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ATLa, an Aspirin-Triggered Lipoxin A4 Synthetic Analog, Prevents the Inflammatory and Fibrotic Effects of Bleomycin-Induced Pulmonary Fibrosis

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Despite an increase in the knowledge of mechanisms and mediators involved in pulmonary fibrosis, there are no successful therapeutics available. Lipoxins (LX) and their 15-epimers, aspirin-triggered LX (ATLa), are endogenously produced eicosanoids with potent anti-inflammatory and proresolution effects. To date, few studies have been performed regarding their effect on pulmonary fibrosis. In the present study, using C57BL/6 mice, we report that bleomycin (BLM)-induced lung fibrosis was prevented by the concomitant treatment with an ATL synthetic analog, ATLa, which reduced inflammation and matrix deposition. ATLa inhibited BLM-induced leukocyte accumulation and alveolar collapse as evaluated by histology and morphometrical analysis. Moreover, Sirius red staining and lung hydroxyproline content showed an increased collagen deposition in mice receiving BLM alone that was decreased upon treatment with the analog. These effects resulted in benefits to pulmonary mechanics, as ATLa brought to normal levels both lung resistance and compliance. Furthermore, the analog improved mouse survival, suggesting an important role for the LX pathway in the control of disease establishment and progression. One possible mechanism by which ATLa restrained fibrosis was suggested by the finding that BLM-induced myofibroblast accumulation/differentiation in the lung parenchyma was also reduced by both simultaneous and posttreatment with the analog (α-actin immunohistochemistry). Interestingly, ATLa posttreatment (4 days after BLM) showed similar inhibitory effects on inflammation and matrix deposition, besides the TGF-β level reduction in the lung, reinforcing an antifibrotic effect. In conclusion, our findings show that LX and ATL can be considered as promising therapeutic approaches to lung fibrotic diseases. The Journal of Immunology, 2009, 182: 5374–5381.

Pulmonary fibrosis is an interstitial disorder of the parenchyma that is a common end-stage sequela of a number of lung diseases, resulting in a disruption of lung architecture that renders gas exchange difficult (1–4). Fibrosis is characterized by diffuse chronic interstitial inflammation, increased fibroblast proliferation, and enhanced extracellular matrix synthesis and deposition (1–6). Fibroproliferative diseases are among the leading causes of morbidity and mortality worldwide. Pulmonary fibrosis has a prevalence of 7–10:100,000 (increasing with age) and a mean survival of 3–4 years (decreasing with age) (7, 8).

In the present study a bleomycin (BLM)3-induced pulmonary fibrosis model was used. BLM is an antibiotic agent with antitumor activity first isolated from Streptomyces verticillus in 1966 (9). BLM induces free radical production, mainly by forming a complex with iron II, which oxidizes to iron III. These products can lead to cell death by DNA breakage, endothelial damage of the lung vasculature, and the production of cytokines and cysteinyi leukotrienes. Depending on the BLM dose and the age of the patient, several pulmonary syndromes can develop, including fibrosis (9–12).

Despite the development of several animal models and the characterization of a variety of key participants, the mediators and mechanisms involved in the pathogenesis of pulmonary fibrosis are not completely defined, a fact that helps explain the limited therapeutic approaches (7, 13–15). Due to the lack of a more effective alternative, the fundamental therapeutic strategy has been the use of corticosteroids, alone or in combination with other immunosuppressive agents; however, this has had little impact on long-term survival (6, 7, 14–18). Lipoxins (LX) are endogenously produced eicosanoids with potent anti-inflammatory bioactivities (19, 20). The original pathways identified for LX formation were via lipoxygenase–prostaglandin E2–5–lipoxygenase interactions. Another pathway for LX synthesis involves aspirin-triggered acetylation of cyclooxygenase-2 and lipoygenase interactions.
activation of 5-lipoxygenase forming 15-epimer LX or aspirin-triggered LX (ATL) (21). These trihydroxytetracene-containing products of arachidonic acid are biothesized in response to specific stimuli, act locally, and are rapidly inactivated enzymatically (22). Changes in LX production can drive the evolution of several diseases such as asthma, fibrosis, cancer, and atherosclerosis (23). Interestingly, diminished levels of LX₄ are detected in the induced sputum of patients with severe asthma that is characterized by irreversible airway remodeling, including increased collagen deposition and smooth muscle proliferation in the bronchial wall (24).

