Activation of liver X receptors inhibits experimental fibrosis by interfering with interleukin-6 release from macrophages

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INTRODUCTION

Fibrosis arises from excessive accumulation of extracellular matrix, disrupts the physiological tissue architecture and causes organ failure. Fibrotic diseases lead to high morbidity and mortality among patients, and represent a major socioeconomic burden accounting for up to 45% of deaths in the developed world. Despite the urgent medical need, effective antifibrotic therapies are not available for clinical routine.1 2

Fibrosis is a pathological hallmark of systemic sclerosis (SSc). In SSc, fibrosis affects the skin and many internal organs, including the lungs, heart and gastrointestinal tract.3-4 Inflammatory cell infiltrates with macrophages, T cells and B cells are a common feature in affected tissues of SSc and other fibrotic diseases. The infiltrating leucocytes release pro-fibrotic cytokines, including interleukin (IL)-6. The pro-fibrotic signals induce the activation of fibroblasts, which in turn express contractile proteins, form stress fibres and release extracellular matrix proteins (eg, collagens).1-4

The activation of fibroblasts can be observed during both normal wound healing and pathological fibrosis. During normal wound responses, the activation of fibroblasts is strictly terminated once wound healing is completed. During pathological fibrosis, however, persistent release of pro-fibrotic signals from inflammatory cells as well as endogenous fibroblast modifications (eg, epigenetic codes, autocrine signalling loops) result in chronic fibroblast activation with excessive release of extracellular matrix proteins.1-4 Although the exact molecular mechanisms of chronic fibroblast activation are only partially revealed, interference with these processes is considered a promising treatment approach for SSc and other fibrotic diseases.1-2

Liver X receptors (LXRs) are nuclear receptors with emerging roles in metabolic and musculoskeletal disorders,3-8 autoimmune diseases,9-17 and neoplasia.18 Based on highly conserved homologies of the nuclear receptor family, LXRs were first identified by their cloned sequences prior to the identification of natural ligands and even prior to the discovery of any functional role. Research over the last 15 years has identified oxysterols and related metabolites of the cholesterol metabolism as potential natural ligands of LXRs, although it remains unclear whether physiological concentrations are able to bind to and activate these receptors. Apart from the identification of potential ligands, further studies demonstrated central roles of LXRs in cholesterol and glucose metabolism as well as in tumour surveillance and inflammatory responses.19 20

In the present study, we evaluated LXRs as potential therapeutic targets in fibrotic disease, in
particular SSc. We observed that activation of LXRs had antifibrotic effects in the models of bleomycin-induced skin fibrosis and in tight skin-1 (Tsk-1) mice. The antifibrotic effects of LXRs were mediated via inhibition of IL-6 release from macrophages.

MATERIALS AND METHODS

Mice and therapeutics

Mouse experiments and the analyses of murine skin are described in the online supplement. T0901317 was obtained from Sigma-Aldrich (Taufkirchen, Germany). The anti-IL-6 antibody 20F3 was provided by Professor S. Rose-John.21 All animal experiments were performed with the approval of the local ethics authorities.

Murine macrophage experiments

Macrophages were isolated from peritonitis exudates of naive 10-week to 12-week-old FVB mice, 72 h after intraperitoneal injection of 2.5 mL of 3% Brewer’s thioglycollate (Sigma-Aldrich). Peritoneal macrophages were harvested by peritoneal lavage with ice-cold 4% fetal bovine serum (FBS) in phosphate buffered saline (PBS) and plated in 48-well plates in a concentration of 1 Mio/mL (250 000 cells per well). Macrophages were allowed to rest overnight at 37°C at 5% CO2 in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS before starting experiments. FBS concentration was then reduced to 0.5% for 24 h. After macrophages were preincubated with T0901317 in a concentration of 5 μM for 3 h, they were stimulated with lipopolysaccharide (LPS) 100 ng/mL (Sigma-Aldrich, Taufkirchen, Germany) for up to 24 h. T0901317 was dissolved in dimethyl sulfoxide (DMSO); the final concentration of DMSO in the experiments did not exceed 0.1%.

Human fibroblast experiments

Fibroblast cultures were obtained from skin biopsies of six SSc patients. All SSc patients presented with diffuse-cutaneous SSc, and 3 mm punches were taken from lesional skin at the volar side of the forearm. Fibroblast isolation and culture were performed as described previously.22–24 All SSc patients provided written informed consent as approved by the institutional ethics committees.

