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Interferon-γ Induces Interleukin-6 Production and Alpha-smooth Muscle Actin Expression in Systemic Sclerosis Fibroblasts

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ABSTRACT

Systemic sclerosis (SSc) is an autoimmune systemic disease that is characterized by immune dysregulation, inflammation, vasculopathy, and fibrosis. Tissue fibrosis plays an important role in SSc and can affect several organs such as the dermis, lungs, and heart. Dysregulation of interferon (IFN) signaling contributes to the SSc pathogenesis and interferon regulatory factor 1 (IRF1) has been indicated as the main regulator of type I IFN.

This study aimed to clarify the effect of IFN-gamma (-γ) and dexamethasone (DEX) on the IRF1, extracellular signal-regulated kinase 1/2 (ERK1/2), and the expression of alpha-smooth muscle actin (α-SMA) in myofibroblasts and genes involved in the inflammation and fibrosis processes in early diffuse cutaneous systemic sclerosis (dcSSc). A total of 10 early dcSSc patients (diffuse cutaneous form) and 10 unaffected control dermis biopsies were obtained to determine IFNγ and DEX effects on inflammation and fibrosis. Fibroblasts were treated with IFNγ and DEX at optimum time and dose. The expression level of genes and proteins involved in the fibrosis and inflammation processes have been quantified by quantitative real-time PCR (RT-qPCR) and western blot, respectively.

IFNγ could up-regulate some of the inflammation-related genes (Interleukin-6; *IL6*) and down-regulate some of the fibrosis-related genes (*COL1A1*) in cultured fibroblasts of patients with early dcSSc compared to the untreated group. Besides, it has been revealed that IFNγ can induce fibroblast differentiation to the myofibroblast that expresses α-SMA.

Concerning the inhibitory effect of IFNγ on some fibrotic genes and its positive effect on the inflammatory genes and myofibroblast differentiation, it seems that IFNγ may play a dual role in SSc.

Keywords: Dexamethasone; Fibrosis; Inflammation; Interferon-gamma; IRF1; Systemic sclerosis

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INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune and multisystem connective tissue disorder described by multiple organ fibrosis, micro-vasculopathy, and immune system abnormalities.[1,2] The etiology of SSc is unknown. There are a few targeted therapy options that can manage the overall disorder activity and the fibrotic complications satisfactorily. Therefore, disease-associated morbidity and mortality stay elevated.[3]

Interferons (IFNs; α, β, and γ) have an essential role in the control of the innate and adaptive immune systems. They regulate immune cell activation, proliferation, and differentiation as well as interleukin production.[4] Previous investigations have provided substantial documentation that dysregulation in type I and II IFNs and IFN-inducible genes (called the "IFN signature") involves the pathogenesis of SSc.[5] Some investigations demonstrated a substantial enhancement in the IFNγ expression in the patient's dermis with SSc.[6] Interferon regulatory factors (IRFs) were initially recognized as transcription factors attaching to sequences in the IFN-α and IFN-β promoters. The IRF family includes IRF1 (Interferon-stimulated gene factor-2; ISGF-2), IRF2 (ISGF-1), IRF3, IRF4 and IRF5, IRF7, interferon-stimulated gene factor-3γ (ISGF-3γ), interferon consensus sequence binding protein for activated T cells (ICSAT), and interferon consensus sequence-binding protein (ICSBP) which develop a complex with transcription factors such as signal transducer and activator of transcription 1 (STAT1) and STAT2 upon IFNα stimulation.[7] IRF1 is expressed in different cells and organs and rolled in inflammatory injuries, fibrotic diseases, regulation of cell growth, and immune and autoimmune responses.[8-10] IRF1 identifies the IFN-stimulated response element (ISRE) in the promoter's area of inflammatory and fibrotic genes such as *IFNA*, *IL6*, and transforming growth factor beta 1 (*TGFB1).*[10]

