

Original article

Interleukin-35 is upregulated in systemic sclerosis and its serum levels are associated with early disease

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Abstract

Objectives. IL-35 is a member of the IL-12 family consisting of p35/IL-12a and EBI3/IL-27b subunits. IL-35 exerts immunomodulatory activities in experimental and human autoimmune inflammatory conditions. Our aim was to assess IL-35 expression in the skin and circulation of SSc patients and to characterize its potential association with SSc-related features.

Methods. Expression of IL-35 in skin and dermal fibroblasts was quantified by quantitative PCR, immunohistochemistry and immunofluorescence. Serum levels of IL-35 (by ELISA), CRP (by turbidimetry), ANA (by immunofluorescence) and autoantibodies of the ENA complex (by immunoblot) were measured in 40 SSc patients. Serum IL-35 was determined in 40 age- and sex-matched healthy controls.

Results. IL-35 expression was increased in SSc skin and dermal fibroblasts in a TGF- β -dependent manner. IL-35 induced an activated phenotype in resting fibroblasts and enhanced the release of collagen. IL-35 serum levels were increased in patients with SSc compared with healthy controls [median 83.9 (interquartile range 45.1–146.1) vs 36.2 (interquartile range 17.2–49.4) pg/ml, $P < 0.0001$]. Serum IL-35 was negatively correlated with disease duration ($r = -0.4339$, $P = 0.0052$). In line with this finding, serum IL-35 was increased in patients with an early SSc pattern on capillaroscopy assessment compared with those with active and late SSc patterns.

Conclusion. The present study demonstrates overexpression of IL-35 in SSc skin, dermal fibroblasts and serum. TGF- β induces IL-35, which in turn activates resting fibroblasts and enhances the release of collagen, thereby contributing to aberrant TGF- β signalling in SSc. Increased serum IL-35 is associated with early, inflammatory stages of SSc.

Key words: interleukin-35, systemic sclerosis, TGF- β , disease duration, capillaroscopy.

Rheumatology key messages

- IL-35 is increased in the skin, in dermal fibroblasts and in serum of patients with SSc.
- The serum levels of IL-35 are elevated in early stages of SSc.

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Introduction

SSc is a severe CTD of unknown aetiology that is considered to be one of the most challenging rheumatic diseases. It is characterized by an excessive accumulation of extracellular matrix in the skin and visceral organs, such as the lungs, heart, kidneys and gastrointestinal tract. SSc is regarded as a prototypic multisystem fibrotic disorder, in which pathologically activated fibroblasts release an excessive amount of collagen and other extracellular matrix components that leads to organ failure and high morbidity and mortality in SSc patients [1]. TGF- β is believed to play a central role in fibroblast activation and tissue fibrosis in SSc. It induces an expression profile in resting normal fibroblasts that is reminiscent of SSc fibroblasts. Aberrant, persistently activated TGF- β signalling has been proposed as the crucial mechanism in the pathogenesis of SSc and other fibrotic conditions [2]. While tissue fibrosis represents the hallmark of later stages of SSc, it has been widely recognized that fibrosis is the end result of the early stage that is characterized by endothelial cell injury, perivascular infiltration by inflammatory cells and a reduction in capillary density [3]. However, the growing knowledge of the pathogenesis of SSc has not yet been successfully translated into effective targeted therapy.

IL-35 is the most recent addition to the IL-12 cytokine family, which also includes IL-12, IL-23 and IL-27. These are heterodimeric cytokines produced by antigen-presenting cells. IL-12 family members exert immunomodulatory activities and are composed of shared α chains (p19/IL-23a, p28/IL-27 and p35/IL-12a) and β chains (p40/IL-12b and EBI3/IL-27b) [4]. IL-35 is distinct from other family members in its immunomodulatory properties and consists of ubiquitously expressed α chain (p35/IL-12a) and β chain (EBI3/IL-27b), which is selectively produced and highly inducible. IL-35 receptor consists of IL12R β 2 (a component of IL-12 receptor) and gp130 (a component of IL-27 receptor), which subsequently activate STAT1 and STAT4 signalling pathways [5]. IL-35 has been studied in tumours and proposed as a promotor of tumour progression and immune evasion and as a biomarker of tumour progression and prognosis [6–9]. In inflammatory conditions, the role of IL-35 remains controversial. While some reports support its anti-inflammatory properties given its effect on Treg, regulatory B cells and associated cytokines in IBD, CIA or other autoimmune conditions [10–14], other groups propose its pro-inflammatory properties in Lyme arthritis or CIA in different experimental settings [15, 16]. Recent reports also propose a potential role of IL-35 in sarcoidosis and SLE [17, 18]. These findings provide solid evidence that IL-35 plays a role in autoimmune inflammatory diseases. However, its role in SSc has not been studied to date. Thus, the aim of our study was to assess IL-35 expression in skin and circulation of SSc patients and to characterize its potential association with SSc-related features.

