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Lipoxin A4 attenuates constitutive and TGF β 1-dependent pro-fibrotic activity in human lung myofibroblasts

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a common, progressive and invariably lethal interstitial lung disease with no effective therapy. The key cell driving the development of fibrosis is the myofibroblast. Lipoxin (LX)A₄ (LXA₄) is an anti-inflammatory lipid, important in the resolution of inflammation and it has potential anti-fibrotic activity. However, the effects of LXA₄ on primary human lung myofibroblasts (HLMF) have not previously been investigated. Therefore, the aim of this study was to examine the effects of LXA₄ on TGF β 1-dependent responses in IPF- and non-fibrotic control (NFC)-derived HLMFs. HLMFs were isolated from IPF and NFC patients and grown *in vitro*. The effects of LXA₄ on HLMF proliferation, collagen secretion, α SMA expression and Smad2/3 activation were examined constitutively and following TGF β 1 stimulation. The LXA₄ receptor (ALXR) was expressed in both NFC- and IPF-derived HLMFs. LXA₄ (10⁻¹⁰ M and 10⁻⁸ M) reduced constitutive α SMA expression, actin stress fiber formation, contraction and nuclear Smad2/3, indicating regression from a myofibroblast to fibroblast phenotype. LXA₄ also significantly inhibited FBS-dependent proliferation, and TGF β 1-dependent collagen secretion, α SMA expression, and Smad2/3 nuclear translocation in IPF-derived HLMFs. LXA₄ did not inhibit Smad2/3 phosphorylation. In summary, LXA₄ attenuated pro-fibrotic HLMF activity and promoted HLMF regression to a quiescent fibroblast phenotype. LXA₄ or its stable analogues delivered by aerosol may offer a novel approach to the treatment of IPF.

Keywords

Idiopathic Pulmonary Fibrosis (IPF); fibrosis; lung; myofibroblasts; Lipoxin A₄; differentiation; Smad 2; Smad 3

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive disease with a median survival of only 3 years(1, 2). Patients present with breathlessness and disabling cough, progressing to

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respiratory failure and a distressful death. The cause is not known, but alveolar cell injury coupled with fibroblast/myofibroblast proliferation and activation are critical in the pathophysiology(3, 4). There is little effective treatment, and there is therefore an urgent unmet clinical need for novel modulators of lung fibrosis and tissue remodelling.

The key cell driving the development of fibrosis is the myofibroblast(5, 6). Myofibroblasts are intermediate in phenotype between fibroblasts and smooth muscle cells, expressing α -smooth muscle actin (α SMA) and exhibiting contractile activity, but they are also the principle cell responsible for the synthesis and deposition of the fibrotic matrix in IPF(7). The increased numbers of myofibroblasts found within IPF lungs occurs in part through the differentiation of resident fibroblasts(8); this involves reorganization of the actin cytoskeleton, increased expression of α -smooth muscle actin (α SMA) and incorporation of actin stress fibers(9), a process regulated by the TGF β 1/Smad pathway(10, 11). Furthermore, myofibroblasts derived from IPF lungs demonstrate enhanced proliferation(12), migration(13), collagen production(14), α -smooth muscle actin (α SMA) expression(15) and actin stress fibre formation(15). The myofibroblast is therefore a highly attractive target for the treatment of IPF. As myofibroblasts rarely persist in healthy lungs, their differentiation is considered a key event in the pathogenesis of IPF, and it is likely that a therapy capable of reversing this phenotypic change would be efficacious.

A family of lipid mediators known as lipoxins, resolvins, protectins and maresins (collectively called resolving mediators for convenience)(16-18), are important for the resolution of inflammation, and are generated soon after a tissue insult. Lipoxins are generated from membrane arachidonic acid through biochemical synthesis involving the enzymes 5- and 15-lipoxygenase (5-LOX, 15-LOX)(18). Several molecules are generated through transcellular synthesis with 15-LOX active in one cell such as an epithelial cell, and 5-LOX active in a second cell such as an inflammatory leukocyte. In addition, aspirin-triggered (AT) forms of these molecules exist (epi-lipoxins, AT-resolvins), where acetylated COX-2 generates the initial metabolite which is then modified further by 5-LOX. Several molecules can also be formed endogenously in the absence of aspirin, possibly via a cytochrome P450-dependent pathway(17), and 5-LOX-independent pathways for the production of protectins and maresins also exist with unicellular synthesis evident(18).

A key feature of these molecules is that they promote resolution of inflammation at low nM concentrations, but are not innately immunosuppressive as they also activate anti-bacterial mechanisms(17, 19). Lipoxin (LX)A₄ also has anti-fibrotic activity in a number of model systems. For example, it inhibits PDGF-dependent TGF β 1 production and pro-fibrotic gene expression by renal mesangial cells(20), inhibits mesangial cell proliferation(21), and experimental renal fibrosis(22). LXA₄ also inhibitions EMT in renal epithelial cells(23), while knockout of the 12/15-LOX pathway prevents experimental dermal fibrosis(24). With respect to lung fibrosis, LXA₄ inhibited connective tissue growth factor-dependent proliferation of a human lung fibroblast cell line(25), while a stable epi-LXA₄ analogue reduced bleomycin-induced pulmonary fibrosis in mice(26).

The effects of LXA₄ on the function of healthy and IPF-derived primary human lung myofibroblast (HLMF) function are unknown. We hypothesized that LXA₄ inhibits

constitutive HLMF pro-fibrotic responses and TGF β 1-driven pro-fibrotic activity through the Smad signalling pathway. We therefore investigated the effects of LXA₄ on constitutive and TGF β 1-dependent HLMF Smad 2/3 activity, gene transcription, and pro-fibrotic HLMF processes such as contraction, collagen secretion, proliferation and differentiation.

MATERIALS AND METHODS

Human lung myofibroblast isolation, characterisation and culture

Non-fibrotic control (NFC) HLMFs were derived from healthy areas of lung from patients undergoing lung resection for carcinoma at Glenfield Hospital, Leicester, UK. No morphological evidence of disease was found in the tissue samples used for HLMF isolation. IPF HLMFs were derived from patients undergoing lung transplant at the University of Pittsburgh Medical Center, USA, and were shown to have usual interstitial pneumonia (UIP) on histological examination. Myofibroblasts were grown, cultured and characterised as previously described(27). All NFC patients gave informed written consent and the study was approved by the National Research Ethics Service (references 07/MRE08/42 and 10/H0402/12). Written informed consent was also obtained from all IPF subjects, with the protocol approved by the University of Pittsburgh Institutional Review Board.

All cultures demonstrated the typical elongated spindle-shaped fibroblast morphology. All cultures underwent further extensive characterisation via flow cytometry and immunofluorescence. They were found to be predominantly a myofibroblast rich population (99% expressing α SMA) as described previously by us and others (15, 27-30). They expressed α SMA, CD90, fibroblast surface protein (FSP1), and collagen type 1. No contaminating cells were found. Immunostaining for macrophages (CD68), T-cells (CD3), and progenitor cells (CD34) was negative. There was no evidence of cobblestone shaped epithelial cells amongst the cultures. Results of this detailed characterisation have been shown previously(27, 30).

LXA₄

LXA₄ (5(S),6(R)-Lipoxin A₄) (Cayman Chemicals, Michigan, USA) was used at concentrations of 10⁻¹⁰ M and 10⁻⁸ M.

Flow cytometry

HLMFs were grown in T25 flasks and serum-starved for 24 h prior to experiments. The myofibroblasts were incubated for 24 h and either left unstimulated or stimulated with TGF β 1 (10 ng/ml)(R&D Systems, Oxford, UK).