Fibroblasts from passages 4 to 8 were used for the experiments. Fibroblasts were seeded in 6-well plates and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS until cells reached confluence. FBS was reduced to 0.1% for 48 h. Fibroblasts were pretreated with T0901317 in a concentration of 5.0 μM for 3 h and then stimulated with recombinant transforming growth-factor-β (TGF-β) (10 ng/mL; R&D Systems, Abingdon, UK). Forty-eight hours after TGF-β stimulation, supernatants were collected (to measure collagen content) and cells lysed in RAI buffer (for RNA analysis; NucleoSpin RNA II extraction system). T0901317 was dissolved in DMSO; the final concentration of DMSO in the experiments did not exceed 0.1%.

Human macrophage experiments

Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of five SSc patients using Lympholot® (Bio-Rad, Hercules, California, USA) according to the manufacturer’s recommendations. All SSc patients provided written informed consent as approved by the institutional ethics committees. PBMCs were seeded in RPMI supplemented with 0.5% FBS in a concentration of 1×10^6/mL. Monocytic cells were allowed to adhere to the plastic ground for 2 h at 37°C, and non-adherent cells were washed away with warm PBS. Afterwards, monocytes were kept in RPMI with 10% FBS and 10% autologous serum for 7 days to allow the differentiation into macrophages. At day 8, the medium was replaced by fresh RPMI with 0.5% FBS. Twenty-four hours later, studies were performed according to the murine macrophage experiments.

IL-6 ELISA

IL-6 was determined in the supernatants from the murine macrophage experiments with the mouse IL-6 DuoSet ELISA (R&D Systems, Minneapolis, Minnesota, USA).

Multiplex bead array

Cytokine levels were measured in the supernatants from the human macrophage experiments by multiplex bead array technology (Bender MedSystems, Vienna, Austria) as described previously.23,26

Quantitative real-time PCR (qPCR)

Gene expression was quantified by SYBR green real-time PCR on a Stratagene Mx3005 qPCR System (Agilent Technologies, Santa Clara, California, USA). Nonspecific signals caused by primer dimers were excluded by dissociation curve analysis and use of non-template controls. To normalise for loaded cDNA, β-actin was used as an endogenous control.

Statistical analysis

All data are presented as median with IQR. Differences between the groups were tested for their statistical significance by two-tailed Mann–Whitney U non-parametric test using GraphPad Prism (V.5.03) except indicated otherwise. p values less than 0.05 were considered significant.

RESULTS

Activation of LXRs inhibits bleomycin-induced skin fibrosis

To investigate the role of LXRs in experimental fibrosis, we studied the effects of the LXR agonist T0901317 in the model of bleomycin-induced skin fibrosis. When we treated bleomycin-challenged mice with T0901317, we observed potent, dose-dependent antifibrotic effects on skin thickening, hydroxyproline content and myofibroblast numbers (figure 1A–D). In the group of mice receiving T0901317 in a dose of 25 mg/kg once daily, we found a decrease in skin thickening of 64.1% (CI 21.6% to 114.2%), a reduction in the hydroxyproline content of 90.7% (CI 14.9% to 275.1%) and a decrease in myofibroblast counts by 91.3% (CI 51.4% to 139.0%) (figure 1B–D). Apart from the potent antifibrotic effects of LXR activation, we observed a strong decline in inflammatory infiltrates by 60.2% (CI 11.9% to 154.6%) in the group of mice receiving 25 mg/kg/d T0901317 (figure 1E). The potent antifibrotic and anti-inflammatory effects of LXR activation were accompanied by excellent tolerability: throughout all bleomycin experiments, mice tolerated both doses of T0901317 well as indicated by constant weight, normal texture of the fur and normal activity (data not shown).

LXR activation inhibits fibrosis in the model of sclerodermatous GvHD (scGvHD)

Given the extent of skin fibrosis and the substantial overlap in gene expression profiles, murine scGvHD is considered an elegant model to study inflammatory, diffuse-cutaneous SSc.27,28 By contrast to their syngenic controls, mice receiving allogenic bone marrow transplantation developed clinical signs,
including hair loss and superficial skin ulcerations, as well as severe skin fibrosis (figure 2A–E). Treatment with the LXR agonist T0901317 was started on day 21 after transplantation when first clinical signs became obvious, and it was continued to day 42 when mice were sacrificed. We observed that LXR activation reduced weight loss, improved clinical signs (data not

Figure 1  Activation of liver X receptors by T0901317 inhibits the development of bleomycin-induced skin fibrosis in a dose-dependent manner. (A) Representative images of Masson’s trichrome staining with blue staining for collagens. Mice were challenged with bleomycin subcutaneously and received daily per os feeding with T0901317 in different doses. Pictures are shown at 100-fold magnification. Scale bar, 100 µm. (B) Skin thickening as determined in trichrome stainings. (C) Hydroxyproline (HP) content. (D) α-smooth muscle actin-positive myofibroblasts. (E) Inflammatory infiltrates as determined in H&E stainings. The groups consisted of ≥6 mice each.