Methods

Skin biopsies and fibroblast cultures

Dermal fibroblasts were isolated from biopsies of lesional skin from SSc patients ($n=19$) and from age- and sex-matched healthy volunteers ($n=12$) as described [19, 20]. All SSc patients fulfilled both the 1980 ACR classification criteria for SSc and the 2013 ACR/EULAR classification criteria for SSc [21–23]. Sixteen patients were female, three were male. Of the 19 SSc patients, 7 had lcSSc and 12 had dcSSc. The median age of SSc patients was 52 (range 19–69) years, and their median disease duration (measured from the onset of the first non-Raynaud's symptom attributable to SSc) was 4 (range 0.5–11) years. Clinical and demographic characteristics of the patients and healthy individuals are shown in supplementary Table S1, available at *Rheumatology* Online. None of the patients was receiving DMARDs, glucocorticoids or NSAIDs at the time of biopsy. The study was approved by the local institutional review board of the University of Erlangen-Nuremberg, and all patients and control subjects signed an approved consent form. Fibroblasts from passages 4–8 were used for the experiments.

Patients and healthy controls in serum IL-35 analysis

A total of 40 Caucasian patients [35 female and 5 male; median age 57 years (range 30–71) and mean disease duration 3 years (range 0.5–18)] who met both the 1980 ACR and the 2013 ACR/EULAR criteria for SSc and 40 Caucasian healthy individuals [35 female and 5 male; median age of 55.5 years (range 30–70)] matched by age and sex were included in this study [21–23]. Clinical and demographic characteristics of the patients and healthy individuals are shown in Table 1. SSc-related manifestations were obtained from the Czech Registry of patients with SSc. Interstitial lung disease, pulmonary arterial hypertension, oesophageal dysmotility, cardiac and renal involvement, nailfold capillaroscopy pattern and RP were recorded. Assessments and definitions of these organ manifestations are detailed by Becvar *et al.* [24]. Skin changes of patients with SSc were assessed using the modified Rodnan skin score [25]. Disease activity was assessed using the EULAR Scleroderma Trials and Research group (EUSTAR) SSc activity score [26]. The study was approved by the local ethics committee at the Institute of Rheumatology in Prague, and each patient signed an informed consent form. None of the SSc patients and healthy controls used for serum IL-35 analysis underwent skin biopsy for the purpose of this study and vice versa.

Stimulation with IL-35 and TGF- β

In selective experiments, cells were incubated with human recombinant TGF- β (10 ng/ml; PeproTech, Hamburg, Germany) or human recombinant IL-35 protein at different concentrations (50 pg/ml and 1 ng/ml; ALEXIS Biochemicals, Enzo Life Sciences, Lausen, Switzerland) for 9 or 6 h, respectively.

Quantitative real-time PCR

Total RNA was isolated with a NucleoSpin RNA II extraction system (Macherey-Nagel, Dueren, Germany) and reverse transcribed into complementary DNA, as previously described [27]. Gene expression was quantified by SYBR Green real-time PCR using an ABI Prism 7300 Sequence Detection System (Life Technologies, Darmstadt, Germany). Specific primer pairs for each gene were designed with Primer3 software. The sequences of the human primers used are shown in supplementary Table S2, available at *Rheumatology* Online. A predeveloped β -actin assay (Life Technologies) was used to normalize for the amounts of loaded cDNA. Dissociation curve analysis, samples without enzyme in reverse transcription (non-RT controls) and no-template controls were used as negative controls to exclude genomic DNA contamination and formation of primer dimers. Differences were calculated with the threshold cycle and the comparative threshold cycle method for relative quantification.

Quantification of collagen protein

The amount of soluble collagen in cell culture supernatants was quantified using the SirCol collagen assay (Biocolor, Belfast, UK) as described previously [28].

Immunohistochemistry and immunofluorescence staining

Formalin-fixed, paraffin-embedded skin sections or 4% paraformaldehyde-fixed and 0.25% Triton X-100-permeabilized dermal fibroblasts were stained with rabbit anti-EBI3 antibodies (Lifespan Biosciences, Seattle, WA, USA), mouse anti-IL12a (anti-p35) antibodies (Santa Cruz, Santa Cruz, CA, USA), mouse anti-prolyl-4-hydroxylase- β (P4H) antibodies (Acris Antibodies, Herford, Germany) or mouse anti- α -smooth muscle actin (anti- α SMA) antibodies (clone 1A4; Sigma-Aldrich, Steinheim,

Germany). Horseradish peroxidase-conjugated or Alexa Fluor antibodies (Life Technologies) were used as secondary antibodies. Irrelevant isotype-matched antibodies were used as controls. Selected immunohistochemistry staining of paraffin-embedded skin sections for EBI3, p35 and α SMA was visualized with diaminobenzidine peroxidase substrate solution (Sigma-Aldrich). Stress fibres were visualized with rhodamine-conjugated phalloidin (Sigma-Aldrich) as previously described [29]. In addition, cell nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI; Santa Cruz Biotechnology, Heidelberg, Germany). Stained skin sections and cells were visualized using a Nikon Eclipse 80i microscope (Nikon, Badhoevedorp, Netherlands). The intensity of immunohistochemistry and immunofluorescence staining was quantified using ImageJ software version 1.44.

