroblasts. In contrast, only a very low parenchymal labeling was observed in NOX4-deficient mice after bleomycin exposure (Fig. 5b). Of note, myofibroblasts were seen in all sections in the media of the vessels.

In addition to Smad signaling, activation of MAP kinases has been suggested to contribute to lung fibrosis after bleomycin treatment (11). Consequently, we analyzed phosphorylation of ERK, JNK, and p38 MAP kinases in lung homogenates, 21 days after bleomycin exposure. In WT mice, both ERK and JNK were phosphorylated, whereas p38 phosphorylation was unchanged. Interestingly, bleomycin-induced phosphorylation of ERK and JNK was not altered in NOX4-deficient lungs (Fig. 5c; JNK phosphorylation at day 7 after bleomycin was also not affected by NOX4 deficiency; data not shown). Thus, bleomycin-induced Smad phosphorylation and accumulation of myofibroblasts depends on NOX4.
NOX4 is crucial in bleomycin-induced alveolar epithelial cell death

Next, we investigated a crucial proximal step in the pathogenesis of lung fibrosis, namely, epithelial cell apoptosis (22, 43). To this end, we first analyzed the time course of lung cell death by TUNEL staining in WT and NOX4-deficient mice (Fig. 6a). Instillation of saline had no or only minor effects on the number of TUNEL-positive cells up to 20 days either in WT or in NOX4-deficient mice. Instillation of bleomycin in WT mice led to a massive increase in TUNEL-positive cells during the first 2 weeks, returning to baseline levels after 3 weeks. In contrast, no significant increase in TUNEL-positive cells was observed in NOX4-deficient mice. Note that the differences between bleomycin-treated WT and NOX4-deficient mice were statistically significant ($p < 0.01$ on days 2 and 7, and $p < 0.05$ on day 12). Prevention of apoptosis by NOX4 deficiency
was also observed by analyzing cleaved caspase-3 (Fig. 6b). To understand the temporal relationship between the epithelial cell apoptosis occurring in bleomycin-treated WT and the subsequent development of fibrosis, we analyzed the alveolar perimeter (a sensitive measure of lung fibrosis) as a function of time after bleomycin exposure. Bleomycin did not affect this parameter at day 7 (i.e., when maximum apoptosis occurs), but only at later time points (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/ars). Thus, in the bleomycin model, epithelial cell apoptosis precedes the development of fibrosis.

To identify the type of cells undergoing cell death, we performed costaining of lung sections with TUNEL and cell type-specific markers (Fig. 6c). TUNEL-positive cells in WT mice were negative for the endothelial cell marker, but positive for alveolar epithelial type I and type II cell markers. Thus, NOX4 is essential for bleomycin-induced death of alveolar epithelial cells.

We also investigated NOX4 expression in lung homogenates of WT mice after bleomycin exposure (day 7). Interestingly, we observed a decrease in NOX4 expression 2.6±0.39 in saline-treated mice, as compared to 1.03±0.04 in bleomycin-treated mice; p=0.02. This most likely reflects the apoptotic loss of NOX4-expressing alveolar epithelial cells. As whole lung homogenates are a crude mixture of many cell types, precise information about the relationship between NOX4 expression and apoptosis in alveolar epithelial cells requires the work with primary cells.

**NOX4 deficiency and acute inhibition of NOX4 prevents TGF-β1-induced cell death in primary alveolar epithelial cells**

To further determine the relationship between NOX4 and cell death, we analyzed isolated primary epithelial type II cells. Death of alveolar epithelial cells in pulmonary fibrosis is thought to be mediated, at least in part, by TGF-β1 (26). Stimulation of epithelial type II cells with TGF-β1 increased NOX4 mRNA expression (Fig. 7a). Even more striking, NOX4 deficiency almost entirely blunted TGF-β1-induced ROS generation (Fig. 7b). Analysis of cell death yielded interesting results. Stimulation with TGF-β1 for 24 h increased significantly the number of TUNEL-positive alveolar epithelial type II cells from WT mice. By contrast, a much lower number of cells were positive when derived from NOX4-deficient mice under TGF-β1 stimulation (Fig. 7c). This suggests that NOX4-dependent ROS generation contributes to TGF-β1-induced death of epithelial type II cells.