Figure 2  Activation of liver X receptors by T0901317 inhibits the development of systemic fibrosis in sclerodermatous graft-versus-host disease (scGvHD). (A) Representative images of Masson’s trichrome staining with blue staining for collagens. Mice were subject to allogenic stem cell transplantation and received daily per os feeding with T0901317 from day 21 to 42 after transplantation. Pictures are shown at 100-fold magnification. Scale bar, 100 µm. (B) Skin thickening as determined in trichrome stainings. (C) Hydroxyproline (HP) content. (D) α-smooth muscle actin-positive myofibroblasts. (E) Inflammatory infiltrates as determined by H&E stainings. The syngenic group consisted of four mice, the two other groups of six mice each.
shown) and inhibited skin fibrosis in mice receiving allogenic stem cell transplantation (figure 2B–E). In detail, treatment with the LXR agonist T0901317 reduced skin thickening by 69.4% (CI 15.3% to 143.1%), hydroxyproline content by 122.6% (CI 19.6% to 458.3%), myofibroblast counts by 89.9% (CI 32.6% to 306.9%) and leucocyte infiltration by 60.5% (CI 40.4% to 206.2%). Together with the data from the bleomycin model, these results highlight that LXR activation effectively inhibits inflammation-driven fibrosis induced by both exogenous, profibrotic toxins and intrinsic autoimmune processes.

**LXRs activation is not required to maintain skin homeostasis**

Next, we analysed whether knockout of LXR might exacerbate bleomycin-induced skin fibrosis. Both LXRα-knockout and LXRβ-knockout mice showed similar responses to bleomycin as wildtype mice with comparable increases in skin thickening, hydroxyproline content and myofibroblast counts (see online supplementary figure S1A–F). To exclude that lack of one isoform could be compensated by the other one, we generated LXRα/β-double-knockout mice. Similar to the single knockout animals, the double knockout mice showed a comparable response to the bleomycin challenge as wildtype animals (figure 3A–E). Since we observed low expression levels of the LXR target gene ABCA-1 in both NaCl- and bleomycin-challenged wildtype mice, we concluded that LXR signalling may be characterised by low baseline activation in skin tissue (figure 5C). Treatment with T0901317, however, resulted in a strong increase in expression of ABCA-1 demonstrating the high responsiveness of fibrotic skin towards LXR agonists (figure 5C). While low baseline activity suggested the dispensability of LXRs for normal tissue homeostasis of the skin, specific LXR activation was effective in stimulating an anti-fibrotic signalling cascade and inhibiting skin fibrosis.

Taking advantage of LXRα/β-double knockout mice, we confirmed that the antifibrotic effects of T0901317 in bleomycin-induced dermal fibrosis were indeed mediated via LXRs and not caused by off-target effects of T0901317. In contrast to wildtype mice, treatment with T0901317 was ineffective to reduce skin thickening, hydroxyproline content, α-smooth muscle actin-positive myofibroblasts or leucocyte counts in LXRα/β-double knockout mice challenged with bleomycin (figure 3A–E).

**Activation of LXRs inhibits skin fibrosis in the Tsk-1 mouse model**

Bleomycin-induced skin fibrosis and scGvHD both reflect inflammatory subtypes of SSc. To also mimic other subsets of SSc patients with less pronounced inflammation, we investigated the antifibrotic effects of LXRs in the Tsk-1 mouse model. Although Tsk-1 mice show B cell activation and develop auto-antibodies against SSc-antigens, inflammatory infiltrates are absent or scarce in this model.28 In Tsk-1 mice, activation of LXRs with T0901317 had only modest antifibrotic effects (figure 4A–D). Although statistically significant, reductions of skin thickening, hydroxyproline content and myofibroblast counts were far less prominent as observed in the inflammatory models of bleomycin-induced skin fibrosis and scGvHD.