Enzyme-linked immunosorbent assay

Serum levels of IL-35 in SSc patients and healthy donors and the levels of IL-35 in the supernatants of cultured dermal fibroblasts were measured using a high-sensitivity ELISA kit (USCN Life Sciences Inc., Hubei, China) in a blinded manner. The assay recognizes natural human IL-35 protein, and its limit of detection is 6 pg/ml.

Clinical laboratory analysis

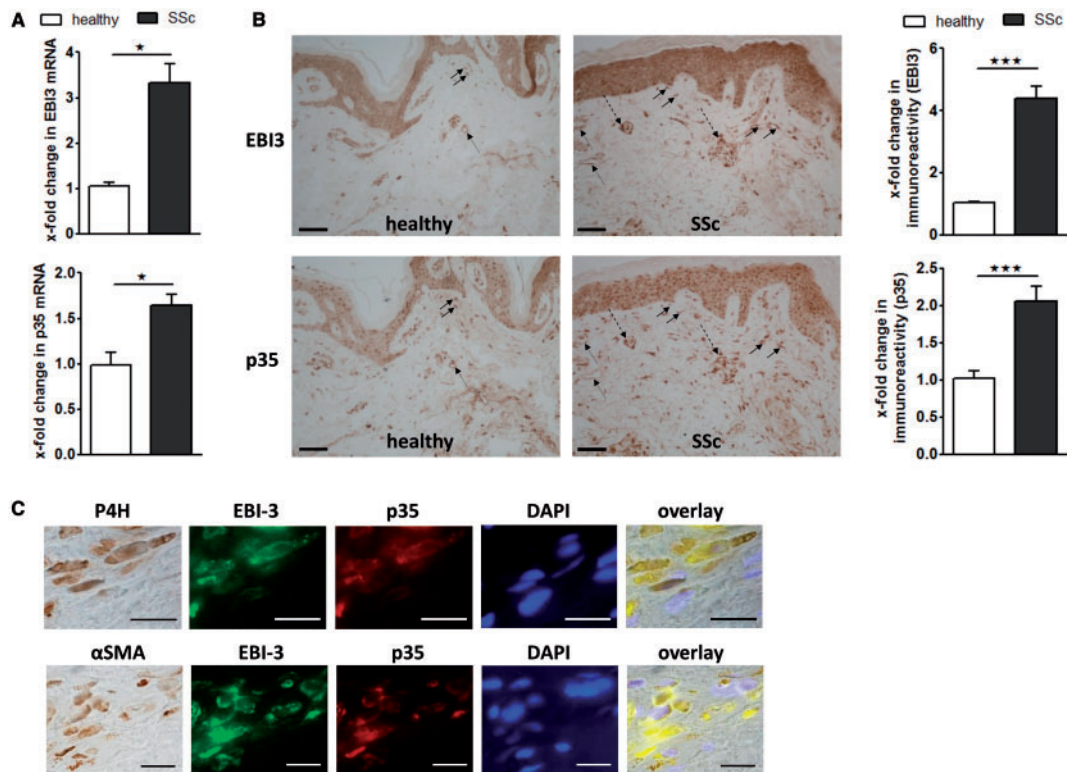
ANAs were detected using indirect immunofluorescence on HEP2 cells, and the autoantibodies of the ENA complex (anti-U1RNP, anti-Ro, anti-La, anti-DNA-topoisomerase I, anti-Jo-1, anti-P protein, anti-Sm and anti-centromere) were assayed by immunoblot. CRP concentration was assessed using turbidimetry, and ESR was measured according to the Fahraeus and Westergren method.

TABLE 1 Clinical and demographic characteristics of patients with SSc and healthy controls used in serum IL-35 analysis

Characteristics	SSc (n = 40)	Controls (n = 40)	P-value
Age, median (range), years	57 (30–71)	55.5 (30–70)	0.7520
Sex, female/male	35/5	35/5	0.9933
Disease duration, median (range), years	3 (0.5–18)	NA	NA
SSc subset, lcSSc/dcSSc	30/10	NA	NA
EUSTAR SSc activity score, median (range)	4 (0–8.5)	NA	NA
Active disease, %	73	NA	NA
SSc-related manifestations, %, ILD/PAH/OD/CI/RI/PCP/RP	63/20/83/45/10/78/100	NA	NA
Autoantibodies, % positive, ANA/Scl-70/ACA	95/33/23	—	NA
MRSS, median (range)	10.5 (0–29)	—	NA
CRP, median (range), mg/l	3.2 (0.2–80.6)	—	NA
ESR, median (range), mm/h	14 (1–78)	—	NA
Previous treatment, %, GC/CYC/D-Pen	83/23/28	NA	NA