To determine the expression of the LXA₄ receptor ALXR, HLMFs were detached using 0.1% trypsin/0.1% EDTA and gated using fibroblast surface antigen, Thy-1 (Merck, Hertfordshire, UK). Myofibroblasts were then labelled with APC-conjugated mouse monoclonal anti-FPRL1 (Formyl-peptide receptor-like 1 [FRPL1], also known as ALXR) (R&D Systems) or APC-conjugated isotype control IgG2b antibody (R&D Systems).

To study the inhibitory effects of LXA₄ on α SMA expression, HLMFs were incubated in the presence of serum-free medium alone or 0.1% ethanol vehicle control or LXA₄ at 10⁻¹⁰ M and 10⁻⁸ M. Cells were detached using 0.1% trypsin/0.1% EDTA, washed, then fixed and permeabilized in 4% paraformaldehyde plus 0.1% saponin (Sigma-Aldrich, Poole, Dorset, UK) for 20 minutes on ice. Myofibroblasts were labelled with either: FITC-conjugated mouse monoclonal anti- α SMA (Sigma) or isotype control FITC-conjugated mouse IgG_{2a}; secondary antibodies labelled with FITC were applied if appropriate. Analysis was performed using single colour flow cytometry on a FACScan (BD biosciences, Oxford, UK).

Immunofluorescence

HLMFs were grown on 8-well chamber slides and serum-starved for 24 h prior to the experiment. The cells were left unstimulated and incubated in the presence of 0.1% ethanol vehicle control or LXA₄ at 10⁻¹⁰ M and 10⁻⁸ M for 48 hours. Cells were then immunostained as described previously(27) using FITC-conjugated mouse monoclonal anti- α SMA (F3777, 10 μ g/ml, Sigma) and isotype control FITC-conjugated mouse IgG_{2a} (X0933, 10 μ g/ml, Dako, Ely, UK). Cells were mounted with fluorescent mounting medium and cover-slipped. Original images were captured on an epifluorescent microscope (Olympus BX50, Olympus UK Ltd, Southend-on-Sea); grey scale intensity was examined using Cell F imaging software (Olympus UK Ltd). Matched exposures were used for isotype controls.

Actin stress fibres were calculated using a specialised macro on image J designed by Dr Kees Straatman, University of Leicester(15). The macro is capable of providing a quantitative, unbiased score of the number of stress fibres per individual cell by determining the fluctuations of grey scale intensity created by the α SMA staining within the stress fibres.

Collagen Gel Contraction Assay

HLMFs were serum-starved for 24 h and then pre-treated for 24 h with serum-free medium alone, 0.1% ethanol control, LXA₄ 10⁻¹⁰ M or LXA₄ 10⁻⁸ M. Cells were detached and embedded in collagen gels as described previously (31). TGF β 1 was then added to appropriate wells to a final concentration of 10 ng/ml. Photographs were taken at 0 and 24 h. The surface area was measured at each time point using ImageJ software (<http://rsbweb.nih.gov/ij/>).

Smad2/3 nuclear localisation

HLMFs were grown on 8-well chamber slides and serum-starved for 24 h prior to the experiment. The cells were either unstimulated or stimulated with TGF β 1 (10 ng/ml) in the presence of serum-free medium alone, 0.1% ethanol control, LXA₄ 10⁻¹⁰ M or LXA₄ 10⁻⁸ M. After 1 h cells were immunostained using rabbit monoclonal anti-Smad2/3 (0.174 μ g/ml, Cell Signalling). Secondary antibody labelled with FITC (F0313, Dako) was applied and the cells counterstained with DAPI (Sigma). Cells were mounted with fluorescent mounting medium and cover-slipped. Images were analysed as above. The intensity of nuclear Smad2/3 staining was quantified by measuring the grey scale intensity of DAPI positive nuclei to whole cell staining.

qRT-PCR

HLMF RNA was isolated using the RNeasy Plus Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions. Primers were designed for α SMA (ACT2A), forward TTCAATGTCCCAGCCATGTA and reverse GAAGGAATAGCCACGCTCAG, product size 222bp from NCB1 Reference sequence NM_001141945.1; Collagen type 1 (COL1A1), forward TTCTGCAACATGGAGACTGG and reverse CGCCATACTCGAACTGGAATC, Collagen type IV (COL4A1), forward GGACTACCTGGAACAAAAGGG and reverse GCCAAGTATCTCACCTGGATCA, product size 240bp from reference sequence NM_001845.4; β -actin primers were analysed using gene-specific Quantitect Primer Assay primers (Qiagen), HS_ACTB_1_SG. All expression data were normalized to β -actin and corrected using the reference dye ROX. Gene expression was quantified by real-time PCR using the Brilliant SYBR Green QRT-PCR 1-Step Master Mix (Stratagene, The Netherlands). PCR products were run on a 1.5% agarose gel to confirm the product amplified was the correct size, and each of the products were sequenced to confirm the specificity of the primers.

Collagen Secretion Assay

HLMFs were cultured in serum-free medium alone or 0.1% ethanol control, and stimulated with TGF β 1 10 ng/ml in the presence of ethanol control, and LXA₄ at 10⁻¹⁰ M or 10⁻⁸ M for 24 h. Soluble collagen released by HLMFs was quantified using the Sircol collagen assay (Biocolor, County Antrim, UK) according to the manufacturer's instructions (27, 32, 33).

Proliferation Assay

HLMFs were seeded into 6 well plates, and when 50% confluent cells were serum starved for 24 hours in serum-free medium. Cells were then stimulated with serum-free medium plus 0.1% ethanol, 10% FBS medium plus 0.1% ethanol, or 10% FBS plus LXA₄ at 10⁻¹⁰ M and 10⁻⁸ M. After 48 hours cells were mobilized with 0.1% trypsin/0.1% EDTA and counted using a standard haemocytometer. Cell viability was assessed by Trypan blue exclusion. Results were counted by 2 blinded observers with high agreement (intra-class correlation of 0.969). All conditions were performed in duplicate.

Western blot for Smad proteins

HLMFs were grown in T75 flasks, serum-starved for 24 hours, and stimulated with TGF β 1 (10 ng/ml) in the presence of either serum-free medium alone, 0.1% ethanol control, LXA₄ 10⁻¹⁰ M or LXA₄ 10⁻⁸ M for 1 hour. HLMFs were detached with 0.1% trypsin/EDTA and washed. Protein was isolated using the RIPA buffer lysis system (Santa Cruz, Germany) and total protein concentration was determined using the DC Bio-Rad protein Assay (Bio-Rad, UK). 30 μ g of protein was resolved using 10% Mini-Protean TGX precast gels (Bio-Rad) and then transferred to an immunobilon-P polyvinylidene difluoride membrane, using Trans-blot Turbo transfer packs (Bio-Rad). Membranes were blocked with 5% milk and incubated with rabbit monoclonal anti-phosphorylated-Smad2/3 (0.231 μ g/ml, Cell Signalling) or rabbit monoclonal anti-Smad2/3 (0.0087 μ g/ml, Cell Signalling). Protein bands were identified by horseradish peroxidase-conjugated secondary antibody and enhanced

chemiluminescence reagent (Amersham, UK). Immunolabelled proteins were visualized using ImageQuant LAS 4000 (GE Healthcare Life Sciences, UK).

Statistical Analysis

Experiments from an individual donor were performed either in duplicate or triplicate and a mean value was derived for each condition. Data distribution across donors was tested for normality using the Kolmogorov-Smirnov test. For parametric data the 1-way ANOVA or repeated measures ANOVA for across-group comparisons was used followed by the appropriate multiple comparison post hoc test; otherwise an unpaired or paired t-test was used. For non-parametric data the Friedman test was used for across group comparisons followed by the appropriate multiple comparison post hoc test, or the Wilcoxon signed rank test was used where there paired groups. GraphPad Prism for windows (version 6, GraphPad Software, San Diego California USA) was used for these analyses. A value of $P < 0.05$ was taken to assume statistical significance and data are represented as mean (\pm SEM) or median (IQR).