Metabolically stable synthetic analogs represent useful tools to evaluate the potential of pharmacological manipulation of the inflammatory process as a means to develop new and selective anti-inflammatory therapies with reduced toxic side effects (25, 26). Among the various ATL analogs studied, 15-epi-16-((para-fluoro)phenoxy)- LX₄ (ATLa) and related molecules have been shown to be active in vivo in several models of inflammatory disease (27, 28), including cystic fibrosis (29).

In this study we demonstrated the prevention, and more importantly, the reversion of BLM-induced pulmonary fibrosis in mice treated with ATLa. Moreover, we also observed that ATLa treatment conferred an improvement in the lung function and resistance to BLM-induced mortality.

Materials and Methods

Animal model of fibrosis

C57BL/6 mice at 6–7 wk of age were provided by the Oswaldo Cruz Foundation Breeding Unit (Rio de Janeiro, Brazil). Mice were caged with free access to food and fresh water in a temperature-controlled room (22–24°C) on a 12-h light/dark cycle. For the induction of pulmonary fibrosis, mice (n = 5–6) were administered BLM (0.05, 0.1, or 0.5 µg/mouse dissolved in 30 µl of saline; Sigma-Aldrich) by the intratracheal (i.t.) route with or given sterile saline (30 µl) as a control (31).

Flow cytometry analysis

Leukocytes were quantified in Giemsa-stained sections for all experimental groups. Alveolar macrophages and neutrophils were determined in 30 fields of 26,000 µm² (10 random fields of three different sections) in each lung. The ×40 objective microscopic field image was observed in an Olympus BH-2 equipped with an eyepiece with a graticule (30).

Flow cytometry analysis

Lungs were collected 21 days after instillation of saline, BLM, and BLM plus ATLa (4 days) and digested with collagenase (0.2% solution; Sigma-Aldrich). Blocking was performed by incubating cells with purified rat anti-mouse CD16/CD32 (FcγRII/III Receptor) mAb. Cells were stained with Abs against CD8 and CD11c (PE conjugated; BD Biosciences Pharmingen) and analyzed. Data were obtained from T cells gated by side scatter/forward scatter using FACSCalibur (CellQuest software; BD Biosciences).

Invasive assessment of respiratory mechanics

Twenty-one days after instillation, mice were anesthetized with Nembutal (60 mg/kg) and neuromuscular activity was blocked with bromide pancuronium (1 mg/kg). Tracheostomized mice were mechanically ventilated and lung function was assessed. The trachea was cannulated and the cannula connected to a pneumotachograph. Air flow and transpulmonary pressure were recorded with a Buxco pulmonary mechanics computer (Buxco Electronics). The computer calculated resistance (Rrs; cmH₂O/l/s) and dynamic lung compliance (ml/cmH₂O) in each breath cycle. Analog signals from the computer were digitized by a Buxco analog converter (33).

ELISA

TGF-β in lung samples was measured at day 21 after BLM challenge using a standardized ELISA technique (R&D Systems) following the instructions of the manufacturer. These samples were prepared from whole lung homogenized in 2 ml of PBS containing a mixture of protease inhibitors (Complete; Sigma-Aldrich) (34).

Hydroxyproline assay

Hydroxyproline contents in lung tissue were used as a quantitative index of fibrogenesis and fibrosis. Lung hydroxyproline levels were determined spectrophotometrically by absorbance at 550 nm as previously reported and the results were expressed as micrograms of hydroxyproline per milligram of lung tissue (35).