The global gene expression profiling of early SSc dermis indicated that IRF1 was the one of top ten phosphorylated transcription elements in the type I IFN signaling cascade.[9,11] Also, it has been reported that the gene expression level of IRF1 is increased in the mononuclear and fibroblast cells of SSc patients.[12] Besides, 2′-5′-oligoadenylate synthesizes like (OASL) is a member of the IFN signature, and mostly acts as an essential antiviral and autoimmune factor induced by IRF1. OASL-IRF1 are essential mediators in the fibrosis of SSc patients by controlling DNA methylation.[12] Additionally, induction of IRF1 could contribute to fibrosis by down-regulation of the Klotho protein and CCAAT/enhancer-binding protein beta (C/EBP-β) factor. So, IRF1 contributes to the pathogenesis of fibrotic disease and IRF1 inhibition could be a potential therapeutic target.[10]

Multiple studies have indicated that IL-6 levels are promoted in the culture medium of skin fibroblasts and plasma from SSc patients.[13,14] Khan et al. verified the IL-6 up-regulation in diffuse cutaneous systemic sclerosis (dcSSc) and supported the IL-6 possibility as a surrogate feature for clinical outcomes in this disorder.[14] However, the role of IL-6 is unclear in the adjustment of skin fibrosis.

In the current research, we explored the effect of IFNγ and dexamethasone (DEX) on the inflammation- and fibrosis-related genes of study and the IRF1, extracellular signal-regulated kinase 1/2 (ERK1/2) signaling cascade and myofibroblast differentiation with the expression of alpha-smooth muscle actin (α-SMA) in early dcSSc dermis fibroblasts which assist to clarify the SSc pathogenesis mechanisms.

MATERIALS AND METHODS

Patients with SSc and Controls

The SSc patients and unaffected controls were enrolled from volunteers in the rheumatology clinic at the Shariati hospital. The patient enrollment and data management were accomplished in the rheumatology research center (RRC), Tehran University of Medical Sciences (TUMS). The disease diagnosis was confirmed by the rheumatologist according to the standards of the American College of Rheumatology (ACR).[15] So, all SSc patients fulfilled the 2013 classification standards for systemic sclerosis. All patients were adults more than 18 years, old who had no prior (last 6 months) or currently taken (tablets or intravenous) cyclophosphamide (Endoxan) and mycophenolate mofetil (CellCept). According to released standards,[16] all of the SSc patients were categorized as having early dcSSc and the disease duration was less than five years. Individuals with a history of any autoimmune diseases and cancer were also excluded from the unaffected control group. All participants were informed regarding the potential outcomes and provided written informed

consent. This study was approved by the medical ethics committee of the Tehran University of Medical Sciences.

Isolation and Primary Culture of Fibroblast Cells

Fibroblasts were isolated from dermis tissue with the non-enzymatic method. Biopsy specimens were minced into small fragments and these fragments were implanted into 6-well plates with pre-treated gelatin 0.1% and then were added 800 µL of Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 20% fetal bovine serum (FBS; Biosera, France) and 1% penicillin/streptomycin (Sigma-Aldrich, USA). After three to four weeks, fibroblast cells with 80 to 90% confluency were also sub-cultured in a T25 flask (SPL, Korea) in the DMEM containing 10% FBS and 1% penicillin/streptomycin and fourth to sixth passage fibroblasts were used for further experiments.

Treatment Condition by IFNγ and Dexamethasone in the Fibroblast Cells

Dermal fibroblast cells with 80 to 90% confluency were harvested from a T25 flask (SPL, Korea) and then were seeded on a 12-well plate at 10^5 cells per well, in 2ml DMEM medium containing 10% FBS and 1% penicillin/streptomycin, in a CO_2 incubator (37°C, 5% CO_2 and under saturating humidity) for 48 hours; then fibroblast cells were treated with 100 IU of IFNγ (285-IF; R&D, USA) or 1 µM of dexamethasone (DEX, D4902; Sigma-Aldrich, USA). The combined treatments were as follows: 100 IU of IFNγ plus 1µM of DEX. The exposure time for all treatment groups was 48 hours.