RESULTS

Human lung myofibroblasts express LXA₄ receptors

LXA₄ acts via activation of the ALXR G-protein-coupled receptor (also known as FPR2, FPRL1, FPRH1, RFP, HM63), in low nM concentrations. The functions of this receptor however are cell-specific; in neutrophils LXA₄-ALXR interactions inhibit migration but in monocytes these can stimulate chemotaxis(34).

NFC- and IPF-derived HLMFs expressed ALXR (FRPL1) with a whole population shift in comparison to the control antibody, $P = 0.0056$, Mann-Whitney test (**Figure 1A**). There was a trend for a higher expression in the IPF-derived myofibroblasts, although this was not statistically significant and expression of ALXR did not change following 24 hours of TGF β 1 stimulation (**Figure 1B**). Thus HLMFs express LXA₄ receptors.

Constitutive α SMA expression and actin stress fibre formation is inhibited by LXA₄

We and others have shown that fibroblasts obtained from human lung parenchyma express high quantities of α SMA and have the typical myofibroblast contractile phenotype(27, 29, 35, 36). Furthermore, these HLMFs derived from IPF donors express higher numbers of actin stress fibres constitutively than cells from NFC donors(15). After 48 hours of treatment with LXA₄ (10^{-10} M and 10^{-8} M), the amount of constitutive α SMA staining was dose-dependently reduced (**Figure 2A-C**). Following treatment with LXA₄ cells were less stellate and became more spindle like, a similar morphology to inactivated fibroblasts (**Figure 2D**). IPF-derived HLMFs again expressed more α SMA stress fibres at baseline in comparison to NFC-derived cells, $P = 0.0177$. LXA₄ reduced the number of actin stress fibres in both IPF- and NFC-derived cells and to a similar extent (**Figure 2E and F**).

Constitutive HLMF contraction is inhibited by LXA₄

One of the main features of a myofibroblast is its ability to drive tissue repair and wound closure by reorganising the ECM via contraction(37). We therefore investigated the effects

of LXA₄ on constitutive HLMF contraction. HLMF contraction was examined over 24 h in the presence of LXA₄ at 10⁻¹⁰ M and 10⁻⁸ M using collagen gels (**Figure 3A**). With both NFC- and IPF-derived HLMFs, basal contraction was dose-dependently reduced by LXA₄ (P=0.0009 and P=0.0007 respectively, for LXA₄ at 10⁻⁸ M) (**Figure 3B**).

Constitutive HLMF Smad2/3 nuclear translocation is inhibited by LXA₄

It has previously been reported that αSMA expression and stress fibre formation in myofibroblasts is regulated in part by the TGFβ1/Smad signalling pathway(10, 11). As LXA₄ inhibited both constitutive αSMA expression and HLMF contraction, we examined whether LXA₄ disrupts constitutive Smad2/3 nuclear translocation using immunofluorescent staining. HLMFs had detectable Smad2/3 within the nucleus at baseline, and the IPF-derived HLMFs displayed a higher proportion of nuclear staining in comparison to the NFC-derived cells (P=0.043), in keeping with previous work(15). This nuclear Smad2/3 immunostaining was reduced and to a similar extent in both IPF- and NFC-derived HLMFs in the presence of LXA₄ (10⁻⁸ M) for 1 h (**Figure 3C and D**).

TGFβ1-induced αSMA expression and contraction are inhibited by LXA₄ in HLMFs

Next we investigated whether LXA₄ inhibits TGFβ1-induced pro-fibrotic activity in HLMFs. Cells were treated with TGFβ1 in the presence of 0.1% ethanol control, or LXA₄ at 10⁻¹⁰ M and 10⁻⁸ M, and flow cytometry was performed. In comparison to vehicle control, TGFβ1 stimulation significantly increased αSMA protein expression in both NFC- and IPF-derived HLMFs, P=0.0070 (**Figure 4A**). The TGFβ1-dependent increase in αSMA expression was dose-dependently inhibited in both NFC- and IPF-derived HLMFs by LXA₄ at 10⁻¹⁰ M (P=0.0106) and 10⁻⁸ M (P=0.0076) (**Figure 4A and B**). This was also confirmed by Western blot analysis, where TGFβ1 increased αSMA expression in IPF-derived HLMFs but not NFC-derived cells, and which was significantly attenuated by LXA₄ at 10⁻⁸ M (P=0.0274)(**Figure 4C**).

Similarly, TGFβ1-dependent stimulation increased αSMA mRNA expression in HLMFs, which was significantly greater in IPF-derived HLMFs compared to NFC-derived cells (P=0.0261). This TGFβ1-dependent αSMA mRNA expression was significantly reduced in IPF-derived HLMFs following treatment with LXA₄ at 10⁻¹⁰ and 10⁻⁸ M (P=0.0479 and P=0.0033 respectively, 2 way ANOVA)(**Figure 4D**), but not NFC-derived cells.

To study the effects of LXA₄ on TGFβ1-dependent HLMF contraction, HLMFs were cultured within collagen gels and their contraction monitored over 24 hours. Pictures were taken at 0 and 24 hours and the contraction measured using Image J software; representative images are displayed in **Figure 4E**. TGFβ1 stimulation increased HLMF contraction in comparison to vehicle control (0.1% ethanol)(P=0.0026), with no differences in response between NFC- and IPF-derived cells (**Figure 4F**). LXA₄ significantly reduced TGFβ1-dependent HLMF contraction at both 10⁻¹⁰ M (P=0.0057) and 10⁻⁸ M (P=0.0006). Thus LXA₄ not only inhibits constitutive HLMF αSMA expression and contraction, but also the increased αSMA expression and contraction induced by the potent pro-fibrotic mediator TGFβ1(38,39).

TGFβ1-induced collagen mRNA expression and collagen secretion is inhibited by LXA₄

To further elucidate the inhibitory effects of LXA₄ we investigated mRNA expression of collagen type I and collagen type IV, which are two of the most abundant collagens found within the IPF lungs (39, 40). Collagen type I mRNA expression was significantly increased by TGFβ1 in both NFC- and IPF-derived HLMFs compared to control, $P=0.0064$ (One sample t test, pooled data)(**Figure 5A**). This increase was significantly reduced by LXA₄ at 10^{-8} M in both NFC- and IPF-derived cells ($P=0.0057$, 1 Way ANOVA corrected by Dunns multiple comparison test)(**Figure 5A**). Similarly, collagen type IV mRNA expression was significantly increased in both NFC- and IPF-derived HLMFs ($P=0.0031$ and $P<0.0001$, respectively, 2 way ANOVA corrected by Dunnetts multiple comparison test). IPF-derived HLMFs expressed significantly more collagen type IV mRNA following TGFβ1 stimulation in comparison to NFC-derived cells ($P=0.0480$, unpaired t test)(**Figure 5B**). LXA₄ dose-dependently inhibited TGFβ1-induced collagen type IV mRNA expression in IPF-derived cells at 10^{-8} M ($P=0.0039$, 2 Way ANOVA corrected by Sidaks multiple comparison test).

The ability of LXA₄ to downregulate TGFβ1-increased collagen mRNA was paralleled by a reduction in total collagen secretion measured using the Sircol Assay. TGFβ1 significantly increased the amount of total collagen secreted by NFC- and IPF-derived cells ($P=0.0022$), which was dose-dependently inhibited by LXA₄ (10^{-10} M, $P=0.0015$; 10^{-8} M, $P=0.0002$) (**Figure 5C**).