Active disease: defined as having EUSTAR SSc activity score ≥ 3 ; CI: cardiac involvement; EUSTAR: The EULAR Scleroderma Trials and Research group; GC: low-dose glucocorticoids; MRSS: modified Rodnan skin score; NA: not applicable; OD: oesophageal dysmotility; PAH: pulmonary arterial hypertension; PCP: pathological capillaroscopy pattern; RI: renal involvement; Scl-70: anti-DNA-topoisomerase I; —: not examined.

Fig. 1 IL-35 chains EBI3 and p35 are overexpressed in SSc skin



(A) The mRNA levels of EBI3 and p35 are increased in SSc skin compared with healthy skin ($n = 6$). **(B)** Increased expression of EBI3 and p35 protein was detected in the skin of SSc patients compared with healthy controls ($n = 15$ and 12 , respectively) and quantified by ImageJ software. The solid-line arrows point at fibroblasts, the dashed-line arrows point at perivascular inflammatory cells and the dotted-line arrows point at endothelial cells (scale bars, $50 \mu\text{m}$). **(C)** Expression of EBI3 and p35 co-localizes with the marker of fibroblasts prolyl-4-hydroxylase (P4H) and myofibroblast marker α -smooth muscle actin (αSMA ; $n = 6$). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (scale bars, $25 \mu\text{m}$). * $P \leq 0.05$; *** $P < 0.001$.

Statistical analysis

Data were analysed and graphs designed using GraphPad Prism 5 (version 5.02; GraphPad Software, La Jolla, CA, USA) and are presented as mean (s.e.m.) unless stated otherwise. The normal distribution of measured variables was assessed using one-sample Kolmogorov-Smirnov test and normality tests based on skewness and kurtosis. IL-35 serum levels in patients with SSc and healthy controls were not normally distributed; therefore, non-parametric Mann-Whitney U -test was used for their comparison, as well as for the differences between the groups in the remaining experiments. Spearman's correlation coefficients were calculated to analyse the relationship between serum IL-35 and other parameters. P -values are expressed as follows: * $P \leq 0.05$; ** $P \leq 0.01$; and *** $P \leq 0.001$. $P < 0.05$ was considered significant.

Results

Expression of IL-35 is increased in SSc skin

The chains of IL-35, EBI3 and p35 were upregulated in the lesional skin of SSc patients compared with healthy

controls at both the mRNA ($P = 0.0286$ and $P = 0.0220$, respectively) and the protein level ($P = 0.0002$ and $P = 0.0009$, respectively; Fig. 1A and B). In healthy skin, EBI3 protein was expressed at low levels in a limited number of fibroblasts and perivascular cells and in the vessels of the dermis. In contrast, in fibrotic SSc skin, prominent staining for EBI3 was detected in an increased number of fibroblasts, perivascular inflammatory infiltrates and the basal layer of epidermis, whereas the intensity of staining in the vessels of the dermis was comparable to that of healthy skin (Fig. 1B). Likewise, expression of p35 in healthy skin was faint and restricted to vessels, keratinocytes and a limited number of fibroblasts and perivascular cells, whereas in clinically fibrotic SSc skin, intense staining for p35 was detected in a larger number of fibroblasts and perivascular inflammatory infiltrates. The staining for p35 in keratinocytes and vessels was comparable in healthy and lesional SSc skin (Fig. 1B). Of particular interest, P4H-positive fibroblasts and αSMA -positive myofibroblasts showed particularly intense staining for both EBI3 and p35 protein (Fig. 1C).

TGF- β induces expression and release of IL-35 in dermal fibroblasts

The overexpression of EBI3 and p35 persisted in cultured dermal fibroblasts from SSc patients, which maintained increased mRNA ($P=0.0160$ and $P=0.0286$, respectively) and protein levels of both chains of IL-35 and released increased levels of IL-35 into supernatants compared with control healthy dermal fibroblasts ($P=0.0190$; Fig. 2). Aberrant TGF- β signalling is a common feature of fibrotic diseases, and persistently enhanced TGF- β has been shown to contribute to the activated phenotype of SSc fibroblasts in culture. We therefore hypothesized that TGF- β may stimulate the expression of IL-35. Indeed, incubation of healthy dermal fibroblasts with TGF- β increased the mRNA ($P=0.0220$ and $P=0.0286$, respectively) and protein levels of both chains of IL-35, EBI3 and p35, and enhanced the release of IL-35 into the supernatants compared with unstimulated fibroblasts ($P=0.0376$; Fig. 3).