To demonstrate that resistance to TGF-β1 is not a chronic adaptation due to the knock-out, we investigated acute NOX4 inhibition using fulvene-5, a compound that inhibits NOX4
and NOX2 (4), and GKT136901, a compound that inhibits NOX1 and NOX4 (24) (but not NOX2). Both inhibitors led to a decrease in TGF-β1-induced apoptosis of epithelial type II cells (Fig. 7d). These results demonstrate that NOX4 activity is important for TGF-β1-induced apoptosis in alveolar epithelium.

**Discussion**

In this study, using NOX4-deficient mice, we demonstrate that ROS generation by the NADPH oxidase NOX4 is crucial for the induction of alveolar epithelial cell death and the subsequent development of lung fibrosis. There is now strong evidence that epithelial cell death is a key event in the pathogenesis of lung fibrosis (22, 43). Indeed, apoptotic cells can be detected in lungs of IPF patients (23, 45), as well as in bleomycin-induced and TGF-β1-induced mouse models of pulmonary fibrosis (19). Inhibitors of apoptosis diminish pulmonary fibrosis in mouse models (6, 47), whereas epithelial cell damage by transgenic expression of diphteria toxin is sufficient to induce pulmonary fibrosis in mice (41). Therefore, strategies aimed at preventing epithelial cell death are increasingly considered as crucial for future treatments of IPF.

ROS generation by NOX NADPH oxidases has a context-dependent impact on cell fate, and may contribute to cell...
survival or to cell death (3). Even for the same NOX enzyme, such as NOX4, it can be involved in enhancement of cell growth (37), cell differentiation (10, 16), and in induction of cell death-independent cellular senescence (14). In the context of bleomycin-induced lung fibrosis in vivo and TGF-β1-induced alveolar epithelial cell death in vitro, NOX4 is clearly involved in the induction of apoptosis (this study). Does it make sense from a biological point of view that NOX4 induces apoptosis in alveolar epithelial cells? In the same situation, NOX4 upregulation might be part of the response to danger signals leading to protective apoptosis as in viral infections (15). However, NOX4 regulation is likely to be more complex and its impact on cell fate might depend on expression levels: limited upregulation of NOX4 might have other functions other than apoptosis (in particular cell signaling), whereas marked upregulation of NOX4 might lead to cell death. Mechanisms by which ROS contribute to cell death are complex: they include nonspecific cell damage as well as specific redox-sensitive pathways for cell signaling and transcriptional control (3). It has been suggested that ROS release from fibroblasts are the cause of epithelial cell death in pulmonary fibrosis (46). Our results do not favor this concept, but suggest cell autonomous effects of NOX4 in epithelial cells.

What is the relationship between lung fibrosis, NOX4, and TGF-β1? Our data demonstrate that NOX4-derived ROS are crucial down-stream mediators of TGF-β1-induced alveolar epithelial cell death. However, there are also indications that ROS might act upstream, contributing to TGF-β1 expression and activation (21), raising the potential of a positive feedback loop between NOX4 and TGF-β1 in lung fibrosis. It is likely that TGF-β1 is not the only factor contributing to NOX4 expression in the bleomycin model. Indeed, NOX4 upregulation may be a part of cellular stress responses, such as endoplasmic reticulum stress (35). If this is true, NOX4 inhibition would be expected to be more efficient than TGF-β1 inhibition in preventing the development of epithelial cell death and the subsequent fibrosis.

NOX4 deficiency did not affect all biological changes in response to bleomycin. In particular, it did not affect either the accumulation of inflammatory cells or the activation of MAP kinases ERK and JNK. Thus, MAP kinase activation in response to bleomycin might occur through activation of other ROS generating systems. Previous work from our laboratory has shown a key role of NOX1 in activation of MAP kinases by hyperoxia (8). This points to an interesting new concept in redox biology, namely, that different NOX enzymes may preferentially activate distinct signaling systems through mechanisms, including distinct subcellular localization (5) or distinct activation mechanisms (40). Note, however, that our results do not exclude a role of MAP kinases in lung fibrosis (1, 29) and epithelial cell death (8); they rather demonstrate that MAP kinase activation is not sufficient to cause sustained fibrosis in the absence of NOX4-dependent ROS generation.