FBS-induced HLMF proliferation is attenuated by LXA₄

HLMF proliferation was assessed after 48 hours of stimulation with 10% FBS which significantly increased proliferation ($P=0.0006$)(**Figure 6**). In both NFC- and IPF-derived HLMFs, FBS-dependent proliferation was significantly decreased by LXA₄ at 10^{-10} M ($P=0.0001$) and 10^{-8} M ($P=0.001$), (**Figure 6**). In summary, FBS-induced HLMF proliferation is attenuated by treatment with LXA₄.

TGFβ1-dependent Smad2/3 nuclear translocation is disrupted by LXA₄

The ability of LXA₄ to attenuate both constitutive Smad2/3 nuclear localisation and several TGFβ1-dependent cell processes in HLMFs suggested that LXA₄ may also attenuate TGFβ1-dependent Smad2/3 nuclear translocation. Phosphorylation is a key initial event in the activation of these Smad proteins. Using Western blot analysis we therefore investigated the effect of LXA₄ on TGFβ1-induced phosphorylation of Smad2/3 and the expression of total Smad2/3 in HLMFs. As previously reported in many studies, TGFβ1 significantly increased Smad2/3 phosphorylation in HLMFs ($P=0.0110$). However, LXA₄ at 10^{-10} M and 10^{-8} M had no significant effect on the TGFβ1-dependent increase in Smad2/3 phosphorylation in either NFC- or IPF-derived HLMFs (**Figure 7A and B**).

Once TGFβ1 has induced phosphorylation of Smad2/3, they interact with Smad4, and this complex translocates to the nucleus to initiate gene transcription. Although Smad2/3 phosphorylation was not inhibited, TGFβ1-dependent Smad2/3 nuclear translocation was significantly attenuated by LXA₄ (**Figure 7C, D and E**). Thus LXA₄ appears to significantly disrupt TGFβ1-dependent nuclear translocation of Smad2/3, but not Smad2/3 phosphorylation.

DISCUSSION

The myofibroblast is implicated as the key cell driving the progression of IPF through the synthesis of excess fibrotic extracellular matrix and tissue contraction. The myofibroblast is therefore an attractive target for novel anti-fibrotic therapies. Here we have demonstrated that HLMFs express LXA₄ receptors, and that LXA₄ attenuates i) constitutive HLMF α SMA expression, stress fibre formation, contraction and Smad2/3 nuclear localisation, ii) TGF β 1-dependent increases in HLMF α SMA expression, contraction, collagen mRNA expression, collagen secretion, and Smad2/3 nuclear localisation, and iii) serum-dependent HLMF proliferation.

Fibroblasts and myofibroblasts demonstrate marked functional and phenotypic heterogeneity between tissues and across species. As an example, there is a marked difference in the phenotype of fibroblasts grown from human airway and lung parenchyma, with those from lung spontaneously displaying a myofibroblast phenotype(29, 35, 36). When considering novel therapeutic targets, it is therefore important to study cells from the species, tissue compartment and disease of interest. Expression of the LXA₄ receptor ALXR (FRLP1) has been demonstrated previously in human synovial fibroblasts by RT-PCR(41), and these cells and rat fibroblast cell lines respond to LXA₄ implying functional receptor expression (22-41). Here we have demonstrated that ALXR protein is expressed on the surface of the majority of parenchymal HLMFs derived from both NFC and IPF tissue.

In a rat fibroblast cell line LXA₄ at 10⁻⁹ M attenuated TGF β 1-dependent Smad2 phosphorylation (but not Smad3 phosphorylation) and MAP kinase activation, and inhibited TGF β 1-dependent gene transcription (22). We also found that LXA₄ inhibited TGF β 1-dependent gene transcription, but in contrast to Börgeson and colleagues, we could not detect inhibition of Smad2/3 phosphorylation, although there was reduced TGF β 1-dependent nuclear Smad2/3 translocation. The means by which LXA₄ would prevent nuclear translocation of activated Smads without affecting phosphorylation is intriguing and will require further work to elucidate the mechanism. Nevertheless, the ability of LXA₄ to inhibit Smad nuclear localisation is in keeping with its ability to inhibit TGF β 1-dependent gene transcription, and the downstream production of collagen and α SMA in HLMFs.

Several phenotypic differences have been observed previously between control and IPF-derived HLMFs in culture, suggesting that there may be genetic and/or epigenetic changes that promote a pro-fibrotic HLMF phenotype in patients who develop IPF. Interestingly, we found that LXA₄ not only reduced TGF β 1-dependent pro-fibrotic responses, but promoted HLMF differentiation towards a fibroblast phenotype by reducing constitutive α SMA actin expression and actin stress fibre formation. This in turn is likely to explain the reduced constitutive HLMF contraction evident in collagen gels. This occurred in conjunction with a reduction in nuclear Smad2/3 immunostaining which occurred within 1 hour of LXA₄ exposure, suggesting there is a component of constitutive Smad2/3 signalling in HLMFs at rest. This ability of LXA₄ to promote de-differentiation towards a fibroblast phenotype suggests that if there is a degree of “constitutive” pro-fibrotic HLMF activity in IPF lungs due to genetic/epigenetic factors, LXA₄ or a stable analogue might be a particularly useful therapy.

In addition to the inhibition of TGF β 1-dependent stimulation, we found that LXA₄ also inhibited HLMF proliferation induced by serum, and this is in keeping with previous work showing that LXA₄ inhibited CTGF-dependent proliferation of a human fibroblast cell line(25). Previous studies using LXA₄ in cell culture systems suggest it is highly active in the nM range. Our data are consistent with this, with effects readily evident in HLMFs at 10⁻¹⁰ M (0.1 nM), although more consistent at 10⁻⁸ M.

In summary we have shown that LXA₄ inhibits many TGF β 1-dependent pro-fibrotic responses in healthy and IPF-derived HLMFs, which may result from the inhibition of Smad2/3 nuclear translocation. Furthermore, LXA₄ promotes HLMF de-differentiation in the resting state, suggesting it may have the potential to reverse the fibrotic process. In support of this, and of particular relevance to this study, a stable epi-LXA₄ analogue markedly inhibited bleomycin-induced pulmonary fibrosis in mice when administered either preventively or curatively, with reversal of fibrosis evident in the latter (26). This was associated with a reduction in the accumulation and differentiation of myofibroblasts in the lung parenchyma. Thus there are consistent *in vitro* and *in vivo* data from primary HLMFs and the mouse bleomycin model respectively, indicating that LXA₄ or its stable analogues may not only prevent the progression of lung fibrosis, but potentially reverse it. This indicates that clinical trials of LXA₄ analogues should be considered in patients with IPF.