RNA Extraction, cDNA Synthesis, and SYBR Green Real-time PCR

The total RNA of fibroblast cells was isolated using an RNA Kit (Roche, Germany) based on the manufacturer's protocol; 1- The fibroblast cells are lysed by a lysis solution and RNases are inactivated. 2- The fibroblast nucleic acids are bound to the glass fibers pre-packed in the filter tube. 3- Residual contaminating DNA is digested by DNase I. 4- Bound nucleic acids are washed with a wash solution to get rid of RT-PCR inhibitory contaminants. Further washing of bound nucleic acids purifies them from salts, proteins, and other cellular contaminants. Finally, RNA is eluted from the filter using the elution solution. The quantity and purity of RNA were verified by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Subsequently, 250 ng of RNA was subjected to reverse transcription to make complementary DNA (ROJE Technologies, Iran) founded on the manufacturer's instruction. To determine mRNA levels, 150 ng of cDNA was employed as a template for an RT-qPCR reaction with the RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) by the Applied Biosystems StepOnePlus Real-Time PCR System (Foster City, CA, USA). RT-qPCR was designed using the following PCR program: stage one: pre-denaturation: 95°C for 10 min; stage two: denaturation: 95°C for 15 sec, annealing: 60°C for 1 min (40×). The relative expression of genes was presented by the $2^{-\Delta\Delta CT}$. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was employed as a reference gene to standardize the mRNA levels. All of the experiments were carried out in triplicate.

Total Protein Isolation and Western Blot

Western blot analysis was performed to assess the IRF1, ERKs, pERKs, and smooth muscle alpha-actin (α-SMA) protein levels. After 48 hours of treatment, the fibroblast cells were harvested with trypsin (Sigma-Aldrich, USA) and washed with PBS buffer. The radioimmunoprecipitation assay (RIPA) lysis buffer plus protease inhibitor (Roche, Germany) was employed to extract total proteins, and the total protein of cell lysates was measured by the Lowry method. Total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Sigma-Aldrich, USA) and then transferred to a polyvinylidene difluoride (PVDF; Sigma-Aldrich, USA) membrane. The free spaces were blocked with blocking buffer (5% skim milk in 1X Tris-buffered saline with Tween (TBST; Sigma-Aldrich, USA)) for one hour at room temperature and then incubated with the primary antibodies (anti-phosphorylated-ERK1/2; GTX78988, anti-ERK1/2; GTX50560, anti-α-SMA; GTX100034, anti-GAPDH; GTX100118 [GeneTex; USA for all antibodies], and anti-IRF1; orb373694 [Biorbyt; USA]) at 4˚C for overnight (1:1000 for all antibodies). After three washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (BA1054-2 [Boster; USA], 1:3000) for two hours at room temperature. Eventually, the blots were visualized with an enhanced electrochemiluminescence (ECL) detection reagent. GAPDH protein was employed as an internal control to standardize the protein levels.

Statistical Analysis

Continuous variables were presented as mean±standard error of the mean (SEM) and categorical variables were presented as N (%). Statistical analysis was performed by SPSS version 25.0 and GraphPad Prism version 9.4 (Insightful Science; USA). We used ImageJ version 1.38e analysis to quantify the density and size of the blots. The normality test was checked using the Kolmogorov-Smirnov test for the continuous variables. The independent t-test or Mann-Whitney U analysis was used to compare the measurements between baseline groups. Spearman's rank correlation coefficient was performed to indicate the correlation between genes. In addition, the Friedman analysis was performed for comparison among multiple paired groups, and analysis between two groups was applied using Wilcoxon signed-rank correction. So, *P* values for multiple comparisons were adjusted by multiplying each *P* value by the number six. The *P* values less than 0.05 were considered statistically significant.

RESULTS

Study Subjects' Characteristics

The clinical and demographic features of all participants are shown in Table 1. The mean age of dcSSc patients and unaffected controls were 44.3±5.21 and 37.1 ±2.78 years, respectively. A total of 10 dcSSc patients were enrolled in this investigation, comprising 2 men and 8 women. Also, 10 of the unaffected controls were 3 men and 7 women. However, in this study, unaffected controls were matched to the dcSSc patients by gender and age (p=0.5 and p=0.24 respectively). In these dcSSc patients, the most common medication prescriptions were atorvastatin, prednisolone, alendronate, azathioprine, bosentan, and methotrexate. There was no significant difference in gene expressions based on their medications.

Gene Expression Pattern in Diffuse Cutaneous SSc Patients Compared to Unaffected Controls

The mRNA expression levels of the selected genes were analyzed in dcSSc patients and unaffected controls. As shown in Figure 1A, mRNA levels of *IL6*, *TGFB1*, *COL1A2*, and *C/EBPB* were significantly higher in the unaffected control group. In contrast, mRNA levels of *IRF1*, *COL1A1*, and fibronectin (*FN1)* had no significant changes between these two groups.