IL-35 induces fibroblast activation and collagen synthesis

To analyse the functional consequences of the upregulation of IL-35 in SSc, we stimulated the healthy dermal fibroblasts with human recombinant IL-35 protein using concentrations of 50 pg/ml and 1 ng/ml. These concentrations were selected to cover the average concentrations of IL-35 in the supernatants of cultured dermal fibroblasts and in the sera of SSc patients and healthy individuals. Stimulation with human recombinant TGF- β served as a positive control. Indeed, the formation of stress fibres and α SMA protein levels increased upon stimulation of healthy dermal fibroblasts with recombinant IL-35 protein in a dose-dependent manner, indicating that IL-35 can induce myofibroblast differentiation in resting fibroblasts (Fig. 4A). Furthermore, incubation of resting fibroblasts with recombinant IL-35 protein upregulated the mRNA levels of TGF- β target genes plasminogen activator inhibitor-1 and connective tissue growth factor. Stimulation with recombinant IL-35 also increased the mRNA levels of COL1A1 and COL1A2 and enhanced the release of collagen protein into the supernatants in a dose-dependent manner compared with control unstimulated fibroblasts (Fig. 4B–D).

IL-35 is increased in sera of SSc patients and is associated with early disease

Serum levels of IL-35 were increased in patients with SSc compared with healthy controls {median 83.9 pg/ml [interquartile range (IQR) 45.1–146.1] vs. 36.2 pg/ml (IQR 17.2–49.4), $P<0.0001$ }. IL-35 serum levels were higher in dcSSc patients than in lcSSc patients, but this difference did not reach statistical significance. Nevertheless, serum IL-35 levels of both subsets, lcSSc and dcSSc, were significantly higher than those of healthy controls (Fig. 5A). Interestingly, serum levels of IL-35 were negatively correlated with disease duration in SSc patients ($r=-0.4339$, $P=0.0052$; Fig. 5B). In line with this finding, we also observed significantly higher levels of serum IL-35

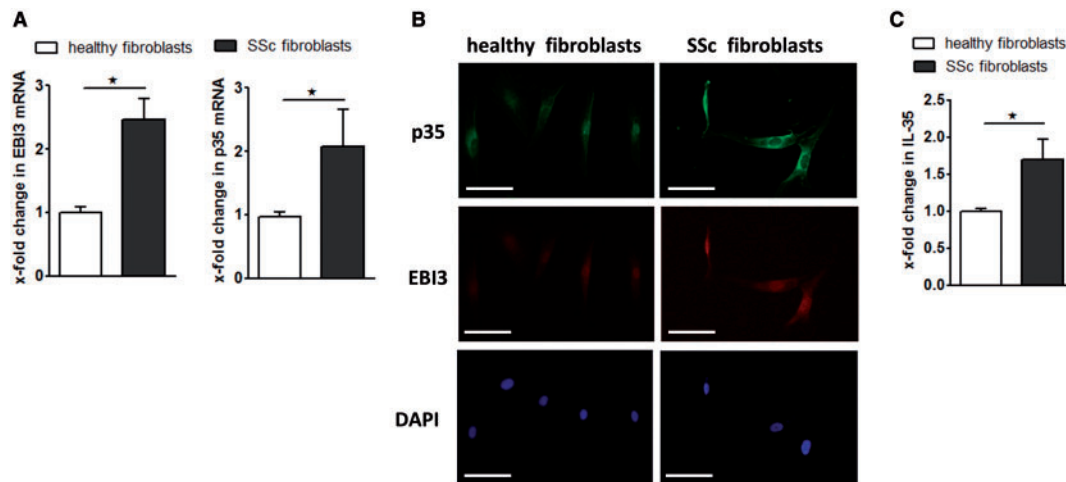
in SSc patients with the early SSc pattern on capillaroscopy assessment in comparison with those with active and late SSc patterns [148.6 (IQR 80.8–660.3) vs 52.5 (IQR 37.3–85.4) and 79.5 (IQR 19.8–116.8), $P=0.0441$ and $P=0.0485$, respectively; Fig. 5C]. However, after exclusion of the highest outlying value from the group with early SSc pattern, this difference was no longer significant. Nevertheless, the early SSc capillaroscopy pattern is known to be associated with the early stages of SSc [30]. Given that the values of serum IL-35 in lcSSc patients showed a dichotomous distribution, an additional statistical analysis was performed in this clinical subset. Herein, the negative correlation of serum IL-35 with disease duration was maintained; furthermore, increased IL-35 levels were found in lcSSc patients with higher ANA titre (data not shown). In all patients with SSc, besides disease duration and capillaroscopy pattern, no correlations of the serum levels of IL-35 with age, gender, EUSTAR SSc activity score, modified Rodnan skin score, lung function tests, ESR, CRP, the presence of autoantibodies (ANA, ACA and anti-DNA-topo I), previous treatment or organ involvement were observed (data not shown).

