RESEARCH ARTICLE

Interleukin-15 attenuates transforming growth factor-β1-induced myofibroblast differentiation in human fetal lung fibroblasts

Dirk M. Wuttge[1], Marie Wildt[1], Agneta Scheja[1], Gunilla Westergren-Thorsson[2]

[1] Department of Clinical Sciences, Lund, Section of Rheumatology, Lund University, Lund, Sweden
[2] Department of Experimental Medical Science, Division of Vascular and Respiratory

Correspondence: D.M. Wuttge, Department of Clinical Sciences, Lund, Section of Rheumatology, Skåne University Hospital, Lund, SE-221 85 Lund, Sweden
<dirk.wuttge@med.lu.se>

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ABSTRACT. ***Objective.*** **Fibroproliferative diseases are common causes of morbidity and mortality. Interleukin-15 (IL-15) is a pleiotropic cytokine with multiple effects on cells of the immune system. Although IL-15 is also expressed in mesenchymal cells, its effects on the development of fibrosis are unknown. We have previously described an association between serum IL-15 levels and the extent of pulmonary fibrosis in the connective tissue disease systemic sclerosis, suggesting that IL-15 may have profibrotic effects. To test this hypothesis, we studied the effects of IL-15 on myofibroblast differentiation, an *in vitro* model of fibrosis development. *Methods.* We used human fetal lung fibroblasts for the cytokine stimulation. As a marker of myofibroblast differentiation, α-smooth muscle actin (α-SMA) was analyzed by western blot and quantitative real-time PCR. The well-known profibrotic cytokine, transforming growth factor-β1(TGF-β1), was used for comparison, and TGF-β signaling paths were also studied. *Results.* IL-15 did not induce α-SMA expression, a marker for myofibroblast differentiation. Unexpectedly, IL-15 counteracted TGF-β1-mediated α-SMA expression. Moreover, TGF-β1-induced expression of collagen, fibronectin and connective tissue growth factor was attenuated by addition of IL-15. There was no effect of IL-15 on early events in the TGF-β signaling cascades. *Conclusion.* IL-15 has anti-fibrotic properties that, speculatively however, may be insufficient in systemic sclerosis.**

Keywords: fibrosis, IL-15, TGF-β1, α-smooth muscle actin, CTGF, SMAD7

Chronic fibroproliferative diseases, including cardiovascular disease, pulmonary fibrosis, and systemic sclerosis, are important causes of morbidity and mortality [1]. Central to the development of fibrosis is chronic inflammation, which leads to continuous remodeling of the extracellular matrix and formation of permanent scar tissue that may result in organ failure and death [1]. A key feature in this process is the acquisition of smooth muscle cells characterised by fibroblasts that differentiate into myofibroblasts. These myofibroblasts have been recognized as the driving force behind granulation tissue contraction [2], and account also for the excessive accumulation of extracellular matrix, *i.e.* collagens and fibronectin [3].

Interleukin-15 (IL-15) is a cytokine of 14–15 kD that belongs to the 4-α-helix bundle cytokine family [4], which also includes cytokines such as IL-2, IL-4, and IL-21. IL-15 binds with high affinity to the IL-15 receptor α chain, and recruits the transducing complex involving the IL-2 receptor β and common γ (γc) chains [5, 6]. The IL-2 receptor β/γc complex binds IL-15 with intermediate affinity, whereas the IL-2 receptor β and γc chains alone, only bind IL-15 with low (β chain) or very low (γc) affinities. IL-15 is a survival and growth factor for several hematopoetic cells [7-9], and it has also emerged as an important molecule in autoimmunity and transplantation [10, 11]. However, IL-15 is also expressed in mesenchymal cells such as smooth muscle cells, fibroblasts, and epithelial cells, as well as in keratinocytes [12-14].

Although IL-15 is also expressed in mesenchymal cells, it has not yet been studied in the context of fibrosis, and its effect on the development of fibrosis is unknown. However, we have recently shown an inverse association between serum IL-15 levels and pulmonary function test in patients with systemic sclerosis, suggesting that there may be a profibrotic effect of IL-15 in systemic sclerosis [15]. In addition, IL-15 mRNA is constitutively expressed in fibroblasts [14], and we have previously shown the presence of IL-15 in vascular smooth muscle cells in mouse atherosclerotic lesions [12]. These data suggest that IL-15 may have an influence on myofibroblast differentiation, and thus on the development of fibrosis. Importantly, anti-IL-15 antibodies have already been tested in phase-II clinical trials for the treatment of

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rheumatoid arthritis [16]. If IL-15 does indeed have an influence on the development of fibrosis, a promising treatment for fibroproliferative diseases could therefore be in the offing.

In contrast to IL-15, transforming growth factor-beta (TGF-β) has been well studied in the context of fibrosis. TGF-β has gained attention as a profibrotic cytokine that both induces the expression of α-smooth muscle actin (α-SMA) [17], and stimulates the production of extracellular matrix by fibroblasts [18]. TGF-β activity is mediated by several intracellular signaling pathways [19]. Phosphorylation of SMAD2/3 and activation of the SMAD2/3/4 signaling pathway is a major target of the heterodimeric TGF-β receptor complex upon binding of TGF-β. SMAD2/3 signaling has been linked to α-SMA expression in fibroblasts [20, 21]. SMAD2/3 signaling is also involved in the activation of several genes of myofibroblast-associated connective tissue metabolism, such as those for collagen type I, connective tissue growth factor (CTGF), and platelet activation inhibitor (PAI)-1 [22, 23]. Conversely, alternative signaling mechanisms may regulate TGF-β-induced gene expression in fibroblasts independently of SMAD signaling [19]. Inhibition of the MAP kinase c-Jun-NH2-terminal kinase (JNK) has recently shown that JNK may be involved in TGF-β-induced α-SMA expression in human fetal lung fibroblasts [24]. JNK signaling may also be involved in TGF-β-induced expression of CTGF in the human fetal lung fibroblast line HFL-1 [25] and in the induction of fibronectin expression [26].

In this study, we tried to determine whether IL-15 has profibrotic effects in human fetal lung fibroblasts. Surprisingly, IL-15 was not found to affect myofibroblast differentiation. IL-15 reduced TGF-β1-mediated expression of α-SMA and collagen type I, independently of SMAD or MAP-kinase signaling in HFL-1 cells. IL-15 had a dual effect on TGF-β1-mediated CTGF mRNA expression, and caused a reduction in SMAD7 mRNA expression after 24 hours.