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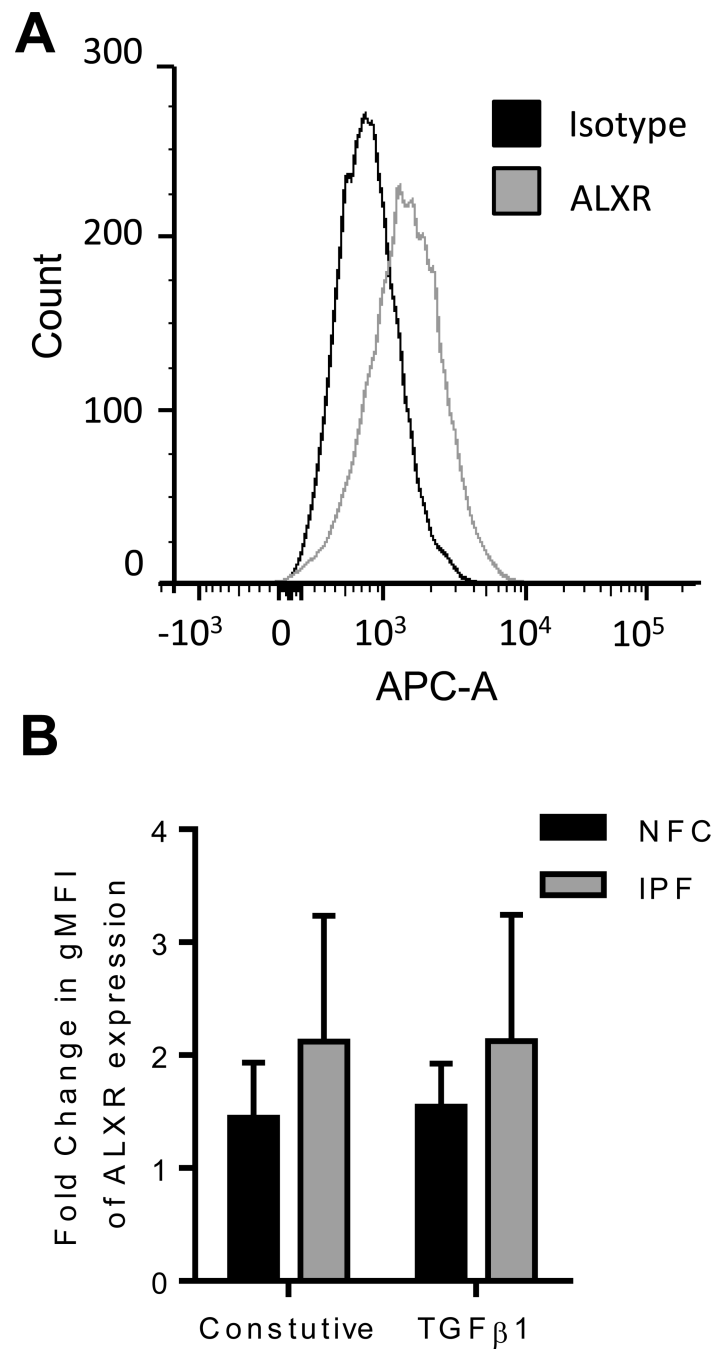


Figure 1. HLMFs express ALXR

(A) Representative histogram for ALXR staining assessed by flow cytometry in HLMFs obtained from an IPF donor. (B) Both NFC (n=3) and IPF-derived (n=3) HLMFs expressed ALXR constitutively, P=0.0056 (pooled data). ALXR expression was not increased following 24 hours of treatment with TGF β 1.

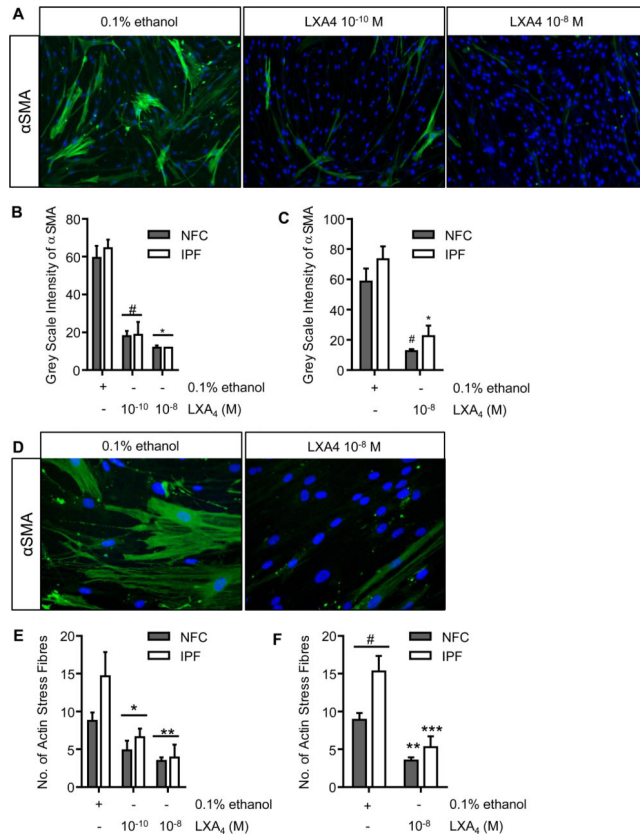


Figure 2. Constitutive α SMA and stress fibre formation is attenuated by LX A₄
(A) Representative immunofluorescent images from a NFC donor showing the decrease in α SMA expression in HLMFs following incubation with LX A₄ (10^{-10} M and 10^{-8} M). **(B)** α SMA expression was assessed by measuring grey scale intensity. LX A₄ decreased α SMA expression at 10^{-10} M and 10^{-8} M in NFC-derived and IPF-derived HLMFs (n=6 and n=3 respectively, data pooled) (P<0.0001, repeated measures ANOVA, *p=0.0001 and **P<0.0001, corrected by Sidaks multiple comparison test). For each donor a minimum of 10 cells per field were measured and the mean taken. **(C)** In both NFC- (n=6) and IPF (n=6)-derived HLMFs α SMA was attenuated by LX A₄ 10^{-8} M (2 way ANOVA corrected by Sidaks multiple comparison test, NFC #P=0.0004 and IPF *P=0.0002). **(D)** Cells were noticeably more spindle like following treatment with LX A₄; a representative image from an IPF donor is shown. **(E)** An Image J macro was used to quantify the number of actin filaments and a minimum of 10 cells from each donor was assessed and averaged. LX A₄ decreased the number of actin filaments at both 10^{-10} M and 10^{-8} M in both NFC- and IPF-derived cells (n=6 and n=4 respectively, data pooled) (P=0.0003, repeated measures ANOVA, *P=0.0135 and **P=0.0005, corrected by Sidaks multiple comparison test). **(F)** IPF-derived HLMFs expressed increased numbers of smooth muscle actin filaments (stress fibres) in comparison to NFC-derived cells, #P=0.0177. Actin filaments in both NFC- (n=6) and IPF (n=6)-derived HLMFs were attenuated by LX A₄ 10^{-8} M (2 way ANOVA corrected by Sidaks multiple comparison test, NFC **P=0.0005 and IPF ***P<0.0001).