Discussion

In the present study, we demonstrated upregulation of the IL-35 chains p35 and EBI3 in the dermis of the skin of patients with SSc compared with that of healthy controls. Overexpression of IL-35 persisted in cultured dermal fibroblasts isolated from the lesional SSc skin. In addition, IL-35 expression was induced by stimulation of healthy fibroblasts with TGF- β . Furthermore, IL-35 itself induced activation of resting fibroblasts and enhanced the release of collagen. In line with these data, we detected increased levels of IL-35 in the sera of SSc patients compared with healthy controls. Interestingly, elevated serum IL-35 was associated with the early stage of the disease, defined by short disease duration and early SSc pattern on capillaroscopy assessment. These data suggest a potential role for IL-35 in the pathogenesis of SSc. Our findings may point to a role of IL-35 in initiation of the disease, rather than towards a role in disease progression. However, further studies are required to elucidate the potential molecular mechanisms.

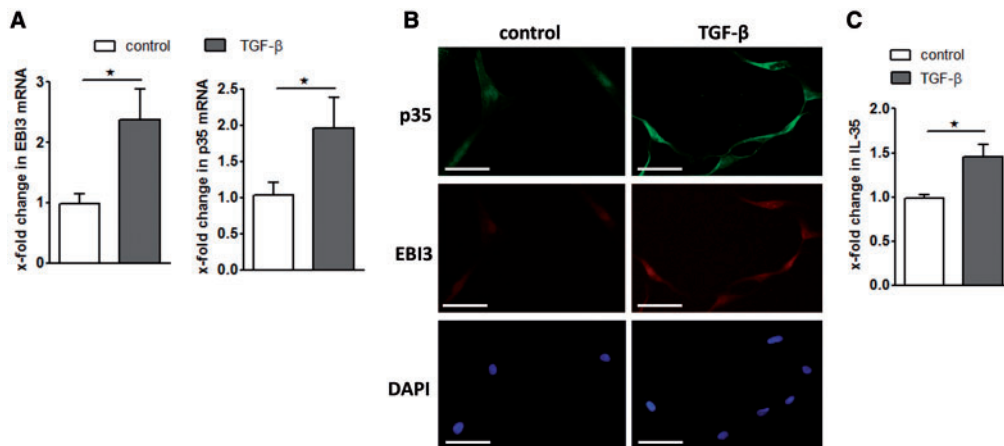
This study is the first showing that both subunits of IL-35, p35 and EBI3, are overexpressed in the dermis of the lesional SSc skin. IL-35 subunits were detected predominantly in dermal fibroblasts and perivascular inflammatory cells. Of particular interest, P4H-positive fibroblasts and α SMA-positive myofibroblasts showed prominent staining for both subunits of IL-35. While the p35 subunit has been demonstrated to be constitutively expressed at low levels in a broad spectrum of cells [31], EBI3 is selectively produced and highly inducible, and its expression was found in placental trophoblasts, dendritic and plasma cells, macrophages, endothelial cells, Hodgkin and Reed–Sternberg lymphoma cells and B-cell lymphomas [32–35]. Our recent data on the role of IL-35 in RA show localization of the p35 and EBI3 subunits in macrophages,

Fig. 2 Increased expression of EBI3 and p35 persists in cultured SSc fibroblasts



(A) The mRNA levels of EBI3 and p35 are increased in cultured SSc dermal fibroblasts compared with healthy dermal fibroblasts ($n = 6$). **(B)** Cultured SSc fibroblasts express increased levels of EBI3 and p35 protein compared with healthy fibroblasts ($n = 6$). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (scale bars, 25 μ m). **(C)** The levels of IL-35 are increased in the supernatants of SSc fibroblasts in comparison with healthy fibroblasts ($n = 6$). * $P \leq 0.05$.