METHODS AND MATERIALS

Materials

Recombinant IL-15, IL-2, and TGF-β1 were obtained from R&D Systems (Minneapolis, MN, USA). Monoclonal mouse anti-human α-SMA (clone 1A4; Dako A/S, Glostrup, Denmark), polyclonal rabbit anti-human α-SMA (Abcam plc, Cambridge, UK), anti-fibronectin (Abcam plc), and anti-β-actin (clone AC-15; Sigma-Aldrich, St Louis, MO, USA), were obtained from the companies stated. Anti-phospho-JNK, anti-phospho-ERK, anti-ERK, anti-phospho-P38, and anti-phopho-SMAD2/3 antibodies were all obtained from Cell Signaling Technology (Danvas, MA, USA). Horseradish peroxidase- and alkaline phosphatase-conjugated secondary polyclonal anti-mouse and anti-rabbit antibodies were from obtained from Dako.

Cell culture

Normal, diploid, human fetal lung fibroblasts (HFL-1) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells displayed a stable phenotype when used for experiments between passages 12 and 24, and they had features that resembled those of lung biopsy-derived fibroblasts [27, 28]. Cells were maintained at 37°C in 25 cm^2 dishes in complete Modified Eagle's Medium (Life Technologies, Rockville, MD, USA) containing 2 mM L-glutamine, penicillin and streptomycin (Sigma-Aldrich), and 10% donor bovine serum (Gibco/BRL, Grand Island, NY, USA), in an atmosphere of 5% CO_2. For the experiments, the cells were serum-starved in complete Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) containing 0.4% fetal calf serum (Fetal Clone III; HyClone Laboratories, Logan, UT, USA) for 24 h, and incubated thereafter in complete Dulbecco's Modified Eagle's Medium containing 1% fetal calf serum with cytokines, for the times indicated.

Flow cytometry staining for receptor protein expression

Cells were detached with Accutase (PAA Laboratories GmbH, Linz, Austria) and blocked with 100 μg/mL porcine IgG (Sigma-Aldrich, St. Louis, MO,USA) for 30 minutes, before incubation with 2.5 μg/mL goat serum against human IL-15 receptor α , IL-2 receptor β and γc chains or control serum (all from R&D Systems) for 30 minutes. Cells were visualized with secondary phycoerythrine-lablled porcine anti-goat antibody (BD Bioscience, San Jose, CA, USA). Two thousand events in the gated cell population were analyzed in fluorescence channel two of a Cytomics FC500 (Beckman Coulter, Brea, CA, USA), using the CXP Software for acquisition and analysis.

RNA extraction and cDNA synthesis

Total RNA was isolated from cells using RNeasy (Qiagen GmBH, Hilden, Germany) according to the manufacturer's instructions. The quantity of RNA was measured by spectrophotometry using a NanoDrop ND-100 (Nano Drop Technologies, Delaware, MD, USA). Total RNA (1 μg) was reverse-transcribed using superscript II according to the manufacturer's manual (Invitrogen, Carlsbad, CA, USA), and stored at - 70°C.

Real-time RT-PCR

Five μl of cDNA (diluted 1:250) was mixed with 15 μL 1x SYBR-green mixture (PE Biosystems, Foster City, CA, USA) and amplified by real-time RT-PCR using an ABI 7900 (AME Bioscience A/S, Toroed, Norway). Initially the samples were held for 2 min at 50°C, then for 10 min at 90°C; they were then cycled for forty cycles of 15 sec at 90°C, 1 min at 50°C, and 1 min at 72°C. Each sample was analyzed in triplicate. Reactions were performed in MicroAmp optical, 96-well reaction plates (PE Biosystems). All primers were constructed using the online Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and they were ordered from A/S DNA Technology (Risskov, Denmark). The following primers were used. α-SMA forward: GAA GGA ATA GCC ACG CTC AG, and reverse: TCA ATG TCC CAG CCA TGT A. Collagen type I alpha I forward: ACG TCC TGG TGA AGT TGG TC, and reverse:

CAG GGA AGC CTC TTT CTC CT. PAI-1 forward: CAA CTT GCT TGG GAA AGG AG, and reverse: GGG CGT GGT GAA CTC AGT AT. Fibronectin forward: CGA TCA CTG GCT TCC AAG TT, and reverse: TCC GAG CAT TGT CAT TCA AG. SMAD7 forward: GAG GGC TCC TGG ACA CAG TA, and reverse: CTT AGC CGA CTC TGC GAA CT. CTGF forward: TTA GAG CCA ACT GCC TGG TC, and reverse: TGG AGA TTT TGG GAG TAC GG. S18 forward: CGA ACG TCT GCC CTA TCA AC, and reverse: TGC CTT CCT TGG ATG TGG TA. All primers were tested for specificity by sequence alignment in the PubMed nucleic acid database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Immunoblotting

Cells were grown to subconfluence, serum-starved overnight, and stimulated with the cytokines at indicated concentrations, for varying times. They were washed three times in ice-cold PBS before lysis buffer was added. Lysis buffer for analysis of phosphorylated proteins contained 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 3 mM EDTA, 2 mM sodium pyrophosphate, 25 mM β-glycerolphosphate, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton, 1 μg/mL aproptinin, 1 μg/mL leupetin, 1 μg/L PMSF, and 200 mM sodium orthovanadate. Cells were incubated in the appropriate lysis buffer on ice for 10 min, scraped off with inverted sterile pipette tips and centrifuged at 14,500 g at 4°C for 15 min. Supernatants were stored at - 70°C until use. Protein concentration was measured using the BCA protein assay according to the manufacturer's instructions (Pierce Chemical, Rockford, IL, USA). Aliquots of 40 μg protein/lane were loaded for analysis of the phosphorylated proteins. The phosphorylated proteins were analyzed on a NuPage 12% Bis-Tris discontinuous SDS-PAGE gel (Invitrogen). Gels were washed with washing buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20), blocked with washing buffer containing 5% skimmed milk powder for 1 h, and incubated with primary antibody overnight at 4°C at concentrations as follows: anti-α-SMA (1:100), anti-fibronectin (1:1,500), anti-phospho-JNK (1:400), anti-phospho-ERK (1:400), anti-phospho-P38 (1:400), anti-phopho-SMAD2/3 (1:400). Thereafter, the gels were incubated in relevant HRP-conjugated secondary antibodies (1:1,000), in blocking buffer at room temperature for 1 hour, developed with the ECL development system (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and photographed using Konica Minolta AX film (Oriola Oy, Espoo, Finland). The bands were scanned with a GS-800 densitometer (Bio-Rad Laboratories, Hercules, CA, USA). The gels were then stripped by incubation with 7 M guanidine-HCl for 10 min and restained with an anti-ERK antibody (1:1,000) overnight at 4°C, and the bands visualized as described above.