The gene expression level of *COL1A2* in dcSSc patients was correlated positively with *IRF1* expression level (p=0.008). In addition, there was a positive correlation but no significance (p=0.06) between *IL6* and *IRF1* mRNA levels (Figure 1B, C).

IFNγ Stimulates and Dexamethasone Inhibits IRF1 Gene and Protein Expression

In this study IFNγ, as expected, significantly enhanced the expression of the *IRF1* gene (Figure 2A and B). SSc fibroblast cells treatment with IFNγ led to the increased expression of IRF1 up to 27 times (Fold change [FC]=27.29, Figure 2C). Also, DEX could inhibit *IRF1* gene expression compared to the IFNγ, combination, and untreated groups.

Western blot analysis of IRF1 protein has shown similar effects of IFNγ and DEX on *IRF1* gene expression (Figure 2D and E). These data indicate that the DEX could inhibit IFNγ-induced IRF1 mRNA and protein expression.

IFNγ Stimulates and Dexamethasone Inhibits Inflammatory Genes

Many studies support the role of IL-6 in the initiation of skin sclerosis through the direct activation of skin fibroblasts. On the other hand, the C/EBP-β could regulate IL-6 production in human epithelial and fibroblast cells.[14,17] As displayed in Figure 3, the gene level of *IL6* was significantly greater in dcSSc fibroblast cells treated with IFNγ which DEX treatment led to a significant decrease of *IL6* expression in SSc fibroblasts. Besides, IFNγ or DEX treatment could not change the expression of *IL6* gene significantly in the unaffected control fibroblasts. In contrast, there were no significant differences in the *C/EBPB* gene level upon IFNγ or DEX in both dcSSc and unaffected control fibroblasts (Figure 3A, B, C, and D).

IFNγ Induces and Dexamethasone Inhibits Myofibroblast Differentiation

To clarify whether IFNγ interferes with the differentiation of myofibroblasts, the α-SMA protein expression was evaluated by western blot. In the fibroblast of the dcSSc patient, IFNγ could increase protein levels of α-SMA compared to the untreated group which is inhibited by DEX treatment. However, we observed similar results in the unaffected control (Figure 4A and B).

Table 1. Demographic and clinical features of the study

Features	SSc patients, N=10	Unaffected control, N=10
Age, (Mean ±SEM, years)	44.3 ± 5.21	37.1 ± 2.78
Gender (Women/Men)	8 / 2	7 / 3
SSc disease duration, (Mean ±SEM, months)	46.67 ± 7.25	-
Diffuse cutaneous SSc	10 (10)	-
Total Rodnan score, (Mean ±SEM)	15.86 ± 1.87	-
Dermis phase, (Active/Atrophy)	8 / 2	-
Prednisolone (Taken/ Not taken)	8 / 2	-
Azathioprine (Taken/ Not taken)	2 / 8	-
Bosentan (Taken/ Not taken)	1 / 9	-
Methotrexate (Taken/ Not taken)	1 / 9	-