**Activation of LXRs reduces the release of the pro-fibrotic IL-6 from macrophages**

The prominent antifibrotic activity of LXRs in the inflammatory bleomycin-model exceeded the effects we observed in the Tsk-1 mouse model. Based on these observations, we considered an indirect, leucocyte-dependent mechanism for the antifibrotic effects of LXR agonists. To test this hypothesis, we first analysed the direct effects of T0901317 on cultured human fibroblasts. Although we found that both isoforms, LXRα and LXRβ, were expressed in healthy and SSc skin as well as fibroblasts isolated from healthy and SSc skin (data not shown), treatment with T0901317 in different concentrations did not alter the release of collagen from resting fibroblasts or fibroblasts stimulated with pro-fibrotic cytokines, such as TGF-β (figure 5A,B).
To investigate the role of leucocytes, we then studied the inflammatory infiltrates in bleomycin-challenged mice and observed a pronounced reduction of infiltrating macrophages upon treatment with T0901317, with decreases of 70.9% (CI 26.4% to 135.0%) for the dose of 25 mg/kg/day (figure 5D). We wondered whether LXR activation may not only reduce the number of macrophages in lesional tissue but also interfere with the release of pro-fibrotic mediators from these cells. Indeed, we observed that LXR activation significantly inhibited the synthesis and release of IL-6 from isolated macrophages upon stimulation with LPS (figure 5F, G). Twenty-four hours after stimulation, IL-6 protein was reduced by 72.0% (CI 30.0% to 135.2%) (figure 5G). Translating these findings in our in vivo model system, we observed decreased IL-6 levels in bleomycin-challenged mice treated with the LXR agonist T0901317 (figure 5E, see online supplementary figure S2). Of note, LXR agonists did not exert additive anti-fibrotic effects in bleomycin-challenged mice treated with anti-IL-6 blocking antibodies. Co-treatment with anti-IL-6 antibodies and T0901317 did not further reduce skin thickness, hydroxyproline (HP) content and α-smooth muscle actin-positive myofibroblasts. The groups consisted of ≥6 mice each. Tsk-1, tight skin-1

**Figure 4** Activation of liver X receptors inhibits spontaneous skin fibrosis in Tsk-1 mice. (A) Representative images of Masson’s trichrome staining with blue staining for collagens. Tsk-1 mice received daily per os feeding with T0901317 in a dose of 25 mg/kg. pa/pa mice were used as controls. Pictures are shown at 40-fold magnification. Scale bar, 250 μm. (B) Hypodermal thickening as determined by trichrome staining. (C) Hydroxyproline (HP) content. (D) α-smooth muscle actin-positive myofibroblasts. The groups consisted of ≥6 mice each. Tsk-1, tight skin-1

DISCUSSION
We identified the nuclear receptors LXRs as novel therapeutic targets for SSc and other fibrotic diseases. We demonstrated that activation of LXRs has potent anti-fibrotic effects in different experimental models of fibrosis. These anti-fibrotic effects are mediated by suppression of macrophage infiltration and decreased release of the pro-fibrotic cytokine IL-6.

Our findings open up a new vein of potential applications for LXRs. So far, research has focused on the roles of LXRs in metabolic and autoimmune diseases, including diabetes, hypercholesterolaemia, multiple sclerosis and rheumatoid arthritis. In rheumatoid arthritis, the role of LXRs is controversial. While some research groups suggested that LXRs could promote disease progression by inducing Th1 and Th17 cytokines, including TNF-α and IL-1β, others found anti-inflammatory and disease-modifying activities of LXRs in experimental models of arthritis. In the context of fibrotic disease, we observed that activation of LXRs ameliorates...
inflammation and fibrosis, mainly via interfering with the IL-6 release from macrophages.

LXRs are master regulators of glucose and cholesterol homeostasis. Activation of LXRs decreases glucose output and increases glucose use by inducing the expression of glucose transporters and enzymes of glycolysis. In addition to fine tuning glucose metabolism, LXR activation reduced streptozotocin-induced diabetic retinopathy and nephropathy. LXRs regulate whole-body cholesterol levels, enhance reverse cholesterol transport and stimulate cholesterol secretion. Within the liver, LXRs protect hepatocytes from cholesterol and bile acid toxicity. LXRs may also inhibit hepatic stellate cell activation upon injury and prevent subsequent fibrotic responses. In this context, loss of LXRα and LXRβ enhanced the activation of hepatic stellate cells and exacerbated CCl4-induced liver injury and fibrosis, while pharmacological activation of LXRs reduced hepatic stellate cell activation. Although these results confirm our findings on the anti-fibrotic role of LXRs, the modes of action of LXRs differ between experimental fibrosis in liver and skin. While LXRs may directly inhibit hepatic stellate cell activation and collagen release in liver fibrosis, we established an indirect anti-fibrotic mechanism of LXRs involving the IL-6 release from fibroblasts. The unique role of hepatic stellate cells in liver fibrosis compared with other fibrotic diseases in which fibroblasts are the key effector cells may explain the differences to our findings in skin fibrosis.