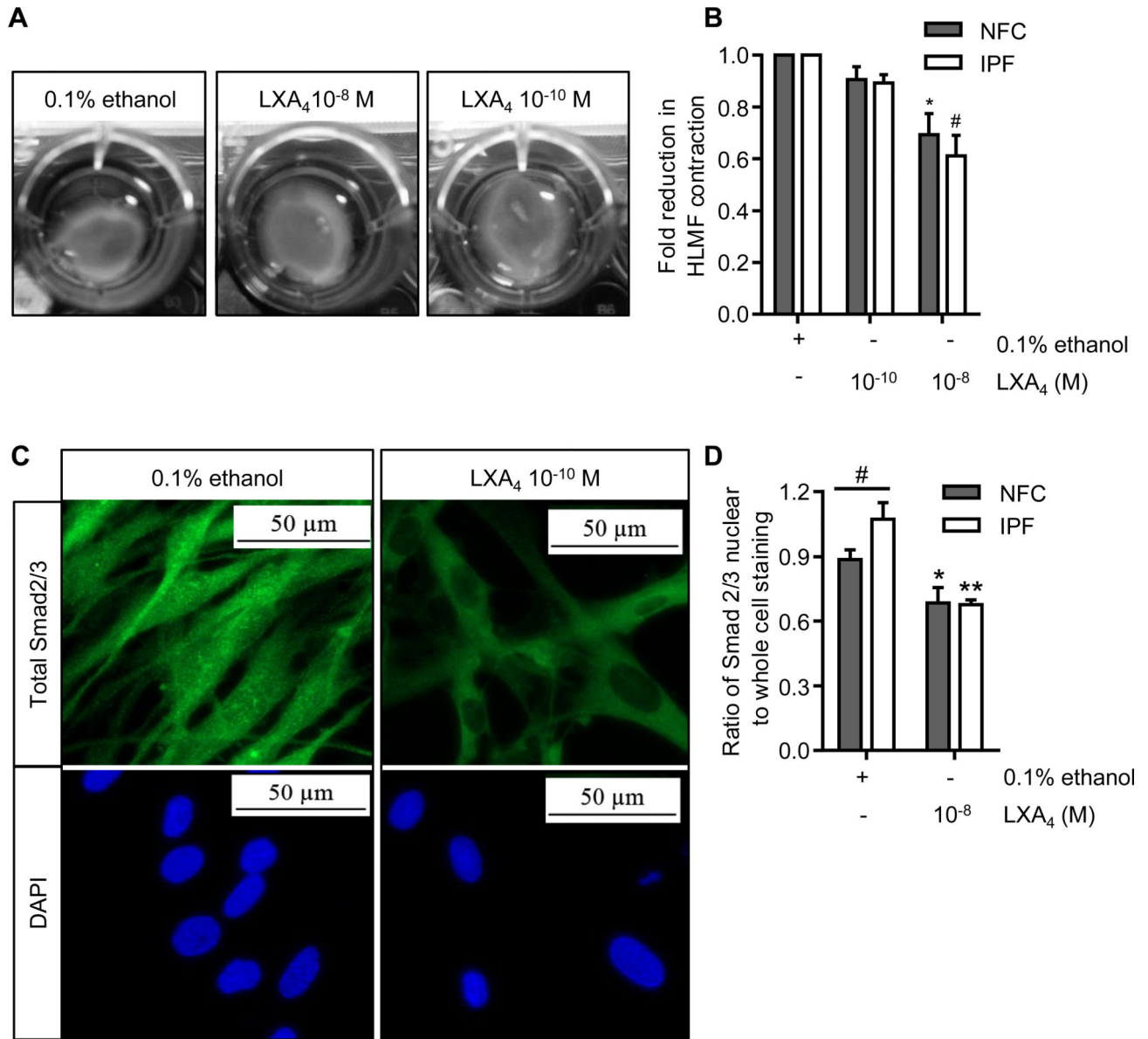


Figure 3. Basal HLMF contraction and Smad2/3 nuclear translocation are inhibited by LXA₄

(A) Representative images from a NFC donor showing the spontaneous contraction of HLMFs within a collagen gel and its suppression by LXA₄. (B) Constitutive HLMF contraction was significantly attenuated by LXA₄ (10⁻⁸ M) in both NFC (n=6) and IPF-derived (n=5) cells (2 way ANOVA corrected by Sidaks multiple comparison test, NFC *P=0.0009 and IPF #P=0.0001). (C) Representative images from an IPF donor displaying total Smad2/3 expression within HLMFs. At baseline the HLMFs have staining both in the nucleus and within the cytoplasm, which is reduced upon incubation with LXA₄ (10⁻⁸ M) for 1 hour. (D) The ratio of total Smad2/3 nuclear to whole cell staining was assessed by measuring grey scale intensity. IPF-derived (n=6) HLMFs demonstrated increased expression of total Smad2/3 within the nucleus in comparison to NFC donors (n=6), (#P=0.043, Mann Whitney U test). LXA₄ (10⁻⁸ M) significantly attenuated the amount of

Smad2/3 localised to the nucleus in both NFC- and IPF-derived cells (2 way ANOVA corrected by Sidaks multiple comparison test, NFC *P=0.0254 and IPF **P=0.0006).

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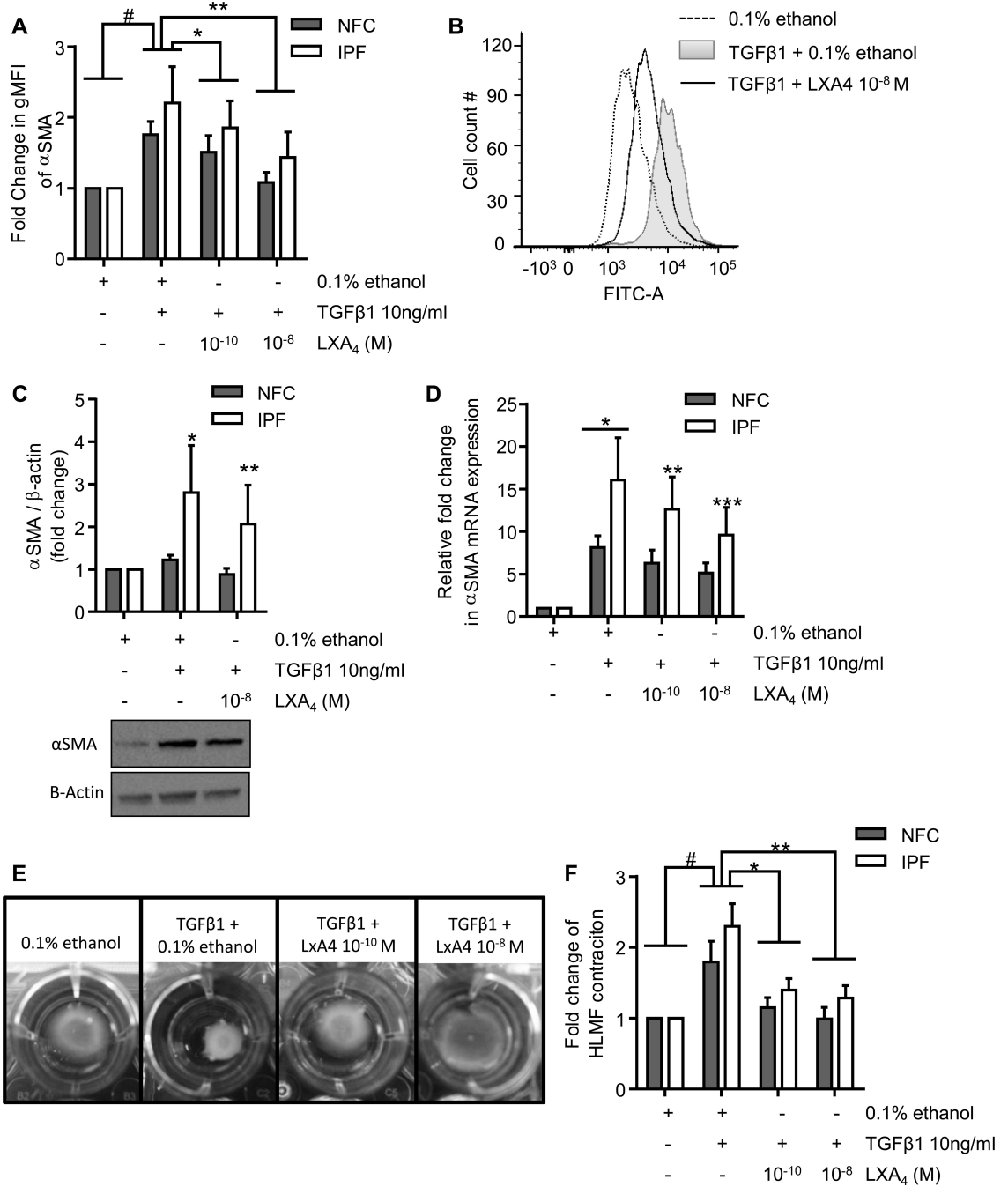