SSc: Systemic Sclerosis, SEM: Standard error of the mean

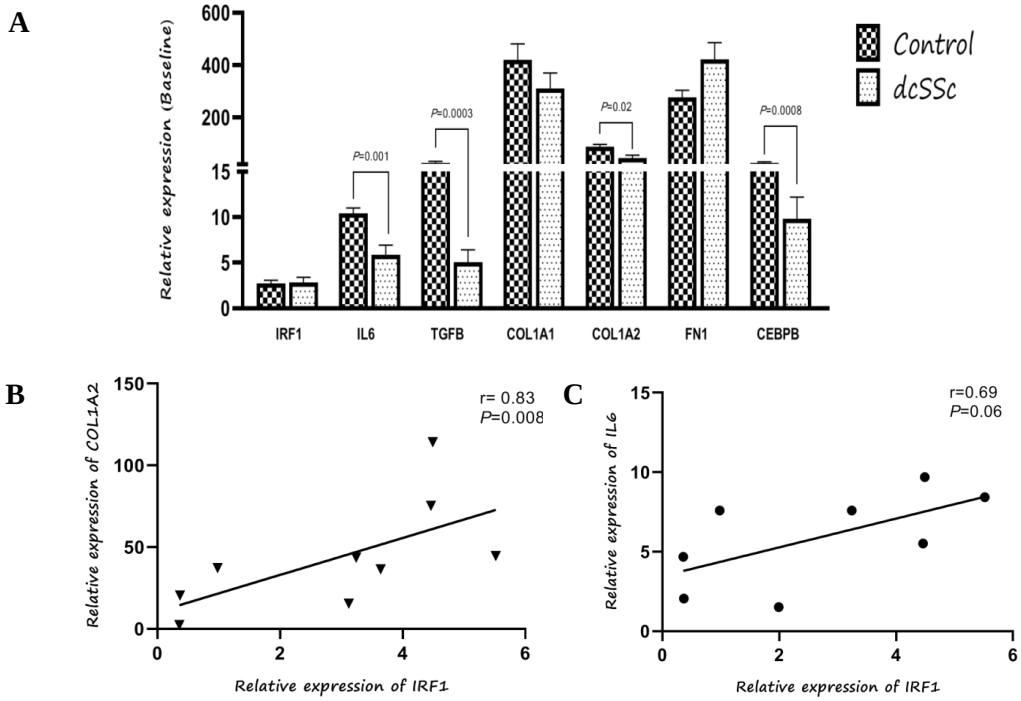


Figure 1. A. Baseline expression of the *IRF1*, *IL6*, *TGFB1*, *COL1A1*, *COL1A2*, *FN1*, and *C/EBPB* in diffuse cutaneous systemic sclerosis (early dcSSc) and unaffected control fibroblast cells. Baseline expression of *IL6*, *TGFB1*, *COL1A2*, and *C/EBPB* indicated considerable differences between the two groups. (B and C): Correlation between expression of *IRF1* and *IL6* (n=8)/*COL1A2* (n=9) levels in early dcSSc, respectively. Correlations of *IRF1* levels and *COL1A2* at baseline were significant (*p*=0.008). **p*<0.05, ***p*<0.01, ****p*<0.001, IRF1: Interferon regulatory factor 1, IL6: Interleukin 6, TGFB1: Transforming growth factor beta 1, FN1: Fibronectin 1, C/EBPB: CCAAT/enhancer binding protein beta, COL1A2: Collagen type I alpha 2 chain, COL1A1: Collagen type I alpha 1 chain.