NOX4 also contributes to TGF-β1-induced α-SMA production and to the contractile function of human fibroblast-like
Therefore, our observation that, in human IPF, NOX4 is not detectable in myofibroblasts of early fibrotic foci (Fig. 1) is unexpected. This might suggest that early steps of myofibroblast formation in IPF require either no or only very low NOX4 activity and that NOX4 rather contributes to the development of the late fibrotic scar.

One of the key findings of our study, namely, the role of NOX4 in epithelial cell apoptosis, was also demonstrated using NOX4 inhibitors. This is important from a scientific point of view, as it proves that acute inhibition of NOX4 suffices to prevent apoptosis, and the resistance of NOX4-deficient epithelial type II cells to TGF-β1-induced cell death was not due to chronic adaptation in NOX4-deficient animals. This is, however, also important in terms of perspectives for IPF treatment. NOX4 inhibition targets epithelial cell death (our study), but also later disease steps such as fibroblast activation (16), and theoretically even IPF-associated pulmonary hypertension (Fig. 1). NOX4 inhibitors are therefore a most promising treatment concept for this severe chronic disease for which no efficient therapies are known.

**Materials and Methods**

See the Supplementary Data for additional details on reagents and methods.

**Immunostaining of lungs from control and IPF patients**

Human lung biopsies of patient suffering from IPF and human normal control lungs were obtained in accordance to an approved protocol by the Institutional Ethics Committee.
of Geneva. Paraffin-embedded sections of lung fixed in 4% paraformaldehyde (PFA) were stained with an anti-NOX4 polyclonal antibody (dilution 1/500; Novus Biologicals) (48) followed by an incubation with a biotinylated goat anti-rabbit Ig (1/100; Vector Laboratories). This reaction was developed using a horseradish peroxidase-avidin/biotin complex solution (1:100; Vector Laboratories) and diaminobenzidine (Invitrogen) before counterstaining with cresyl violet. Negative controls were obtained by incubating the sections with a biotinylated goat anti-rabbit Ig only (1/100; Vector Laboratories). In some control experiments, isotype controls were used, which yielded the same results (data not shown). Endogenous peroxidases were blocked by adding H2O2 and lung sections were subjected to heat-induced epitope retrieval for 15 min in 0.01 M citrate buffer (pH 6.0).

Generation of NOX4-deficient mice and speed back-cross

Embryonic stem cell clones Sv129 NOX4 (IIIG1 and IVA4) were thawed, plated on mitomycin C-inactivated mouse embryonic fibroblasts, and cultivated in Knockout D-MEM (Gibco Inc.) supplemented with leukocyte inhibitory factor and 15% fetal bovine serum (PAN Biotech GmbH). Cells were injected in one session each into C57Bl/6-derived blastocysts. For this purpose, 8-week-old C57Bl/6 female mice were naturally mated to C57Bl/6 breeder males (Charles River). Injections were performed using day 3.5 blastocysts obtained by cultivating day 3.5 morulae overnight at 37°C. Injected blastocysts were transferred into the uterus of pseudopregnant B6CBAF1 females (Charles River). The animals were kept in individually ventilated cages. Generation of knock-out embryonic stem cells as well as blastocyst injection and transfer were performed by PolyGene.

Speed-back cross into a C57Bl/6 background was performed as described (25). Briefly, polymerase chain reaction (PCR) was performed with genomic DNA as template. Three microsatellite marker loci per chromosome were amplified using appropriate PCR primers (Invitrogen), and results were analyzed by acrylamide gel electrophoresis. Mice used for the studies described here were speed back-crossed for five generations and displayed >95% C57Bl/6 microsatellite marker loci.

Animals and experimental design

NOX4-deficient and WT male mice aged 10–12 weeks were inbred in specific pathogen-free conditions on the C57Bl/6J background. All animal experiments were approved by the Institutional Ethics Committee of Animal Care in Geneva and Cantonal Veterinary Office. Male mice weighing 25–30 g were anesthetized with ketamine and xylazine (100 and 5 mg/kg, respectively) and then placed on an inclined surface below cold light. The larynx was observed below cold light and intubated with an endotracheal catheter attached to a 50-ml Hamilton syringe (Hamilton Bonaduz). Bleomycin (2000 I.U./kg, stock solution prepared at 1000 I.U./ml; Zurich) or vehicle (saline) was instilled to WT and NOX4-deficient male mice. Intubation of the trachea was verified by movement of the solution bubble with the animal’s respiratory efforts. After instillation, the catheter was removed, and the mice were returned to their cages, allowed to recover from anesthesia, and provided with free access to food and water. Mice were killed
at days 2, 7, 12, and 21, and lungs processed for subsequent analysis.