Measurement of α-SMA protein by ELISA

Cells were grown to subconfluence, serum-starved overnight, and stimulated with the cytokines at indicated concentrations. They were washed three times in ice-cold PBS before lysis buffer was added. Lysis buffer for analysis of α-SMA, contained 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 2 mM $MgCl_2$, 10% glycerol, and 1% NP-40 with the protease inhibitors aproptinin, leupetin, pepstatin, and PMSF (all at 1 μg/mL). For measurement of α-SMA protein [29], 96-well Nunc-Immuno plates with Maxisorb surface were coated with a polyclonal rabbit anti-α-SMA antibody (Abcam plc, Cambridge, UK), and blocked with 3% BSA. Cell lysates containing 10 μg of total protein were added to each well, and bound α-SMA was detected using the monoclonal, mouse anti-human α-SMA antibody (clone 1A4; Dako A/S), and an alkaline phosphatase-conjugated secondary antibody (Dako A/S). p-nitrophenyl phosphate (pNPP; 2 mg/mL) dissolved in 1 M diethanolamine in 0.5 mM $MgCl_2$, pH 9.8, was added for development: absorbance was measured at 405 nm.

Cell proliferation

Cells (5,000 cells/well) were seeded into 96-well plates in complete medium for 4 h, washed, and starved overnight before stimulation with the cytokines at the concentrations indicated, for 24, 48, or 72 h. After fixation in 1% glutaraldehyde and staining with crystal violet dye, cell proliferation was determined spectrophotometrically at 595 nm in a linear fashion [30]. The crystal violet method has previously been validated and compared with other proliferation assays such as using a cell counter [31].

Analysis of N-terminal propeptide of type I collagen

HFL-1 cells were stimulated with the cytokines in medium containing 10 mM ascorbic acid. Fifty μl of medium was used for analysis of the N-terminal propeptide of type I collagen (PINP) using a commercial radioimmunoassay kit (Orion Diagnostica, Oy, Finland) [32, 33]. Values were divided by protein content of the cell layer for normalisation.

RESULTS

IL-15-receptor α-chain expression in human fetal lung fibroblast (HLF-1) cells

In order to determine the presence of the IL-15 signaling pathway in HLF-1 cells, we analyzed for protein expression of the IL-15 receptor α chain, the IL-2 receptor β chain and the γc chain by flow cytometry. HFL-1 cells expressed all three chains *(figure 1)*, offering the possibility for signal transduction *via* the IL-15-receptor α chain, IL-2-receptor β and γc chain complex, or the IL-2-receptor β and γc chain complex.

IL-15 or TGF-β1, alone or in combination, had no effect on the proliferation of HFL-1 cells

Myofibroblasts derived from pulmonary tissue showed increased growth in response to TGF-β1 [34]. Since it has already been shown that IL-15 also induces proliferation in fibroblast-like synoviocytes derived from patients with rheumatoid arthritis [35], we analyzed the effects of IL-15 and TGF-β1 on the proliferation of HFL-1 cells. Cells treated with IL-15, TGF-β1 or a combination thereof had equal proliferation rates *(figure 2)*.

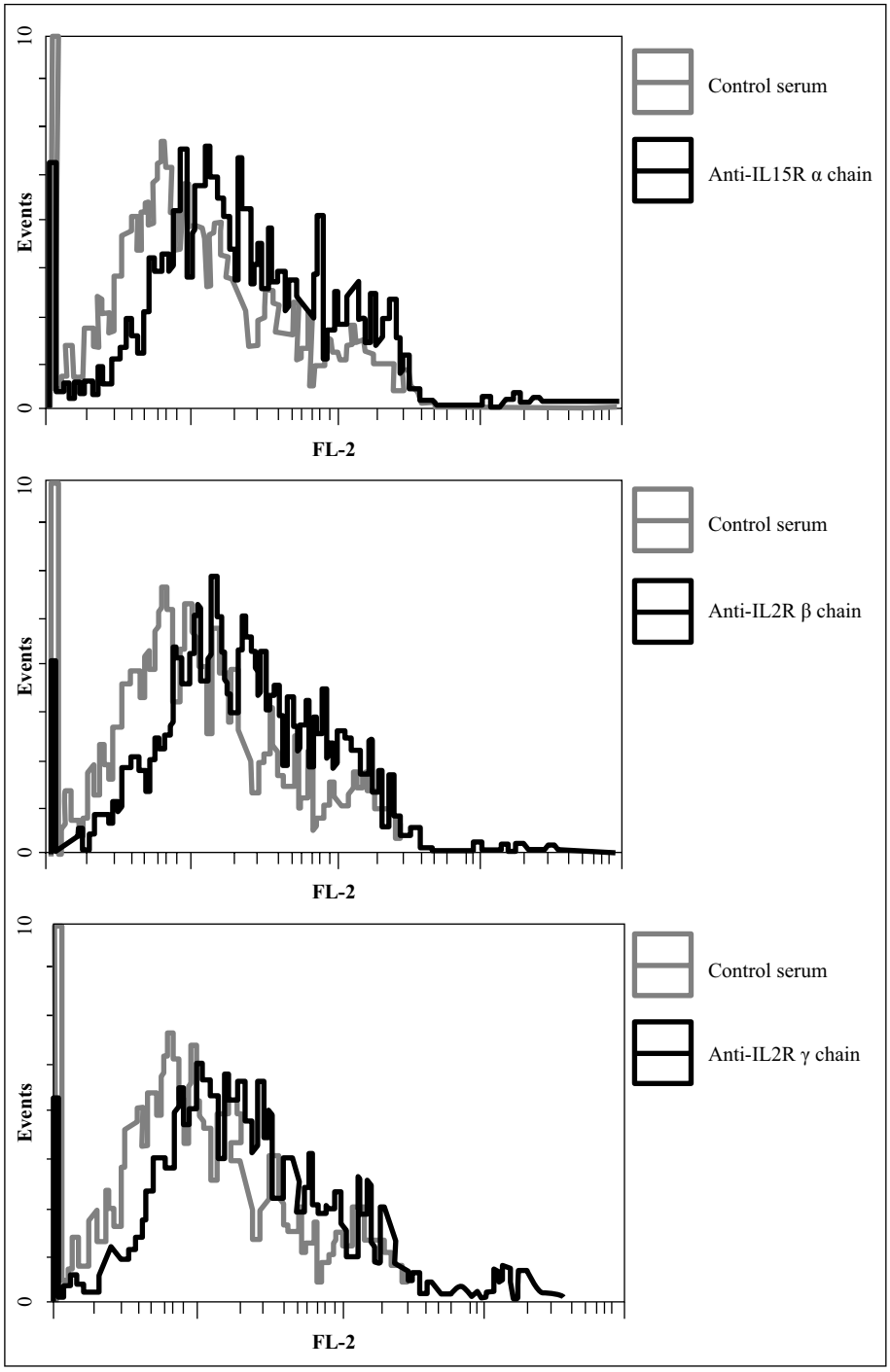

Figure 1

Human fetal lung fibroblasts (HFL-1) express the IL-15 receptor α, IL-2 receptor β and γc chains. Protein expression of the IL-15 receptor chains in HFL-1 cells was analyzed by flow cytometry. The histogram shows the cells as events that were analyzed in fluorescence channel two. Staining with receptor-specific goat serum is shown as a black line for the IL-15 receptor α (upper), IL-2 receptor β (middle) and γc chains (lower). Staining with control goat serum is shown as a grey line. A representative experiment out of three independent experiments is shown.