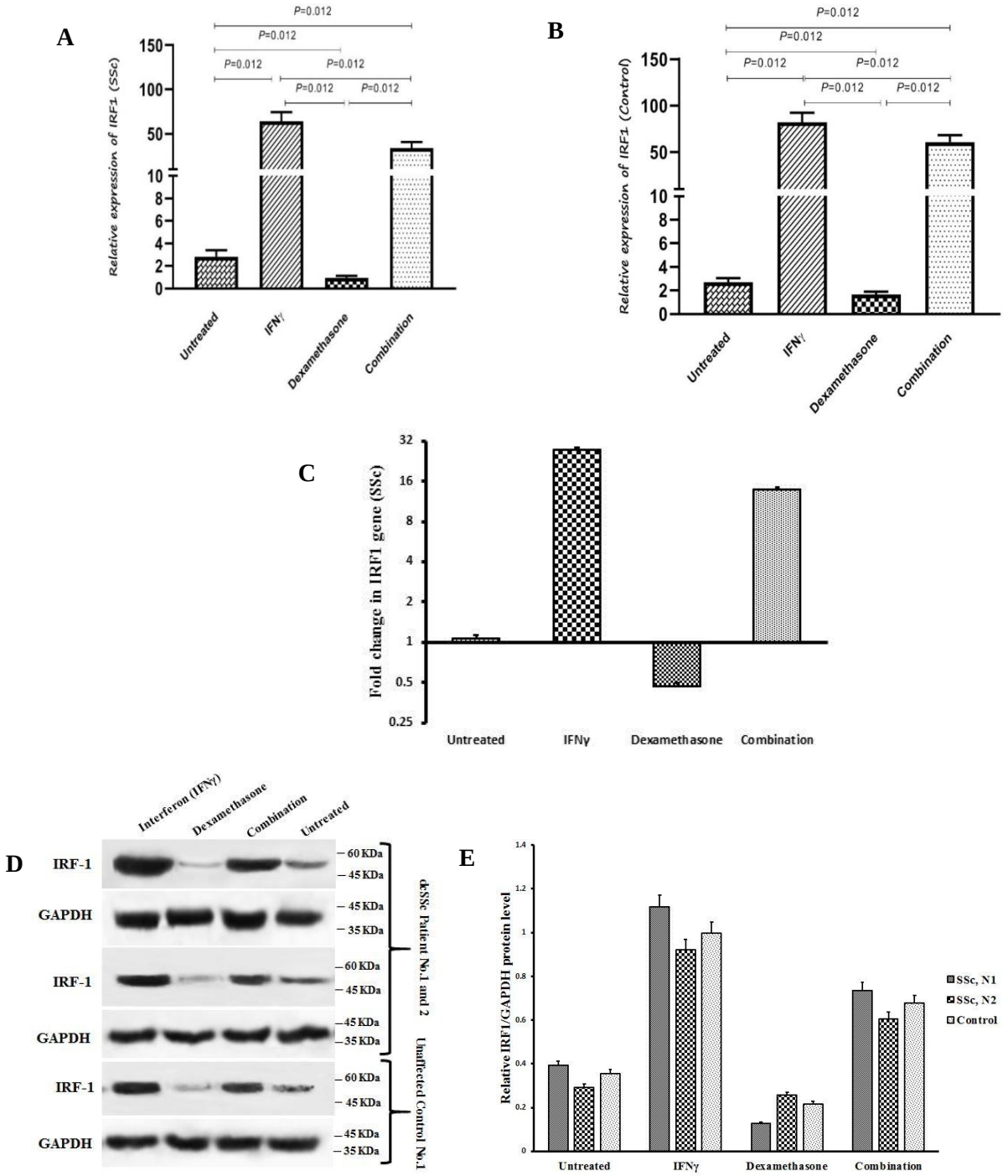


Figure 2. Effect of IFNγ (100 IU) alone and in combination with DEX (1 µM) on the expression of the *IRF1* gene in the fibroblast of early dcSSc patients (A) and unaffected control (B) in comparison with untreated groups. Fold change in *IRF1* gene expression of dcSSc patients (C). The protein levels of IRF1 in fibroblast cells of early dcSSc and unaffected control (D and E), IFNγ could stimulate and DEX could inhibit the protein levels of the IRF1 in the dermal fibroblast cells when compared to untreated groups. *p<0.05, **p<0.01, ***p<0.001, IRF1: Interferon regulatory factor 1, IFNγ: Interferon-gamma, DEX: Dexamethasone, dcSSc: Diffuse cutaneous systemic sclerosis.