**Mouse lung histology and immunohistochemistry**

The right lung was fixed by intratracheal instillation of 4% PFA and embedded in paraffin. Sections (3 μm) were stained with hematoxylin and eosin or with Masson’s Trichrome and examined with light microscopy. For immunohistochemical analysis, sections were incubated with a monoclonal antibody anti-α-SMA (mouse IgG2a, 1:400, a kind gift from Prof. G. Gabbiani) (42). Detection was performed using horseradish peroxidase anti-mouse Envision+ system with diaminobenzidine (Dako SA). Sections were then counterstained with hematoxylin.

**Sircol assay**

Total lung collagen content was determined by measuring total soluble collagen using the Sircol Collagen Assay Kit (Biocolor) at day 21 according to the manufacturer’s instructions. Samples were centrifuged at 15,000 g and supernatants (100 μl) diluted in 0.5 M acetic acid (100 μl), and then 1 ml of Sircol Dye Reagent was added and mixed for 30 min at room temperature in a mechanical shaker. The collagen-dye complex was precipitated by centrifugation at 10,000 g for 10 min. The unfixed dye solution was carefully removed. The precipitated complex was resuspended in 1 ml of alkali reagent, and placed in a 96-well flat-bottomed plate; absorbance at 540 nm was evaluated. Collagen content was calculated by comparing samples values to a standard curve.

**Western blot analysis**

Total lung protein extracts were performed as previously described (34). Proteins blotted on nitrocellulose membrane were incubated with an anti-NOX4 antibody (1:500; Novus Biologicals), an anti-pSmad2 antibody (1:1000; Santa Cruz Biotechnology), an anti-Smad2 antibody (1:1000; Santa Cruz Biotechnology), a monoclonal anti-α-SMA antibody (1:500; Santa Cruz Biotechnology), a polyclonal JNK (1:1000; Cell Signaling), a monoclonal anti-actin (1:1000; Sigma), a polyclonal anti-cleaved caspase-3 (1:500; Cell Signaling), a monoclonal anti-ERKs (1:1000; Cell Signaling), a monoclonal anti-pERKs (1:1000; Cell Signaling), a monoclonal anti-pSMA antibody (1:400, a kind gift from Prof. G. Gabbiani) (42). Detection was performed using horseradish peroxidase-conjugated anti-mouse antibody (1:3000; Bio-Rad Laboratories) or peroxidase-conjugated anti-rabbit antibody (1:3000; Santa Cruz Biotechnology), an anti-Smad2 antibody (1:1000; Santa Cruz Biotechnology), and the medium was replaced after 1 day.

**Detection of superoxide**

For in situ detection, frozen lung tissue from WT and NOX4-deficient mice exposed to saline or bleomycin for 7 days was cryosectioned (20 μm) and collected onto Superfrost plus slides (Menzel GmbH and Co KG) allowed to air-dry at room temperature and stored at −80°C. When needed, slides were placed into phosphate-buffered saline (PBS) for 30 min at room temperature and then stained with DHE (5 μM) diluted in Hank’s Balanced Salt Solution for 30 min in the dark. The slides were rinsed extensively with Hank’s Balanced Salt Solution and cover-slipped, and the images were captured with an inverted microscope (Nikon) and analyzed with Metafluor imaging software (Molecular Devices). Quantification was performed by measuring the fluorescence intensity of all nuclei counted in the lung sections from three different mice.