Fig. 3 TGF- β induces the expression of EBI3 and p35

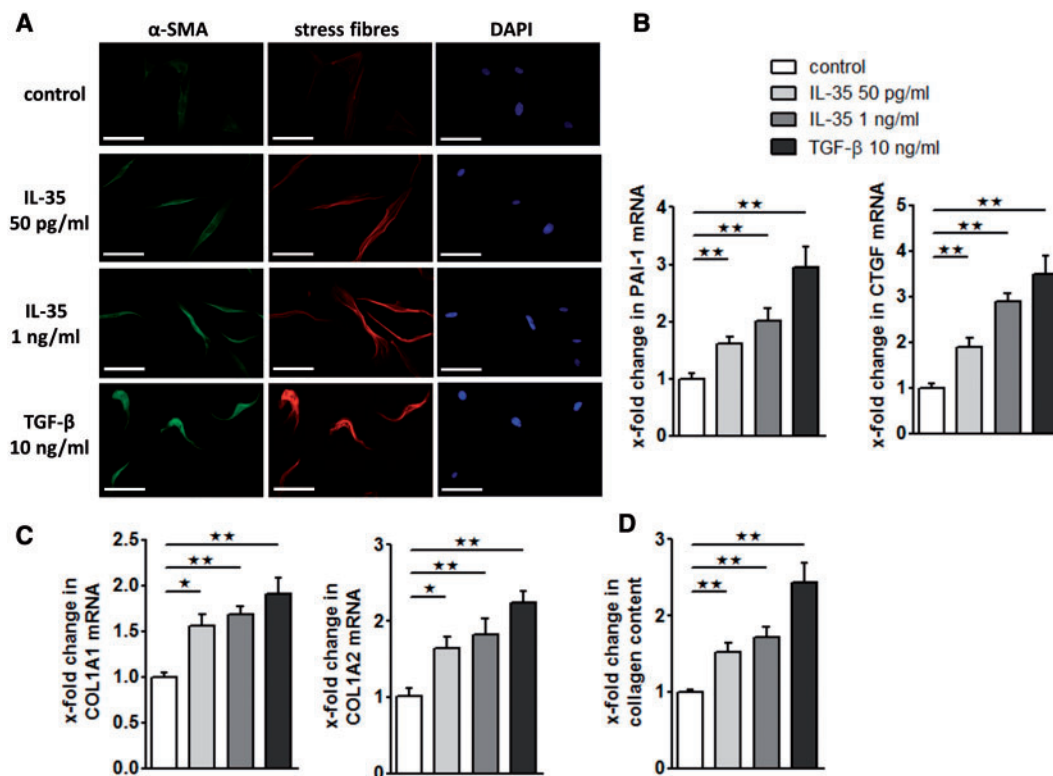


(A and B) Stimulation of healthy dermal fibroblasts with TGF- β increases the mRNA (**A**; $n = 6$) and protein level (**B**; $n = 6$) of EBI3 and p35. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (scale bars, 25 μ m). **(C)** The levels of IL-35 are increased in the supernatants of dermal fibroblasts stimulated with TGF- β compared with unstimulated control fibroblasts ($n = 6$). * $P \leq 0.05$.

dendritic cells, B cells, T cells and synovial fibroblasts in RA synovium [36].

We next demonstrated that increased expression of IL-35 persisted in cultured dermal fibroblasts isolated from the lesional SSc skin and that it could be induced by stimulation with TGF- β . Furthermore, we showed that stimulation of resting dermal fibroblasts with recombinant IL-35 induces their activation and enhances the release of

collagen in a dose-dependent manner. Given that the perivascular inflammatory infiltrate comprising T and B cells, mononuclear cells, macrophages and mast cells is characteristic of early skin involvement in SSc, and type I collagen is abundantly expressed by fibroblasts present in the dermal region adjacent to the inflammatory infiltrate, we hypothesize that perivascular inflammatory cells in early SSc might be the possible source of increased

Fig. 4 IL-35 activates dermal fibroblasts and stimulates the release of collagen

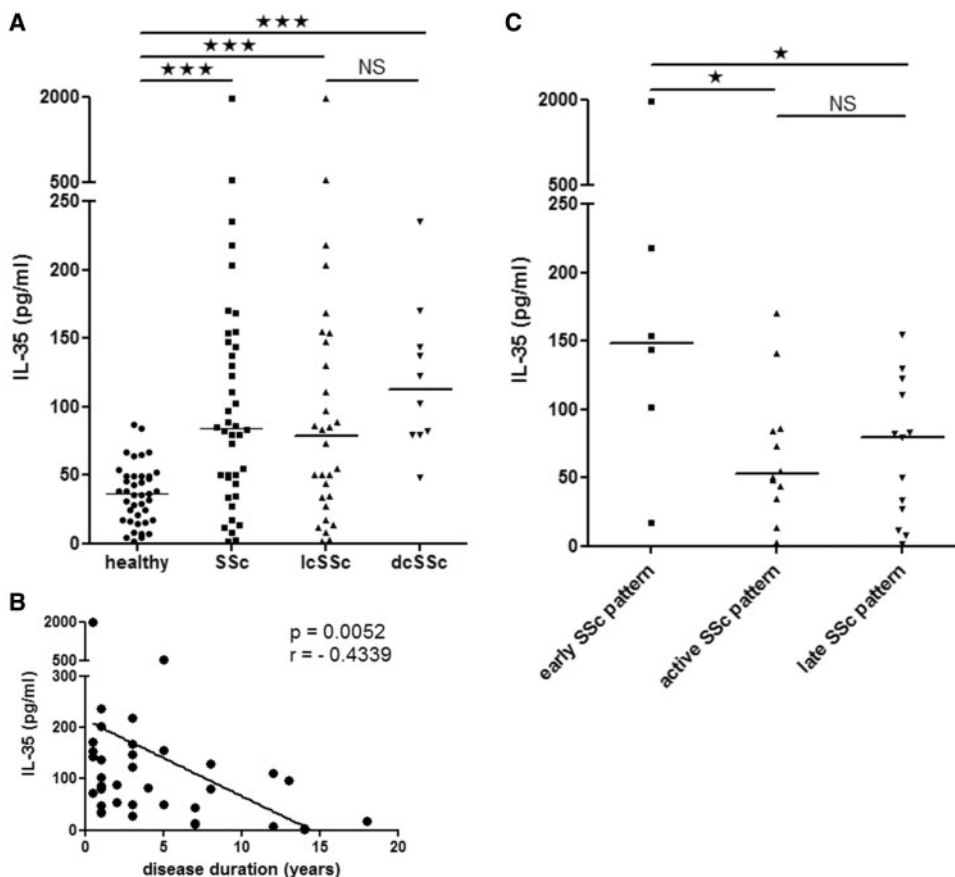
(A–D) Incubation of resting dermal fibroblasts with recombinant IL-35 protein stimulates the expression of α -smooth muscle actin (α SMA) and stress fibres (A; $n = 6$), induces the mRNA expression of TGF- β target genes plasminogen activator inhibitor-1 (PAI-1) and connective tissue growth factor (CTGF; B; $n = 6$), increases the mRNA levels of COL1A1 and COL1A2 (C; $n = 6$) and enhances the release of collagen protein (D; $n = 6$) in a dose-dependent manner. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (scale bars, 25 μ m). * $P \leq 0.05$; ** $P \leq 0.01$.