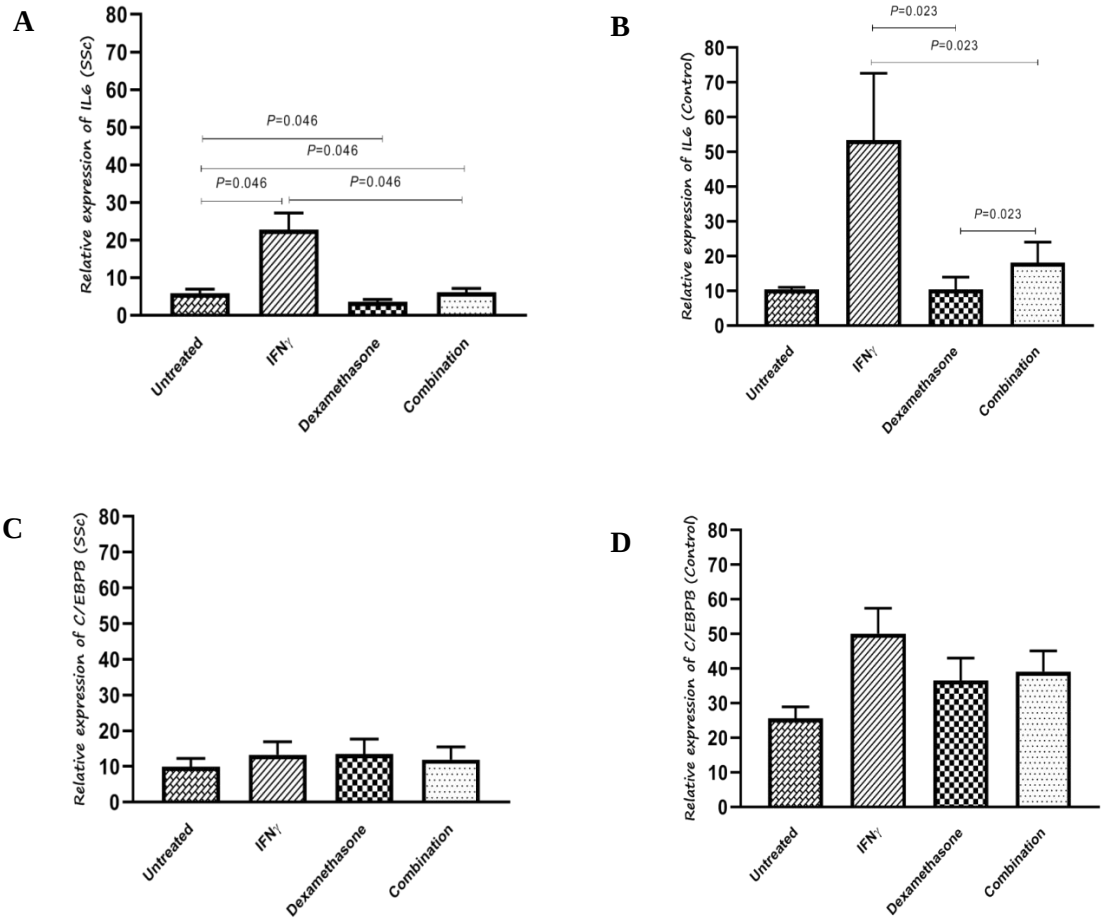


Figure 3. Effect of IFNγ (100 IU) alone and in combination with DEX (1 µM) on the expression of the inflammation related-genes (*IL6* and *C/EBPB*) in the fibroblast of early dcSSc patients (A and C), and unaffected control (B and D) in comparison with untreated groups. **p*<0.05, ***p*<0.01, ****p*<0.001, IL6: Interleukin 6, C/EBPB: CCAAT/enhancer binding protein beta, IFNγ: Interferon gamma, DEX: Dexamethasone, dcSSc: Diffuse cutaneous systemic sclerosis.

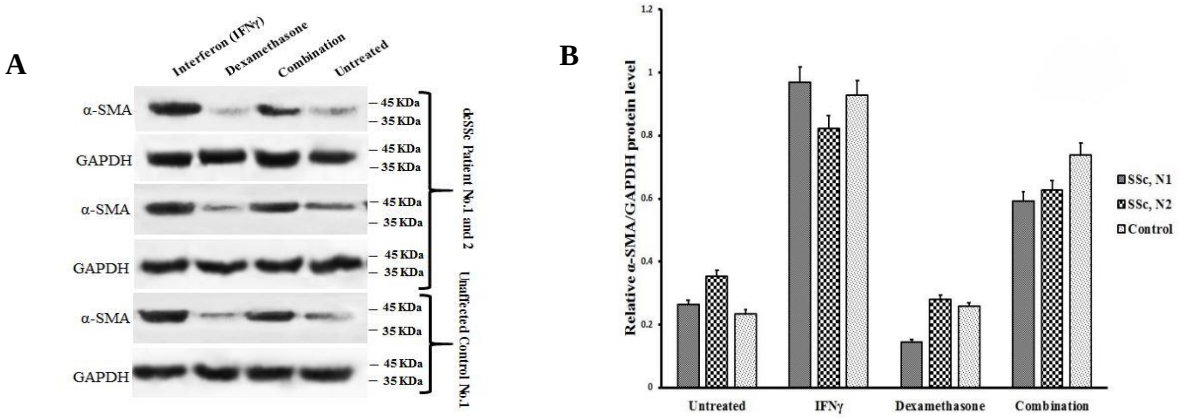


Figure 4. Effect of IFNγ (100 IU) alone and in combination with DEX (1 µM) on protein levels of α-SMA in fibroblast cells of early dcSSc and unaffected controls. (A and B) IFNγ could stimulate and DEX could inhibit the protein levels of the α-SMA in the dermal fibroblast cells when compared to untreated groups. α-SMA: Alpha-smooth muscle actin, IFNγ: Interferon-gamma, DEX: Dexamethasone, dcSSc: Diffuse cutaneous systemic sclerosis.