IL-15-mediated reduction of TGF-β1-induced α-SMA protein and mRNA expression

Differentiation of fibroblasts into myofibroblasts is characterized by a rearrangement of actin filaments. Upon exposure to hard, flat surfaces such as the plastic in our system, fibroblasts gradually rearrange their actin filaments and express α-SMA, which contributes to the rearrangement of these actin filaments [36]. This process is enhanced by addition of TGF-β1. In order to measure myofibroblast differentiation, expression of α-SMA protein was analyzed by ELISA. IL-15 treatment alone did not alter α-SMA protein expression, whereas TGF-β1 (2 ng/mL) induced a 10-fold increase in α-SMA expression, which continued to increase until 72 h of stimulation, as previously reported by others ([37], data not shown). This TGF-β1-stimulated α-SMA expression was reduced, in a dose-dependent manner, upon addition of IL-15 at 72 hours *(figure 3A)*. Addition of IL-15 at 5 ng/mL resulted in a 20-34% reduction, whereas

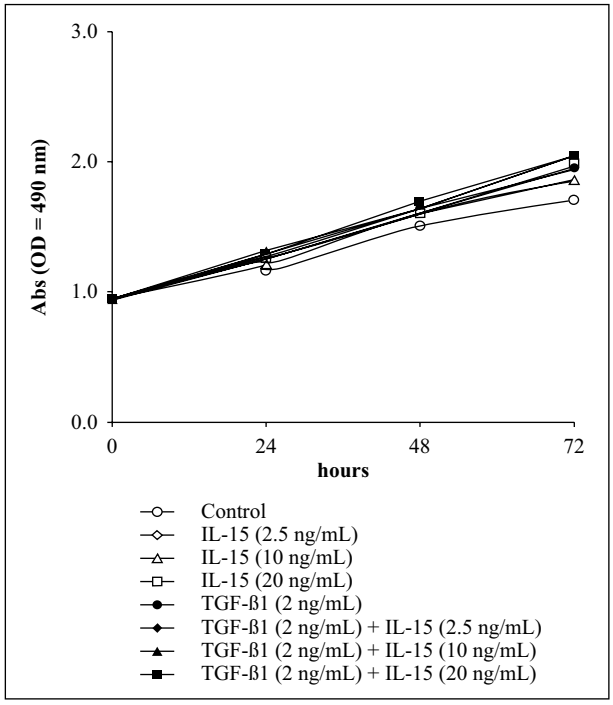

Figure 2
IL-15 and TGF-β1 do not affect proliferation of HFL-1 cells. TGF-β1 and IL-15 were added to HFL-1 cells at concentrations of 2, 2.5, 10, and 20 ng/mL, and the cells grown for 24, 48 and 72 h. Treatment with either TGF-β1 or IL-15 increased proliferation slightly compared to that in control cells. Treatment with both cytokines together did not change the growth pattern relative to addition of TGF-β1 or IL-15 separately. The mean of three independent experiments is shown.

maximal reduction (39-45%) was reached using an IL-15 concentration of 20 ng/mL. Next, we investigated the effect of IL-15 on α-SMA mRNA expression by quantitative, real-time PCR. IL-15 reduced α-SMA mRNA levels by 90% compared to unstimulated cells, with a maximal effect at 48 h, whereas TGF-β1 induced a 25-30 fold increase in α-SMA mRNA levels, with maximal effect after 24–48 h *(figure 3B)*. Combined stimulation with IL-15 and TGF-β1 resulted in an initial 2.8-fold increase in α-SMA mRNA, as compared to a 1.3-fold increase with TGF-β1 alone, at 6 h. Co-stimulation by IL-15 and TGF-β1 led to a reduction of 80% after 24 h, and a reduction of 90% after 48 h relative to results of TGF-β1 stimulation alone.

IL-15-mediated attenuation of TGF-β1-induced production of collagen type I

Myofibroblasts contribute to development of fibrosis through their excessive production of extracellular matrix, and mainly of collagens. We therefore investigated whether IL-15 might alter type I collagen production. To determine collagen protein production, we measured pro-collagen I n-peptide (PINP) levels. IL-15 alone did not stimulate production of PINP, but rather, reduced basal production of PINP by 30%. Ten ng/mL of TGF-β1 induced an increase in PINP levels of 71% compared to the unstimulated control *(figure 4A)*. Combined stimulation with different concentrations of IL-15 and TGF-β1 led to a reduction of TGF-β1-induced PINP synthesis by up to 39% for 10 ng/mL of IL-15, after 48 h, as compared to TGF-β1 stimulation alone. To determine whether the above results for collagen protein production were reflected by mRNA levels, we measured mRNA levels of collagen type I alpha I by quantitative real-time PCR. Stimulation of HLF-1 cells with IL-15 did not affect mRNA levels of collagen type I alpha I compared to control cells that received medium only *(figure 4B)*. TGF-β1 stimulation resulted in a 3.7- to 5-fold induction of collagen type I alpha I mRNA at 24 and 48 h, respectively. Combined stimulation with IL-15 and TGF-β1 led to a reduction of TGF-β1-induced mRNA levels of collagen type I alpha I of 42% after 24 h and of 32% after 48 h, as compared to TGF-β1 stimulation alone. The overall mRNA levels of collagen type I alpha I upon TGF-β1 stimulation were low compared to the mRNA levels of other TGF-β1-stimulated genes, as previously reported [30].

IL-15-mediated abolishment of TGF-β1-induced upregulation of fibronectin and PAI-1 mRNA expression

TGF-β signals through the canonical SMAD signaling pathway, which has been reported to be involved in TGF-β-induced expression of α-SMA and collagen. Recently, the JNK signaling path has also been associated with TGF-β-induced upregulation of α-SMA expression [24]. Thus, we decided to evaluate SMAD and JNK signaling by examination of the effect of IL-15 on TGF-β1-induced gene expression of one SMAD-dependent gene, that for PAI-1, and one JNK-dependent gene, that for fibronectin. In *figure 5A* (upper panel), it can be seen that IL-15 reduced PAI-1 mRNA expression by 65%, whereas TGF-β1 induced a 71-fold upregulation of PAI-1 mRNA expression compared to the control, at 24 h. Combined stimulation with IL-15 and TGF-β1 resulted in a reduction in PAI-1 mRNA levels by 91% compared to TGF-β1 stimulation alone. IL-15 also reduced fibronectin mRNA expression levels by 59%, whereas TGF-β1 induced a 13-fold upregulation in fibronectin mRNA expression compared to the control, at 24 h (*figure 5A*, lower panel). Similarly, combined stimulation with IL-15 and TGF-β1 resulted in a reduction in fibronectin mRNA levels by 73% compared to TGF-β1 stimulation alone.