Figure 4. TGFβ1-dependent increases in αSMA mRNA and protein expression and HLMF contraction are attenuated by LXA₄

(A) Flow cytometry was used to assess the changes in αSMA expression following TGFβ1 (10 ng/ml) stimulation in the presence of LXA₄, in both NFC- (n=4) and IPF-derived (n=3) HLMFs. αSMA expression in HLMFs was significantly increased following TGFβ1 stimulation, #P=0.0070 (paired t test). LXA₄ significantly attenuated TGFβ1-dependent increases in αSMA expression at 10⁻¹⁰ M and 10⁻⁸ M (*P=0.011 and **P=0.008, respectively, Repeated measures ANOVA corrected by Sidaks multiple comparison test, statistics were performed on pooled data). (B) Representative fluorescent histogram showing

α SMA expression in HLMFs under the above conditions. (C) Densitometry results of western blot analysis indicating the increased α SMA expression following TGF β 1 (10ng/ml) stimulation for 24 hours in IPF donors *P=0.0344 (paired t test). This increase was significantly attenuated by LXA₄ 10⁻⁸ M, **P=0.0274 (paired t test). No significant difference was found between NFC and IPF-derived HLMFS in terms of TGF β 1-stimulated α SMA expression (P=0.25). (D) qPCR results displaying the relative expression of α SMA in HLMFs following 24 hours of stimulation with TGF β 1 (10 ng/ml) in the presence of 0.1% ethanol or LXA₄ at 10⁻¹⁰ M and 10⁻⁸ M. TGF β 1 significantly upregulated α SMA mRNA expression in both NFC- (n=6) and IPF-derived (n=5) HLMFs, #P=0.018 (one sample t test). IPF-derived HLMFs demonstrated a significantly greater increase in mRNA expression in comparison to NFC-derived cells, *P=0.0261. TGF β 1-dependent upregulation of α SMA mRNA expression was significantly inhibited in IPF-derived cells in the presence of LXA₄ 10⁻¹⁰ M and 10⁻⁸ M (**P=0.0479 and ***P=0.0033 respectively, 2 way ANOVA, corrected by Sidak's multiple comparison test). (E) Representative images from an IPF donor showing the TGF β 1-stimulated contraction of HLMFs within a collagen gel and its suppression by LXA₄. (F) HLMFs from both NFC (n=6) and IPF (n=5) -derived donors displayed increased contraction following TGF β 1(10ng/ml) stimulation (#P=0.0026, One sample t test, pooled data). LXA₄ significantly attenuated HLMF contraction in both NFC (n=6) and IPF (n=5) -derived myofibroblasts at 10⁻¹⁰ M and 10⁻⁸ M (P=0.0005, repeated measures ANOVA, *P=0.0057 and **P=0.0006, corrected by Dunn's multiple comparison test).

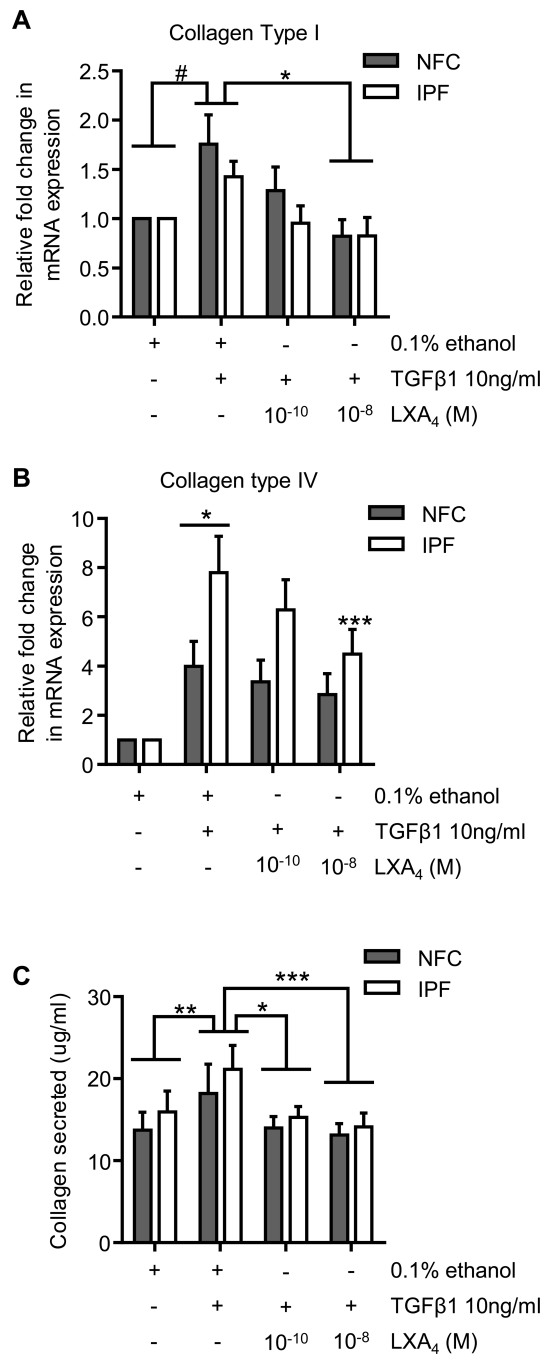


Figure 5. LXA4 significantly decreases TGFβ1-dependent increases in collagen mRNA expression and collagen secretion

(A) Collagen type I mRNA expression was significantly upregulated following 24 hours of TGFβ1 stimulation in NFC- and IPF-derived HLMFs (n=6 and n=5 respectively) (#P=0.0064, one sample t test on pooled data). LXA₄ significantly reduced TGFβ1-dependent up regulation of collagen type I mRNA at 10⁻⁸ M (P=0.0214, 1 way ANOVA, *P=0.0057, corrected by Sidaks multiple comparison test). (B) Collagen type IV mRNA expression was significantly upregulated following 24 hours of TGFβ1 stimulation in NFC and IPF-derived HLMFs (n=7 and n=5 respectively)(P=0.0031 and P<0.0001, respectively,

2 Way ANOVA corrected by Sidaks multiple comparison test). The TGFβ1-dependent increase in collagen type IV mRNA expression was significantly greater in IPF-derived HLMFs in comparison to NFC-derived cells (*P=0.0480, unpaired t-test). The TGFβ1-dependent increase in collagen type IV mRNA expression in IPF HLMFs was significantly reduced by LXA₄ at 10⁻⁸ M **P=0.0039, 2 Way ANOVA, corrected by Sidaks multiple test). (C) The amount of collagen secreted by both NFC- and IPF-derived HLMFs increased significantly following TGFβ1 (10ng/ml) for 24 hours, with no difference between NFC and IPF (**P=0.0022 on pooled data, NFC n=4 and IPF n=4). LXA₄ significantly inhibited collagen secretion at both 10⁻¹⁰ and 10⁻⁸ M (*P=0.0015 and ***P=0.0002, 2-way ANOVA, pooled data).