**Morphometry**

To access uniform and proportional lung samples, 15 fields (five non-overlapping fields in three different sections) were randomly analyzed using a video microscope (Zeiss-Axioplan with a ×20 objective lens and a JVC color video camera linked to a color video monitor; Carl Zeiss) and a cycloid test system superimposed on a monitor screen. The reference volume was estimated by point counting using the points test system (PT). The points hitting the alveoli and collagen fibers (PP) were counted to estimate the volume densities (Vv) of these structures (Vv = PP/PT). A total area of 1.94 mm² was analyzed to determine the volume densities of alveoli (and collagen fibers in H&E and Sirius red-stained sections. The analysis was performed by two investigators a blinded fashion (30).

**Western blotting**

The total protein content in the lung tissue extracts was determined by the Bradford method (32). The lysates were denatured in Laemmli’s sample buffer (50 mM Tris-HCl (pH 6.8), 1% SDS, 5% 2-ME, 10% glycerol, and 0.001% bromophenol blue) and heated in a boiling water bath for 3 min. Samples (30 µg of total protein from whole extracts) were resolved in SDS-PAGE and the proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked with Tween TBS (20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.1% Tween 20) containing 2% BSA and probed with the specific, primary, monoclonal anti-ALX (where ALX is the LX receptor) or polyclonal anti-actin Abs (Genovac). After extensive washing in Tween TBS, polyvinylidene difluoride sheets were incubated with specific biotin-conjugated Ab (1/10,000) for 1h and then incubated with HRP-conjugated streptavidin (1/10,000). Immunoreactive proteins were visualized by ECL kit (Pierce) staining and the bands were quantified by densitometry using Scion Image software.

**Immunohistochemistry**

Sections were deparaffinized and hydrated and the slides were incubated with 10 mM sodium citrate. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Slides were washed in TBS with 0.05% Tween 20 (Sigma-Aldrich), blocked with serum-free protein block (Dako-Cytomation), and immunostained with Vectastain ABC (Vector Laboratories). AB for anti-smooth muscle α-actin (RR-9010) (Lab Vision) was used at a 1/100 dilution in TBS/Tween buffer overnight at 4°C. Color was developed with 3,3-diaminobenzidine tetrahydrochloride (Vector Laboratories) and counterstained with H&E. An isotype IgG was used as negative control (31).
Statistical analysis
Survival rates were expressed as percentages, and a Mantel-Cox log-rank test ($\chi^2$ test) was used to detect differences in mice survival. Data shown are mean $\pm$ SEM and are representative of at least two separate experiments. Differences were analyzed using a Mann-Whitney U test. Statistical significance was set at $p < 0.05$.

Results
Dose-response BLM-induced lung fibrosis
First, we performed a dose-response experiment comparing the effects of 0.05, 0.1, and 0.5 U of BLM on the lungs as assessed by histopathology and morphometry (Fig. 1A and B, respectively). As observed, an extremely severe reaction occurred within the highest doses (Fig. 1A, c and d). The administration of 0.1 and 0.5 U of BLM caused a marked decrease in the number of open alveoli (Fig. 1B), reflecting the histology findings. It is important to highlight that the histological samples of the highest dose of BLM (0.5 U) were obtained from the surviving animals after 14 days (two of eight mice).

Treatment with ATLa impaired BLM-induced lung fibrosis
In view of the results obtained in the dose-response experiment with BLM, the 0.1 U/mouse dose was chosen for additional experiments. The inflammatory component in the development and maintenance of fibrotic process is well recognized (36–40). As observed on representative H&E-stained slides in Fig. 2, a–c, the concomitant administration of ATLa with BLM reduced lung cell infiltration and edema. The hallmark characteristic of fibrosis is the excessive deposition of an extracellular matrix, such as collagen (36, 37). In our experimental condition, ATLa treatment impaired BLM-induced matrix protein deposition as evidenced by Sirius red staining (Fig. 2, g–i). These results were also verified by Gomori staining (data not shown). Furthermore, concomitant ATLa treatment also impaired BLM-induced hydroxyproline accumulation (Fig. 3). The protection conferred by ATLa administration on