IL-15 does not affect early TGF-β1-induced SMAD2/3, JNK, ERK and P38 signaling

The above experiments indicated that IL-15 does not promote myofibroblast differentiation as measured by levels of expression of α-SMA, collagen type I, PAI-1, and fibronectin. By contrast, IL-15 interfered with TGF-β1-mediated, myofibroblast differentiation, the TGF-β1-activated JNK and SMAD signaling pathways being the probable targets. We therefore analyzed the effect of IL-15 on TGF-β1-induced intracellular phosphorylation of SMAD and MAP kinase. TGF-β1 induced the phosphorylation of JNK kinase as early as 5 min, whereas phosphorylation of SMAD2/3 was apparent after 15 min, and was strongest at 30 min. To our surprise, simultaneous addition of IL-15 did not affect the TGF-β1-induced phosphorylation of SMAD2/3, JNK, ERK and P38 until 2 h after stimulation *(figure 5B)*.

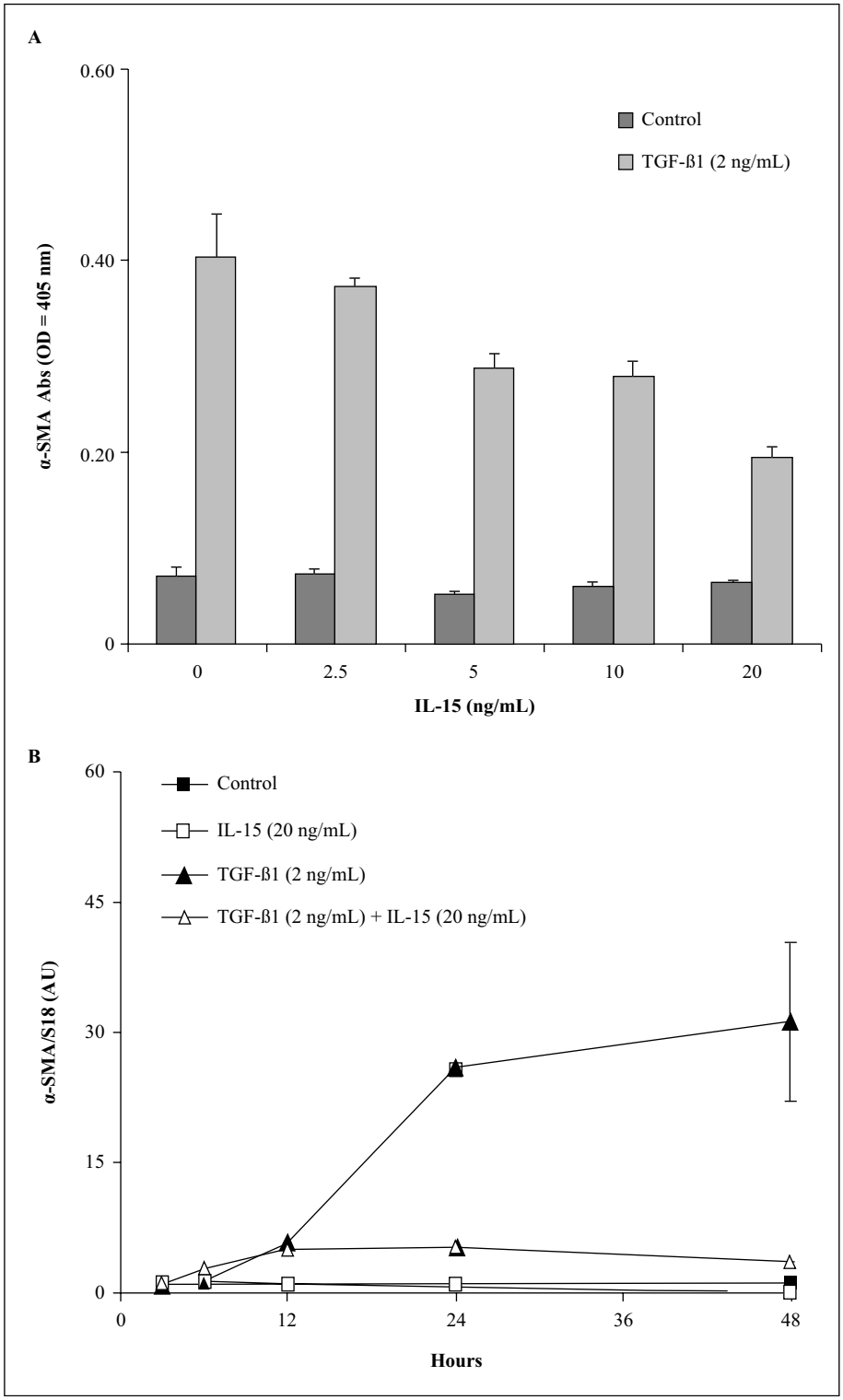

Figure 3

IL-15 attenuates TGF-β1-induced expression of α-SMA on both the protein and mRNA level. **A**) α-SMA protein expression was analyzed by ELISA in HFL-1 cells stimulated with TGF-β1 and IL-15 for 72 h, at the concentrations indicated. The mean ± SEM of duplicate determinations are shown. **B**) HFL-1 cells were stimulated with TGF-β1 (2 ng/mL) and IL-15 (20 ng/mL) for the times indicated, and α-SMA mRNA expression levels were analyzed by quantitative real-time PCR. All data were normalized to expression levels of S18 mRNA and related to the control at 3 h of incubation. The mean ± SEM of triplicate determinations are shown.

IL-15-mediated attenuation of TGF-β1-induced SMAD7 mRNA expression

An acknowledged negative-feedback loop of TGF-β signaling is mediated through SMAD7 expression [38]. Upon TGF-β-induced expression, SMAD7 binds to the TGF-β receptor kinase 2 and inhibits further TGF-β signaling. We therefore investigated whether the inhibitory effects of IL-15 on TGF-β1-induced myofibroblast differentiation might be mediated by SMAD7 gene expression. SMAD7 mRNA levels were determined at various times up to 48 h. IL-15 stimulation resulted in a