levels of IL-35. Its paracrine effect on resting fibroblasts leads to enhanced release of collagen and IL-35, thus creating an autocrine loop and contributing to aberrant TGF- β signalling in SSc [1, 2, 37]. However, further studies are required to elucidate the mechanism of action of IL-35 and its possible sources and targets in SSc. The IL-35 receptor has been described to subsequently activate STAT1 and STAT4 signalling pathways [5]. Various STAT4 variants have convincingly been established as SSc genetic susceptibility risk factors of SSc [38, 39]. Furthermore, Avouac *et al.* [40] recently demonstrated that the transcription factor STAT4 exerts potent profibrotic effects by controlling T-cell activation, proliferation and cytokine release. In addition, other members of the IL-12 cytokine family have recently been implicated in the pathogenesis of SSc. While a genome-wide association study revealed the association of the *IL12RB2* gene (encoding the IL-12R β 2 chain of both IL-12 and IL-35 receptor) with SSc, the IL-23 receptor gene has not been shown to confer risk of SSc and is not associated with SSc disease phenotype [41, 42]. Moreover, IL-27 stimulation increased IgG production by B cells, IL-17 production

by CD4 T cells and proliferation and collagen synthesis by fibroblasts in patients with SSc compared with healthy controls [43].

Finally, we detected increased levels of IL-35 in sera of SSc patients compared with healthy controls. In addition, both subsets of SSc (lcSSc and dcSSc) had higher serum IL-35 than healthy controls, and there was a trend towards increased IL-35 levels in the dcSSc subset compared with lcSSc. Interestingly, elevated serum levels of IL-35 were associated with early stages of the disease, with short disease duration and an early SSc pattern on capillaroscopy assessment. However, increased serum IL-35 in patients with an early SSc pattern should be confirmed in a larger number of patients. Serum levels of other IL-12 family members have been analysed in SSc patients as well. Increased levels of IL-12 were found in SSc sera and may relate to the activation of Th1 cells in SSc and be associated with renal vascular damage [44]. Moreover, treatment with bosentan has been reported to increase serum IL-12 levels in SSc patients with pulmonary arterial hypertension [45]. Serum IL-23 levels were significantly elevated in SSc patients compared with patients with

Fig. 5 Serum IL-35 levels are increased in SSc patients and are negatively correlated with disease duration



(A) Serum levels of IL-35 are significantly higher in SSc patients and both subsets of SSc (lcSSc and dcSSc) compared with healthy controls. **(B)** Serum IL-35 levels in SSc patients are negatively correlated with the disease duration. **(C)** Increased IL-35 serum levels were found in SSc patients with early SSc pattern compared with those with active and late SSc pattern on capillaroscopy assessment. Horizontal bars represent the median. * $P \leq 0.05$; *** $P < 0.001$. NS: not significant.

SLE and healthy controls and were associated with disease duration and the prevalence of pulmonary fibrosis [46]. Lastly, serum IL-27 levels were also raised in patients with SSc compared with healthy controls and were correlated positively with the extent of skin and pulmonary fibrosis and immunological abnormalities [43]. In conclusion, we demonstrated overexpression of IL-35 in SSc skin, dermal fibroblasts and serum. TGF- β induces IL-35, which in turn activates resting fibroblasts and enhances the release of collagen, thereby contributing to aberrant TGF- β signalling in SSc. Increased serum IL-35 is associated with early, inflammatory stages of SSc.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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