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**FIGURE 1.** BLM-induced lung fibrosis. Lungs were removed from surviving animals on the 14th day after treatment with saline or BLM at 0.05 U/mouse, 0.1 U/mouse, or 0.5 U/mouse. A, Histological changes were demonstrated by H&E staining (original magnification, $\times$20). a, Saline; b, 0.05 U of BLM; c, 0.1 U of BLM; d, 0.5 U of BLM. B, Graphics represent the volume densities of alveoli. * $p < 0.05$ compared with saline group; #, $p < 0.05$ compared with BLM 0.05 U group; †, $p < 0.05$ compared with BLM 0.1 U group; n = 5 for each experimental group. Pictures in A are representative of each group.

**FIGURE 2.** ATLa impaired BLM-induced lung fibrosis. Lungs were removed from the animals on the 21st day after treatment with saline, BLM alone (0.1U/mouse), or BLM plus ATLa (added on day 0 (0d)) as described in Materials and Methods. Mice were concomitantly injected with BLM plus ATLa and then boosted i.v. with ATLa at 0.1 µg/mouse on the 7th and 14th days. The coloration was made with H&E (Fig. 2), immunostained for α–actin (d–f), and Sirius red (g–i; original magnification, $\times$20) as described in Materials and Methods; n = 5 for each experimental group. Pictures are representative of each group.

**FIGURE 3.** ATLa reduced lung collagen concentration. Hydroxyproline content from lungs of mice instilled with saline, BLM (0.1U/mouse), or BLM plus ATLa (added on day 0 (0d)) after 21 days. Mice were concomitantly injected with BLM plus ATLa and then boosted with 0.1 µg/mouse i.v. on the 7th and 14th days. Results are expressed as micrograms per milligram of lung tissue. * $p < 0.05$ compared with saline group; #, $p < 0.05$ compared with BLM group. n = 5 for each experimental group.

**FIGURE 4.** ATLa preserves alveolar structure. Lungs were removed from the animals on the 21st day after saline, BLM (0.1U/mouse), or BLM plus ATLa (added on day 0 (0d)) after 21 days. Mice were concomitantly injected with BLM plus ATLa and then boosted with ATLa at 0.1 µg/mouse i.v. on the 7th and 14th days. Graphics represent the volume densities of collagen fibers (A) and the volume densities of alveoli (B). * $p < 0.05$ compared with saline group; #, $p < 0.05$ compared with BLM group. n = 5 for each experimental group.
BLM-induced lung fibrosis was confirmed by morphometric analysis showing a reduction in the percentage of collagen fibers deposited (Fig. 4A) and an increase in the number of open alveoli (Fig. 4B), with similar levels to those obtained with control animals (saline group).

Immunohistochemistry analysis revealed that ATLa administration decreased BLM-induced α-actin expression in the pulmonary parenchyma (Fig. 2, d–f), suggesting an antifibrotic effect for the LX/ATL pathway.

The presence of the lipoxin receptor ALX in the lung was analyzed by Western blotting in saline and BLM-treated groups using whole lung obtained at day 21 after challenge. As shown in Fig. 5, no differences in ALX expression were observed in lung samples of BLM-treated vs saline-treated groups, suggesting that BLM treatment does not change pattern expression.

Treatment with ATLa reversed BLM-induced lung fibrosis
To evaluate whether the analog was also able to modify an ongoing fibrotic process, in a further set of experiments we assessed the effects of ATLa given 4 days after BLM instillation, when both inflammatory and fibrotic processes are already established. Representative slides of these experiments are shown in Fig. 6. Similar to what occurred with the concomitant treatment, ATLa posttreatment reversed BLM-induced inflammation and fibrosis (Fig. 6, a–c). We investigated whether ATLa was able to reduce leukocyte infiltration during lung fibrosis. Fig. 7 shows a reduction of neutrophils and mononuclear cells in the lung tissue at day 21 after BLM challenge as obtained by morphometric analysis, but the differences did not reach statistical significance compared with control. In another set of experiments, lungs obtained 21 days after saline, BLM, and BLM plus delayed ATLa treatment were digested and total cells were analyzed by flow cytometry for CD8