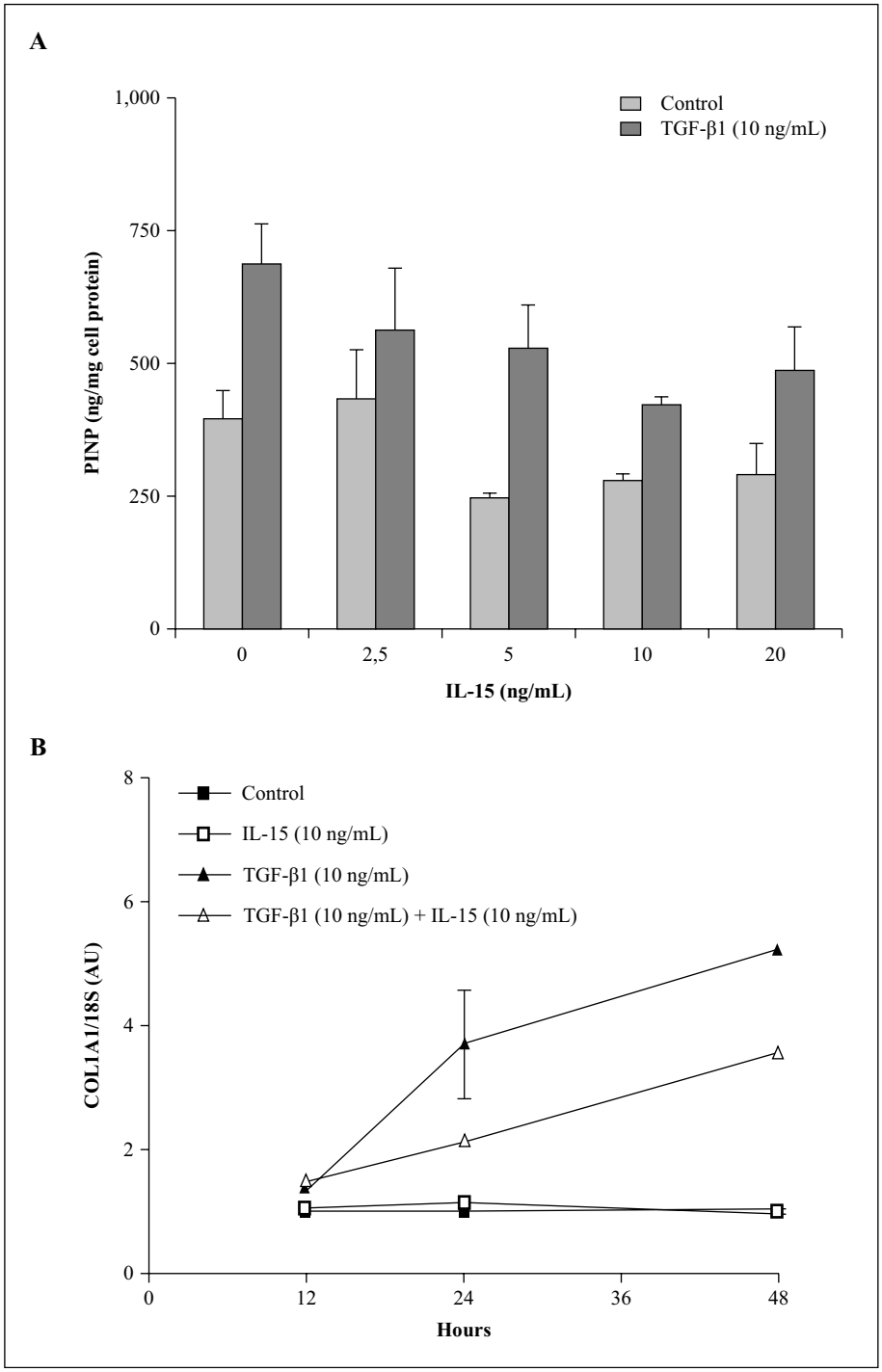

Figure 4

IL-15 attenuates TGF-β1-induced collagen type I expression on both the protein and mRNA level. **A**) HFL-1 cells were stimulated with TGF-β1 (10 ng/mL) with or without IL-15, at various concentrations for 48 h. Collagen production was measure by analysis of procollagen type I N-peptide (PINP). The mean ± SEM of duplicate determinations are shown. **B**) For mRNA analysis, HFL-1 cells were stimulated with TGF-β1 (10 ng/mL) and IL-15 (10 ng/mL) for 12, 24, or 48 h. Collagen type I alpha I mRNA levels were analyzed by quantitative real-time PCR. All data were normalized to expression levels of S18 mRNA and related to the control at 12 h of incubation. The mean ± SEM of duplicate determinations are shown.

slight reduction in SMAD7 mRNA levels relative to the control, whereas TGF-β1 led to an increase in SMAD7 mRNA levels in two phases. The first induction was 3-fold, and peaked at 6 h, whereas the second induction of SMAD7 mRNA levels was 18-fold, and peaked at 24 h. Unexpectedly, addition of IL-15 reduced TGF-β1-induced SMAD7 mRNA levels by 91% at 24 h *(figure 6A)*.

Dual effect of IL-15 on TGF-β1-induced CTGF mRNA expression

Recently, several data have indicated that CTGF is an important mediator of TGF-β signaling. CTGF appears to be involved in TGF-β-mediated α-SMA and collagen production, and thus myofibroblast differentiation [39].