In our study, LXR activation was effective in inhibiting experimental fibrosis with the most prominent effects in the inflammatory models of bleomycin-induced dermal fibrosis and scGvHD. LXR activation reduced inflammatory infiltrates and fibrotic changes in inflammation-driven experimental skin fibrosis by reducing the release of IL-6 from macrophages. Macrophages are key players in physiologic wound healing and pathological tissue fibrosis and have been identified as cellular

**Figure 5** The anti-fibrotic effects of liver X receptor (LXR) activation are mediated via inhibition of macrophage infiltration and interleukin (IL)-6 release. (A) Messenger RNA expression of col1a1 pro-collagen in normal fibroblasts from healthy individuals stimulated with TGF-β 10 ng/mL and pretreated with T0901317 5 μM. Values are expressed as x-folds compared with the control group without TGF-β and T0901317 treatment. N=6 for each group. (B) Collagen content in the supernatant released from normal fibroblasts of healthy individuals stimulated with TGF-β 10 ng/mL and pretreated with T0901317 5 μM. N=6 for each group. (C) Messenger RNA expression of the target gene ABCA-1 in mice challenged with bleomycin and treated with T0901317 25 mg/kg po once daily. N=6 per group. Values are expressed as x-folds compared with the control group receiving subcutaneously NaCl challenge and oral mock treatment. (D) Numbers of F4/80 positive macrophages per high power field in the skin of mice challenged with bleomycin and treated with T0901317 in a dose of 25 mg/kg once daily. N=6 per group. (E) Interleukin-6 staining as assessed by a semiquantitative score in the skin of mice challenged with bleomycin and treated with T0901317 in a dose of 25 mg/kg once daily. N=6 per group. (F) Messenger RNA expression of IL-6 from peritoneal macrophages after pretreatment with T0901317 5 μM and stimulation with LPS 100 ng/mL expressed as x-fold of the untreated and unstimulated control. N=5 for each group. (G) Interleukin-6 release from peritoneal macrophages after pretreatment with T0901317 5 μM and stimulation with LPS 100 ng/mL. N=5 for each group. (H–J) LXR activation by T0901317 does not have additive effects in the bleomycin-challenged mice after IL-6 blockade with a monoclonal IL-6 blocking antibody. The groups consisted of ≥6 mice each. (H) Skin thickening as determined in trichrome stainings. (I) Hydroxyproline (HP) content. (J) α-smooth muscle actin-positive myofibroblasts.

TGF-β, transforming growth factor-β; LPS, lipopolysaccharide
key effectors in SSc.13–17 The numbers of monocytes and macrophages are highly elevated in the affected skin of patients with early SSc, exceeding those of other cell populations, such as T cells.18 In later disease stages, there is good evidence for abnormal differentiation of peripheral mononuclear cells into activated CD163+ or CD204+ macrophages, which reside between the collagen fibres.19 While the role of macrophages as a major source of pro-fibrotic mediators in SSc skin has been well established,20 evidence for a central role of IL-6 in SSc is still emerging.40 Three recent studies reported increased serum levels of IL-6 in SSc, which may correlate with more severe skin disease, cardiac involvement, progression of lung fibrosis and overall long-term survival.41–43 These observations have translational implications for the potential use of LXR agonists in SSc: based on the mode of action, LXR activation might serve as effective personalised therapies for SSc patients in early inflammatory stages or with inflammatory disease subtypes. As established by recent gene expression profiling studies,44–45 these patients are characterised by persistent upregulation of genes associated with T cells, B cells and the monocyte/macrophage lineage.

Taken together, we identified a new role of LXRs in inhibiting experimental fibrosis. Our findings suggest that activation of LXRs may reduce both inflammation and fibrosis in SSc patients. LXRs may therefore be promising therapeutic targets for SSc patients in early stages or with inflammatory disease subtypes. Before translating our findings into clinical practice, however, additional studies investigating the role of LXRs in vascular disease and fibrosis of internal organs are warranted.

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