![FIGURE 5. BLM treatment does not change ALX expression. Lungs were removed from the animals on the 21st day after treatment with saline or BLM (0.1 U/mouse) and homogenized and protein contents were quantified. A, Western blot assay using anti-ALX and anti-α-actin Abs were performed as described in Materials and Methods. B, Blots were analyzed by densitometry and the content of ALX was expressed in arbitrary units normalized by α-actin blot. The blot in A is representative of two identical experiments with similar results.](http://www.jimmunol.org/)

![FIGURE 6. ATLa reversed BLM-induced lung fibrosis. Lungs were removed from the animals on the 21st day after treatment with saline, BLM alone (0.1 U/mouse), or BLM plus ATLa posttreatment (on day 4 (4d)) as described in Materials and Methods. Mice were injected with BLM and 4 days later were treated i.v. with ATLa (1 μg/mouse) and then boosted i.v. with ATLa at 0.1 μg/mouse on the 7th and 14th days. The coloration was made with H&E (a–c), immunostaining for α-actin (d–f), and Sirius red (g–i; original magnification, ×20) as described in Materials and Methods; n = 5 for each experimental group. Pictures are representative of each group.](http://www.jimmunol.org/)

![FIGURE 7. ATLa reduced BLM-induced increase in neutrophils (A) and mononuclear cells (B) in lung tissue. Leukocytes were quantified 21 days after the administration of saline, BLM (0.1 U/mouse), or BLM plus ATLa (added on day 4 (4d)) as described in Materials and Methods. Results are expressed as cells/mm². *, p < 0.05 compared with saline group; n = 5 for each experimental group.](http://www.jimmunol.org/)

![FIGURE 8. ATLa reversed lung collagen deposition. Hydroxyproline content from lungs of mice instilled with saline, BLM (0.1 U/mouse), or BLM plus ATLa (added on day 4 (4d)) is shown 21 days after treatment. Mice were injected with BLM, treated i.v. with ATLa (1 μg/mouse) 4 days later, and then boosted i.v. with ATLa at 0.1 μg/mouse on the 7th and 14th days. Results are expressed as micrograms per milligram of lung tissue. *, p < 0.05 compared with saline group; #, p < 0.05 compared with BLM group; n = 5 for each experimental group.](http://www.jimmunol.org/)
lymphocytes and dendritic cells (CD11c+). Under these experimental conditions, we observed an increase of both CD8+ and CD11c+ cells after BLM challenge (3.8 ± 0.18 to 5.4 ± 0.29 for CD8+ and 8.21 ± 0.67 to 16.39 ± 1.03 for CD11c+; control vs BLM group, respectively) but a reduction in the numbers of both cell types with ATLa treatment (3.6 ± 0.25 and 12.85 ± 0.7 for CD8+ and CD11c+, respectively).

Impaired matrix protein deposition in the lungs of mice instilled with BLM was also observed in animals posttreated with ATLa, as evidenced by Sirius red (Fig. 6, g–i) and Gomori staining (data not shown). In addition, hydroxyproline content analysis revealed the ability of ATLa posttreatment to inhibit/reverse lung fibrosis (Fig. 8). Lastly, morphometric analysis showed that posttreatment with the analog reduced collagen fiber deposition and preserved alveolar structure (Fig. 9).

The α-actin expression, a myofibroblast marker, was also reduced to control levels in the lungs of mice treated 4 days after BLM challenge with ATLa (Fig. 6, d–f).

ATLa reduced the TGF-β levels in the lung tissue

TGF-β is a key mediator during the evolution of pulmonary fibrosis. We examined the effect of the analog on TGF-β levels on the lung tissue at day 21 after BLM challenge. As expected, the cytokine levels in the lungs of BLM-treated animals were highly increased compared with control group, whereas the ATLa-treated group presented a reduction (~32%) in TGF-β compared with mice that received BLM alone (Fig. 10). Although in the present study we only measured TGF-β levels at day 21, other time points such as days 3, 7, and 10 after BLM might also contribute to a better understanding of the process.