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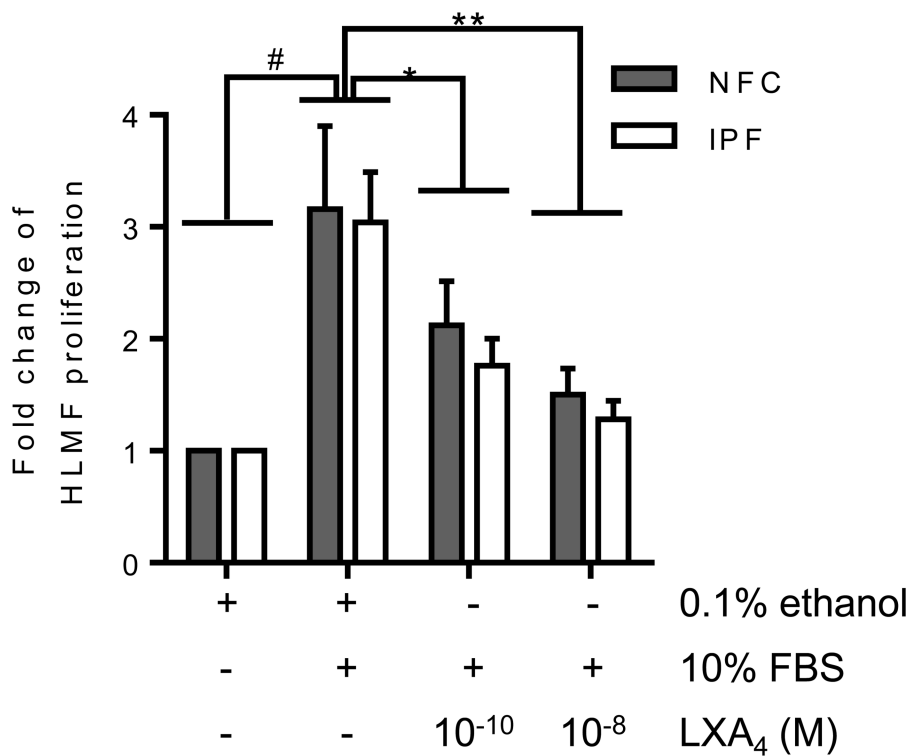


Figure 6. FBS-dependent HLMF proliferation is inhibited by LXA₄

HLMF proliferation was increased following 48 hours of stimulation with FBS and to a similar extent in both NFC- and IPF-derived HLMFs (#P=0.0006, one-sample t test, pooled data, NFC n=5 and IPF n=5). This increase in HLMF proliferation was significantly reduced with LXA₄ at both 10⁻¹⁰ M and 10⁻⁸ M (P=0.0001, repeated measures ANOVA, *P=0.0001 and **P=0.001 respectively, corrected by Dunns multiple comparison test).

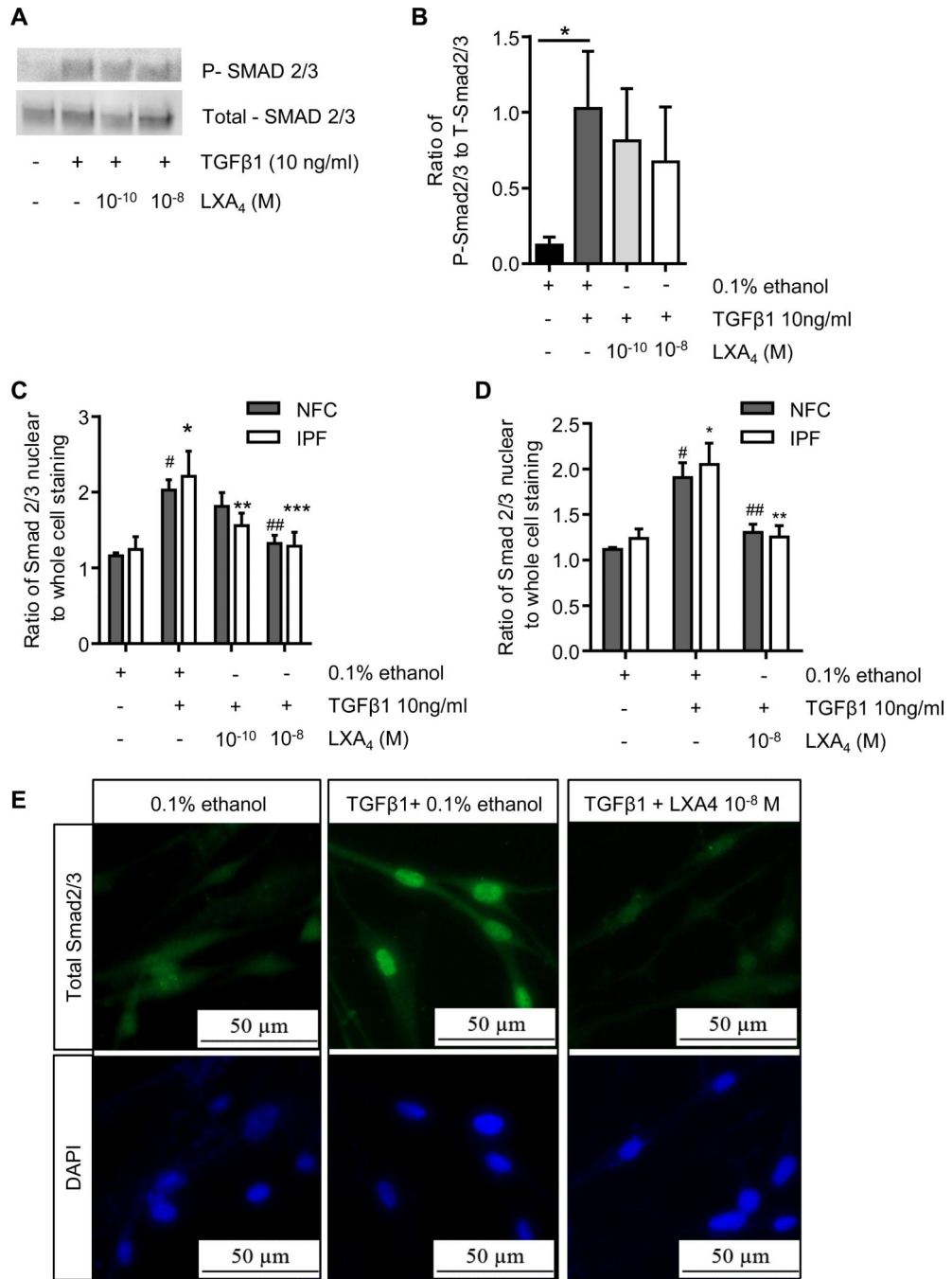


Figure 7. LXA₄ does alter Smad2/3 phosphorylation but exerts its effect through inhibition of Smad2/3 translocation to the nucleus

(A and B) Using Western blot analysis we investigated the effect of LXA₄ on TGFβ1-induced phosphorylation of Smad2/3; a representative image from a NFC donor is shown. TGFβ1 significantly increased Smad2/3 phosphorylation in HLMFs (P=0.0110, NFC n=2 and IPF n=3). However, LXA₄ at 10⁻¹⁰ M and 10⁻⁸ M had no significant effect on the TGFβ1-dependent increase in Smad2/3 phosphorylation in either NFC- or IPF-derived HLMFs. (C) Immunofluorescent analysis of total Smad2/3 nuclear translocation showed a

significant increase in both NFC(n=5) and IPF(n=4) HLMFs (#P=0.0007 and *P=0.0007 respectively). This TGFβ1-dependent increase in total nuclear translocation was significantly and dose-dependently attenuated by LXA₄ (2 way ANOVA corrected by Sidaks multiple comparison test, NFC 10⁻⁸ M ##P=0.0051 and IPF 10⁻¹⁰ M **P=0.0208 and 10⁻⁸ M ***P=0.0011). (D) In both NFC- (n=6) and IPF- (n=6) derived HLMFs TGFβ1-dependent total Smad2/3 nuclear translocation was attenuated by LXA₄ 10⁻⁸ M (2 way ANOVA corrected by Sidaks multiple comparison test, NFC ##P=0.00037 and IPF **P=0.0003). (E) Representative immunofluorescent images from an NFC donor demonstrating the increased nuclear translocation of total Smad2/3 following TGFβ1 stimulation and the inhibition of this by LXA₄ (10⁻⁸ M).