**TUNEL assay and immunostaining**

Frozen lung sections at days 2, 7, 12, and 21 (6 μm) were fixed by instillation with 4% PFA and permeabilized during 2 min on ice with 0.1% Tx-100 in 0.1% sodium citrate. After blocking with 10% normal goat serum and 1% bovine serum albumin in PBS solution, sections were incubated with an antibody anti-digitoxigenin-AP Fab fragments for 30 min at room temperature (1/500; Roche) as described by the manufacturer (TUNEL assay fluorescent kit; Roche). Fast Red substrate system (DaKo) was used to detect the fluorescent staining and nuclei were revealed with 4,6-diamidino-2-phenylindole (DAPI) (1/200; Roche). Slides were mounted with Mowiol and analyzed by confocal microscopy (LSM 510Meta; Zeiss). Quantification of positive staining was...
performed using Metamorph analysis software (20 images per mouse, three mice per group out of three independent experiments).

For double staining, after TUNEL staining according to the manufacturer (TUNEL assay fluorescent kit; Roche), frozen lung sections were washed in PBS and incubated 1 h at room temperature with hamster syrian polyclonal antibody anti-T1 alpha (1:1000; Santa-Cruz), rabbit polyclonal anti-prosurfactant C protein (1:250; Chemicon), or anti-Von Willebrand factor (1:250; DAKO). After washing, sections were incubated with a goat anti-syrian hamster Texas red (1:200; Jackson) or a goat anti-rabbit Texas Red conjugated (1:250; Molecular Probe). The nuclei were stained with DAPI (1:200; Roche Diagnostic). Slides were mounted with fluorosave (VWR) and analyzed by confocal microscopy (LSM 510 Meta; Zeiss).

TUNEL detection in isolated cells was performed as described by the manufacturer (TUNEL assay fluorescent kit; Roche). Briefly, isolated epithelial cells from both mice strains were cultured on matrigel and treated with 10 ng/ml TGF-β1 for 24 h. Cells were fixed with 4% PFA for 1 h at room temperature and then permeabilized during 2 min on ice with 0.1% Tx-100 in 0.1% sodium citrate freshly prepared. Cells were incubated with the labeling mixture, and stained with a rabbit polyclonal anti-prosurfactant protein C (1:250; Chemicon) for 1 h at 37°C. As secondary antibody, a goat anti-rabbit Texas Red conjugated (dilution 1:250; Molecular Probe) was used. The nuclei were stained with DAPI (1:200; Roche Diagnostic). These slides were then mounted with mowiol as described previously. Images were acquired using confocal microscopy (LSM510 Meta; Zeiss) and quantified using Metamorph analysis software (20 images per mouse, three mice per group out of three independent experiments).

Real-time PCR

Spleen, kidney, and pulmonary samples were placed in RNA later (Qiagen). After homogenization with the Tissue Lyser (Qiagen), RNA was extracted with the RNeasy Protect mini kit (Qiagen) and reverse transcribed using the supercript reverse transcriptase (Superscript Choice; Invitrogen). We used the following primer sequences for Col3a1 T-3

\[ \text{T-3: ACC GAC AGT CCT GGC TTA TCT-3} \]

\[ \text{¢-CCG GAC AGT CCT GGC TTA TCT-3} \]

\[ \text{¢-CCG GAC AGT CCT GGC TTA TCT-3} \]

\[ \text{¢-CCG GAC AGT CCT GGC TTA TCT-3} \]

\[ \text{¢-CCG GAC AGT CCT GGC TTA TCT-3} \]

Statistical analysis

Results are expressed as mean ± standard error of the mean and ± standard deviation as indicated and were analyzed either by Wilcoxon Rank test or by analysis of variance, as appropriate. Significance levels were set at $p < 0.05$.

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Author Disclosure Statement


References


Address correspondence to:
Dr. Stephanie Carnesecchi
Department of Pathology and Immunology
Medical School
University of Geneva
1 rue Michel Servet
1211 Geneva 4
Switzerland
E-mail: stephanie.carnesecchi@unige.ch

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Abbreviations Used

- $\alpha$-SMA = $\alpha$-smooth muscle actin
- BALF = bronchoalveolar lavage fluid
- BLM = bleomycin
- DAPI = 4,6-diamidino-2-phenylindole
- DHE = dihydroethidium
- FCS = fetal calf serum
- IPF = idiopathic pulmonary fibrosis
- PBS = phosphate-buffered saline
- PCR = polymerase chain reaction
- PFA = paraformaldehyde
- ROS = reactive oxygen species
- TGF = transforming growth factor
- WT = wild-type