ATLa restored lung function after BLM challenge

To investigate whether ATLa treatment could prevent the deleterious effect of BLM on lung function, animals were mechanically ventilated and the lung resistance and compliance after saline, BLM, and BLM plus later ATLa treatment were evaluated. Unsurprisingly, ATLa restored to normal values (control group) the compliance and resistance of the lung, which were elevated after BLM alone (Fig. 11).

Treatment with ATLa improved mice survival after bleomycin instillation

Intratracheal injection of BLM (0.5 U/mouse) induced a strong lung injury and a significant mortality among mice, which typically occurred as of the 7th day. The concomitant treatment with ATLa increased the survival rate of mice instilled with BLM to 60% vs 0% in the nontreated mice (Fig. 12).

ATLa treatment conferred resistance to BLM-induced mortality. Animals received i.t. administration of 30 μl of saline (SAL), 0.5 U of BLM, or BLM together with ATLa (added on day 0 (0d)) at 1 μg/mouse. ATLa-treated animals were boosted i.v. with ATLa at 0.1 μg/mouse on the 7th day. Animals were followed until the 14th day after BLM administration. *, p < 0.05 compared with the group that received BLM alone; n = 5 for each experimental group.
Discussion

Tissue damage can result from several acute or chronic stimuli (41). The repair process involves two distinct stages: a regenerative inflammatory phase in which the microenvironment attempts to replace injured cells and a fibrotic phase in which connective tissue replaces normal parenchymal tissue (8, 41, 42). However, although initially beneficial, failure to control the healing process can lead to considerable tissue remodeling and the formation of permanent scar tissue (8).

Pulmonary fibrosis is a disease that involves abnormal wound healing and is characterized by lung destruction and dysfunction (42). The mechanisms responsible for the progression of lung fibrosis are complex and, besides fibroblast activation, include alterations in the immune response and its regulation and chronic inflammation. In addition to the well-established role of stimulatory pathways, the importance of natural counter-regulatory mechanisms, which are responsible for the preservation of tissue function through the limitation of inflammatory and fibrotic responses, is being recognized. There is growing evidence that chronic inflammation results from insufficient production of anti-inflammatory and proresolving mediators, whereas fibrosis results from inadequate generation of the suppressive signals that control fibroblast function.

Corticosteroids and other immunosuppressive agents have been used to treat pulmonary fibrosis, but their efficacy has been disappointing and new insights into the pathophysiology and the establishment of a new therapy are urgently needed (43).

The role of LX in the regulation of the inflammatory response is of particular interest, as these novel lipid mediators not only inhibit proinflammatory mediators but actively participate in the resolution of inflammation, preventing an excessive inflammatory response and limiting damage to the host (19, 20).

LX are generated in the lung during a wide range of respiratory illnesses, and both in vitro and in vivo studies showed that LX and ATL display diverse potent anti-inflammatory actions in a variety of respiratory diseases (44–50). Reduced levels of LX have also been found in the airways of patients suffering from cystic fibrosis and appear to play a role in the disease pathophysiology (24, 29, 51). LXA4 receptor expression is induced in vivo in a murine model of airway inflammation, and ALX-transgenic mice are protected from the development of acute inflammation with markedly decreased eosinophil activation and tissue accumulation (52, 53).

In the present study, ALX expression was not induced by BLM challenge evaluated at day 21. Despite the fact that another group (54) demonstrated an increase in ALX expression in the lung after BLM, some differences should be pointed out; the other group observed mRNA instead of protein and the BLM was administered by infusion for 3 wk, whereas we gave one single dose of BLM by an i.t. route.

In this work we show that the inflammatory component of the fibrotic process, characterized by lung cell infiltration and edema, was significantly reduced by the treatment of the mice with ATLa. Cellular analysis demonstrated a beneficial effect of later treatment with the analog, which reduced the number of cells in the lung tissue, suggesting that an influx of several types of cells (e.g., neutrophils, mononuclear cells, CD11c+ cells, and CD8+ cells) plays an important role in the evolution of fibrosis. Detailed experiments evaluating several cell phenotypes and early time points to depict their kinetics and specific role in the disease are under development. It has already been described that LX decreased the production of chemokines in response to connective tissue growth factor and the platelet-derived growth factor-modified profibrotic gene expression in mesangial cells (55, 56).