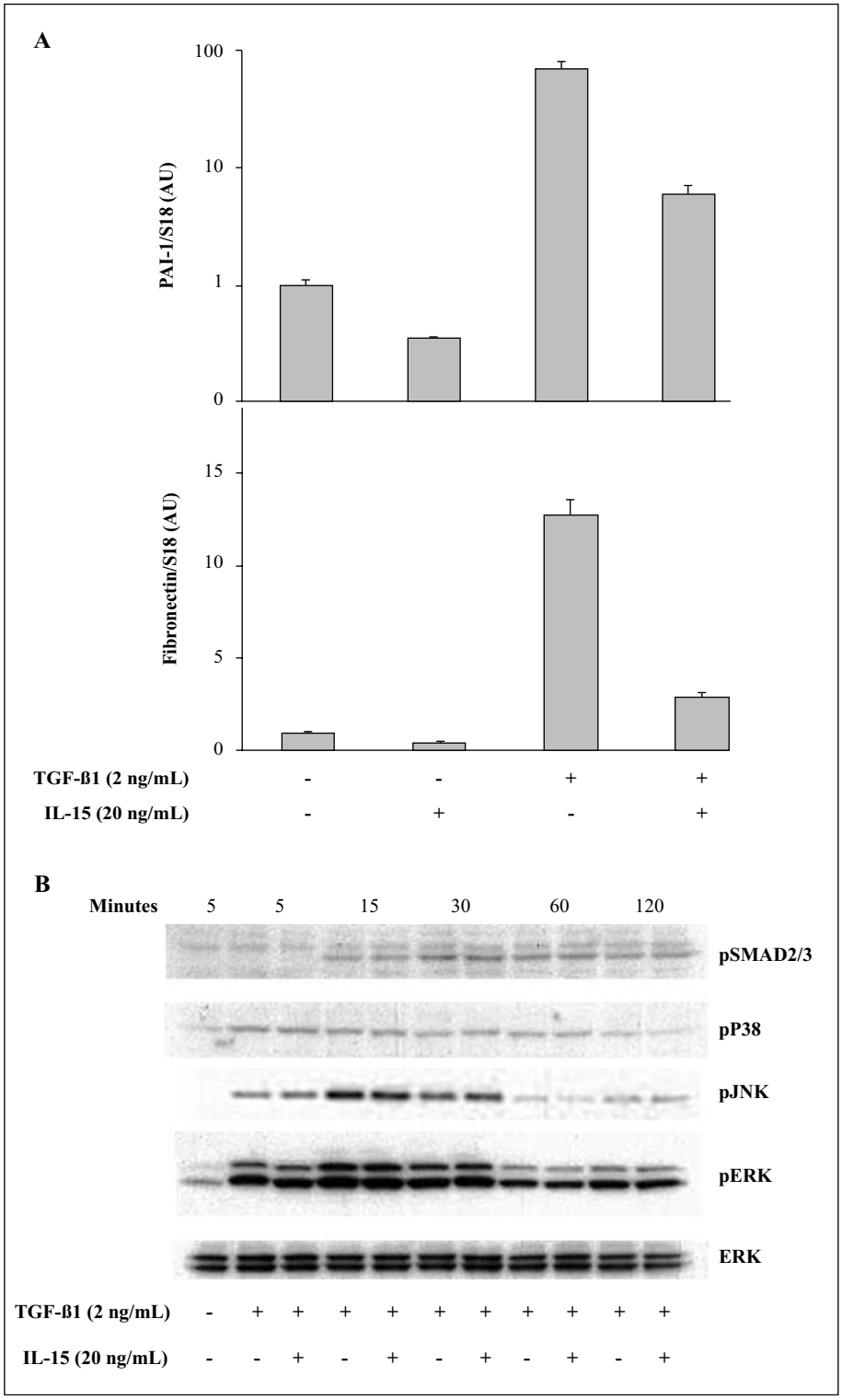

Figure 5

IL-15 reduces TGF-β1-induced PAI-1 and fibronectin mRNA expression, but does not affect TGF-β1-induced phosphorylation of SMAD2/3 and MAP kinases. **A**) HFL-1 cells were stimulated with TGF-β1 (2 ng/mL) and IL-15 (20 ng/mL) for 24 h, and expression levels of PAI-1 mRNA (upper panel) and fibronectin mRNA (lower panel) were analyzed. All data were normalized to expression levels of S18 mRNA and related to the control. The mean ± SEM of triplicate determinations are shown. **B**) HFL-1 cells were stimulated with TGF-β1 (2 ng/mL), with or without IL-15 (20 ng/mL), for the times indicated. Protein levels of phosphorylated SMAD2/3, JNK, ERK, and P38 were determined by western blot. Forty μg of protein were loaded per well. Total ERK protein was used as the loading control.

For that reason, we investigated the effect of IL-15 on TGF-β1-mediated CTGF mRNA expression *(figure 6B)*. IL-15 enhanced TGF-β1-induced CTGF mRNA expression 2-fold, up to 6 h. At 24 h, CTGF mRNA levels in the TGF-β1- and IL-15-stimulated sample were as low as in the negative control, which indicated a reduction in CTGF mRNA levels by 94%, as compared to the case with TGF-β1 alone. In contrast, TGF-β1 stimulation alone led to a 30-fold increase in CTGF mRNA levels that peaked at 24 h.