The hallmark characteristic of fibrosis, namely the excessive deposition of an extracellular matrix such as collagen, was markedly inhibited by ATLa. This result is in agreement with previously reported evidence that LXA4 attenuates TGF-β-driven collagen synthesis in fibroblasts in vitro (54). Additionally, these authors showed that up-regulation of the LXA4 receptor in vivo is associated with reduced collagen accumulation in the BLM-induced model of lung fibrosis (54).

The presence of myofibroblasts in patients with pulmonary fibrosis is documented in lung tissues and also in animal models of the disease (57). It is established that fibroblasts transdifferentiate to myofibroblasts that express elevated levels of α-smooth muscle actin and, consequently, display a markedly enhanced ability to secrete extracellular matrix proteins (57–59). Among several mediators, TGF-β is the major cytokine associated with pulmonary fibrosis; it is involved in the transition of fibroblasts into myofibroblasts (60), the synthesis of matrix proteins, and collagen degradation inhibition (61, 62). Our results show that ATLa reduced BLM-induced myofibroblast accumulation/differentiation in the lung. In addition, in our model the posttreatment of the animals with ATLa also reduced the production of TGF-β, reinforcing a pivotal role of ATLa on the impairment of the fibrogenic process.

LXA4 inhibits the TGF-β-stimulated proliferation of NIH 3T3 fibroblasts and the proliferation of human lung fibroblasts induced by connective tissue growth factor (54, 63). Mesangial cell proliferation that is induced by platelet-derived growth factor, leukotriene D4 or TNF-α is also inhibited by LXA4 or its analogs (64–66). Moreover, high doses of LXA4 can even induce apoptosis of rat renal interstitial fibroblasts (67). Of interest, Sodin-Semrl et al. demonstrated that LX and ATL inhibit the production of IL-6, IL-8, and matrix metalloproteinase-3 induced by IL-1β in human synovial fibroblasts (68).

Given that ATLa reversed several inflammatory and fibrotic processes during pulmonary fibrosis, we investigated whether ATLa treatment was also able to restore BLM-induced damage to pulmonary function. In agreement with the above data, ATLa restored the resistance and compliance of the lung to normal values, reinforcing the promising therapeutic approach for lipoxin and ATL.

The current study clearly demonstrated that ATLa decreased the loss of alveolar structure, conferring resistance to BLM-induced mortality. To our knowledge, this is the first demonstration showing that LX treatment protects against lethal effects of fibrosis. It is also important to point out that the protective effect of ATLa (60% of survival) was observed with very low amounts of the analog (1 µg). Additional studies are necessary to evaluate the potential protective action of ATLa regarding dose-effect experimentation.

In view of the numerous effects of ATLa observed in this study, its mechanism of action could be a combination of several different actions, including modulatory effects on cellular recruitment and/or cellular differentiation or the modulation of TGF-β production. Notably, ATLa probably acts both directly on cells and by indirect mechanisms (via the regulation of inflammatory/fibrotic mediators). Taken together, the present study reveals that ATLa has effects on the improvement of lung function and survival that provide the foundation for future studies to better define LX/ATLa mechanisms.

In summary, the present results elucidate for the first time the antifibrotic effect of an aspirin-triggered LX analog using a relevant in vivo model of lung fibrosis. It is noteworthy that in this report we showed the therapeutic effect of ATLa in addition to the preventive effect, given that in clinical use the therapeutic effect is often more important when the fibrotic changes of various etiologies are already apparent in the patient. These data have important
implications for future efforts in developing an efficient therapeutic strategy for preventing and treating lung fibrosis by targeting LX/A4TL actions. Future experiments are necessary to understand the basic mechanism underlying the antibiogenic effects, which are currently under investigation.

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Disclosures

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