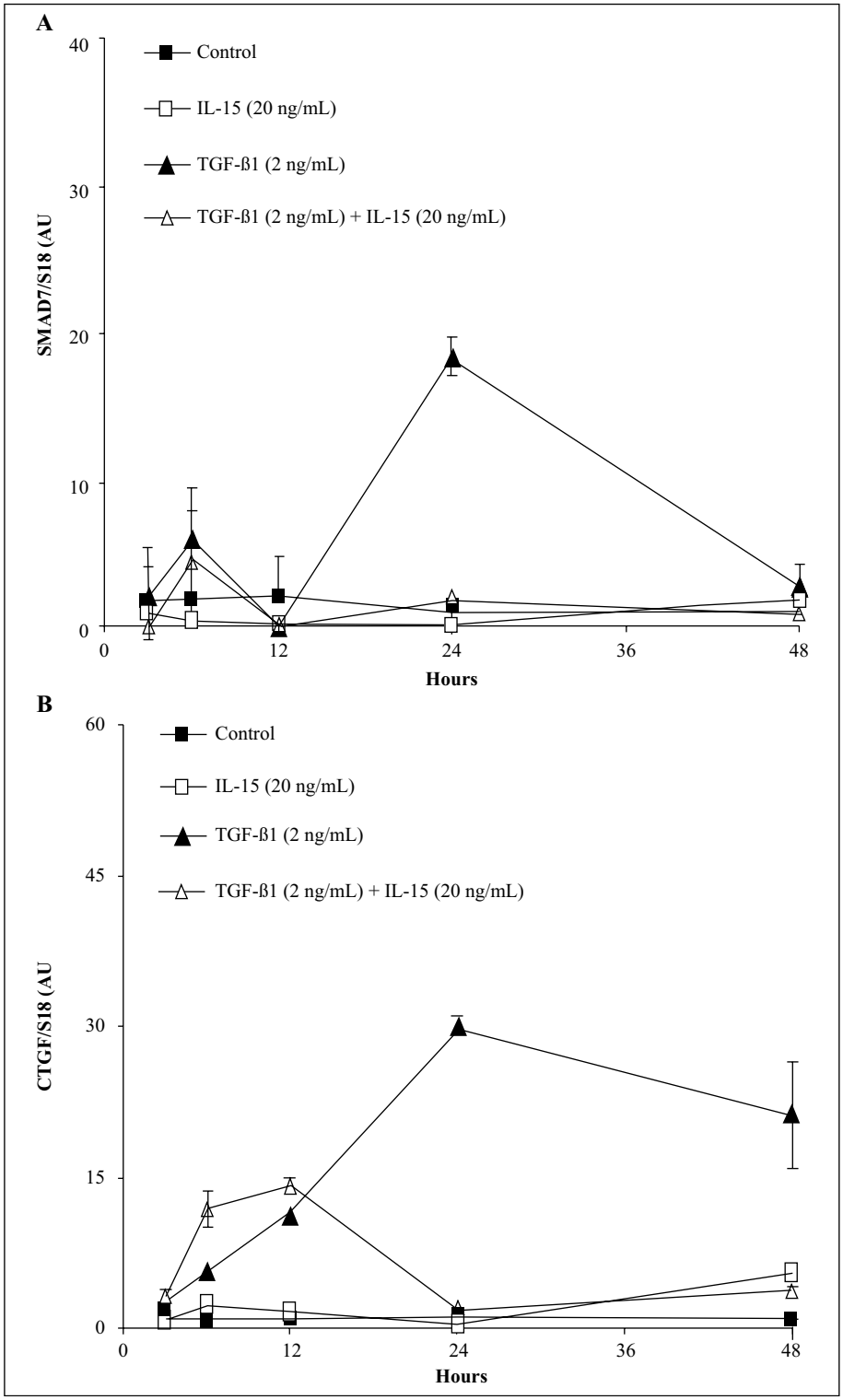

Figure 6

IL-15 abolishes TGF-β1-induced SMAD7 mRNA expression after 24 hours, and has dual effects on TGF-β1-induced CTGF mRNA expression. HFL-1 cells were stimulated with TGF-β1 (2 ng/mL) and IL-15 (20 ng/mL) for the times indicated, and SMAD7 (**A**) and CTGF (**B**) mRNA levels were analyzed. **A**) IL-15 abolished TGF-β1-induced SMAD7 mRNA expression after 24 hours. **B**) IL-15 enhanced TGF-β1-induced CTGF mRNA expression at 6 h, whereas the TGF-β1-induced upregulation of CTGF mRNA expression was abolished after 24 h of stimulation. All data were normalized to expression levels of S18 mRNA, and related to the control at 3 h of incubation. The mean ± SEM of triplicate determinations are shown.

DISCUSSION

This study shows that:

– IL-15 receptor α, IL-2 receptor β and γc chains are expressed at the protein level in HFL-1 cells;

– IL-15 reduces TGF-β1-mediated α-SMA protein and mRNA expression when stimulated for up to 72 h;

– IL-15 reduces TGF-β1-stimulated new synthesis of procollagen type I N-peptide and collagen type I alpha I mRNA expression;

– addition of IL-15, together with TGF-β1, reduces the mRNA levels of fibronectin and PAI-1 compared to TGF-β1 stimulation alone;
– early phosphorylation of SMAD or MAP-kinase signaling, upon stimulation with TGF-β1, remains unchanged in the presence of IL-15;
– TGF-β1-induced stimulation of SMAD7 mRNA levels is unchanged for up to 12 h, but is reduced at 24 h in the presence of IL-15;
– co-stimulation of HFL-1 cells with IL-15 and TGF-β1 results in an initial, additive effect upon stimulation of CTGF mRNA levels, which is abolished at 24 h of stimulation.

We have previously shown that serum IL-15 levels are associated with pulmonary fibrosis in patients with systemic sclerosis, a fibrotic disease that is characterized by vasculopathy, fibrosis, and immunological activation [15]. In this study, we therefore evaluated the effect of IL-15 on myofibroblast differentiation as a key mechanism during the development of fibrosis. Fibroblasts gain smooth muscle-like features and analysis of α-SMA expression is widely used to study the degree of myofibroblast differentiation. IL-15 had no affect on myofibroblast differentiation. On the contrary, the above experiments suggest an antifibrotic effect of IL-15 in TGF-β-stimulated human fetal lung fibroblasts. One interpretation of this *in vitro* experiment is that the upregulation of IL-15 expression in systemic sclerosis represents an attempt to inhibit the increased TGF-β signaling that characterizes the disease. Another explanation would be that IL-15 may contribute to the development of fibrosis in systemic sclerosis by new mechanisms that were not studied in the current experiments, and that remain to be elucidated.

In contrast to IL-15, the effect of TGF-β on the development of fibrosis in general and on α-SMA expression in particular, has been extensively studied in several fibrotic diseases, including systemic sclerosis. TGF-β has been suggested to upregulate α-SMA promoter activity in rat fibroblasts, directly through signal transduction from the TGF-β receptor 2 kinase via the SMAD2/3 signaling pathway [20, 21]. Other publications instead consider TGF-β as a modulator of the activity of other transcription factors [19]. Furthermore, the JNK signaling pathway has been proposed to contribute to TGF-β-mediated myofibroblast differentiation in lung fibroblasts [24]. On the other hand, indirect signaling pathways, including CTGF signaling, have also been suggested. In our study, the addition of IL-15, in conjunction with TGF-β1, reduced α-SMA expression both at the protein level and at the mRNA level, but phosphorylation of either SMAD or MAP kinase was not affected by the addition of IL-15 upon TGF-β1 stimulation. This is in contrast to studies that have shown that activation of the JNK and c-jun signaling by tumor necrosis factor-α or IL-15 inhibits the profibrotic, TGF-β-mediated SMAD2/3 signaling in fibroblasts and T cells, respectively [40, 41]. However, mRNA levels of genes that are commonly upregulated by TGF-β via either SMAD signaling, *e.g.* PAI-1, or JNK signaling, *e.g.* fibronectin, were reduced after 24 h of stimulation in the presence of IL-15. Interestingly, addition of IL-15 had dual effects on TGF-β1-mediated CTGF mRNA transcription. The presence of IL-15 enhanced early TGF-β1-mediated CTGF mRNA expression for up to 12 h, whereas the CTGF mRNA levels of the combined stimulation were as low as the CTGF mRNA levels of the negative control samples at 24 h. Thus, inhibition of TGF-β1-stimulated CTGF mRNA expression by IL-15 may contribute to the reduction in both α-SMA and collagen type I expression when both cytokines are present. Moreover, it is unlikely that IL-15's effect is mediated by a reduced gene regulation of TGF-β receptor chains, which are commonly upregulated through the SMAD signaling path upon addition of TGF-β1. Finally, IL-15 exhibited its most inhibitory effects at 5 to 20 ng/mL, suggesting that the signal transduction may have occurred through the the IL-2 receptor β and γc chain complex [5, 6].

TGF-β signaling leads to phenotypic changes in cells which are not only important for the immune system, but also for biological development [42]. Owing to its potent biological effect, several negative-feedback loops exist to limit the activity of TGF-β. One prominent negative regulator of TGF-β signaling is SMAD7, which, upon TGF-β-mediated expression and protein transcription, binds to the TGF-β receptor chain II and inhibits further TGF-β receptor II kinase activity and signaling [38]. Our observation that IL-15 abolished TGF-β-mediated SMAD7 upregulation may have interesting implications for myofibroblast activation in diseased tissue. A prolonged TGF-β1/IL-15-induced down regulation of SMAD7 could turn out to be deleterious, and promote the development of fibrosis in a scenario of repeated exposure to growth factors. Whether or not fibroblasts isolated from the skin of patients with systemic sclerosis have lower SMAD7 expression, compared to fibroblasts from healthy individuals, remains a matter of debate [43, 44].

In conclusion, IL-15 may prevent TGF-β1-mediated myofibroblast differentiation in the human fetal lung fibroblast. The mechanism appears to be independent of both the early TGF-β1-mediated SMAD2/3 and MAP-kinase signaling cascades, and of the SMAD7 feedback loop. Further studies are needed to identify the mechanism by which IL-15 antagonizes TGF-β1-mediated myofibroblast differentiation, and to determine whether IL-15 also exerts its antifibrotic effect on fibroblasts that are derived from diseased tissue, such as from patients with systemic sclerosis.

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