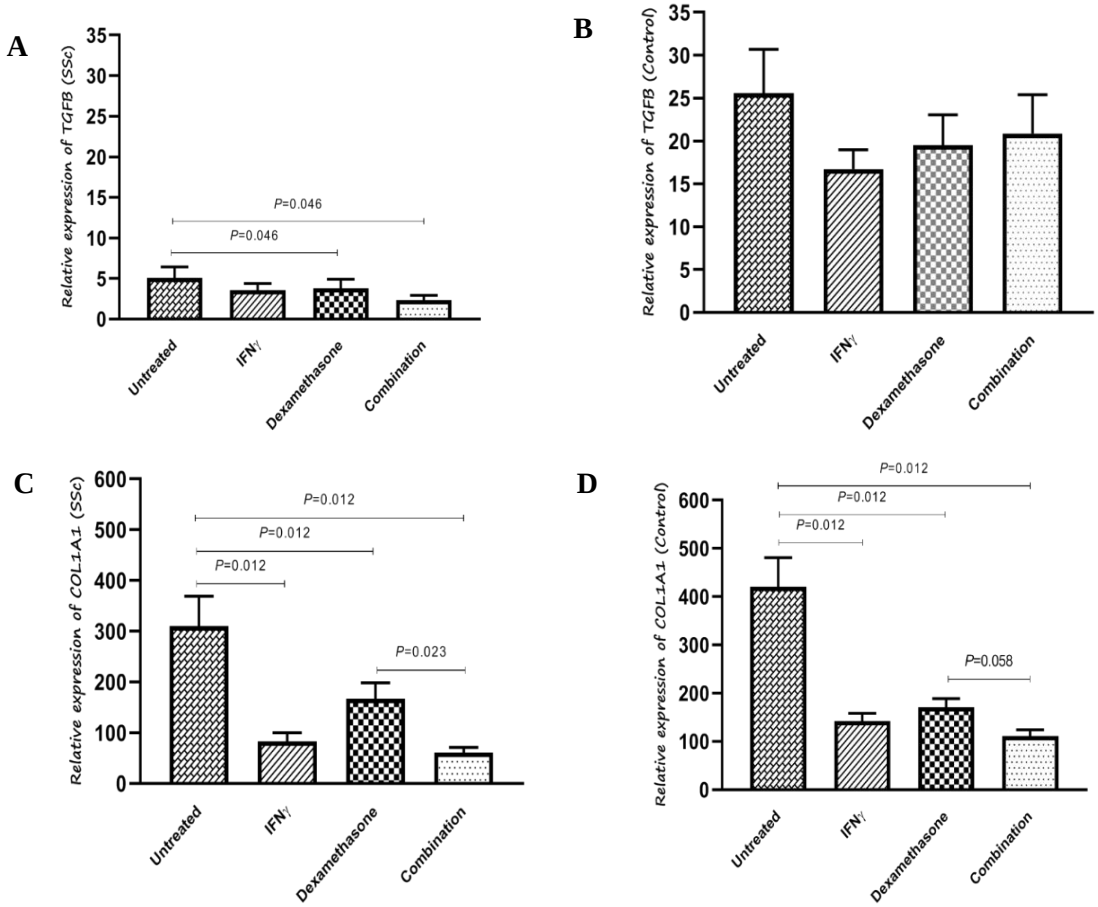
IFNγ and Dexamethasone More Effectively Inhibit Fibrotic Genes

TGF-β1 has an important role in SSc pathogenesis through the induction of fibrotic genes. IFNγ treatment could not change *TGFB1* expression in both groups (dcSSc and unaffected controls), while the expression of *TGFB1* is significantly decreased upon DEX treatment only in dcSSc fibroblasts. Furthermore, IFNγ significantly attenuated the mRNA expression level of fibrosis-related genes (*COL1A1* and *FN1*) compared to the untreated group but had no significant effect on the *COL1A2* expression. In addition, DEX treatment significantly decreased the mRNA expression level of *COL1A1* and *FN1* compared to the untreated group. On the other hand, DEX had no significant impact on the *COL1A2* expression (Figure 5 A, C, E, and G). IFNγ and DEX treatment of unaffected control fibroblasts can just down-regulate the *COL1A1* expression and do not affect *FN1* and *COL1A2* genes (Figure 5 B, D, F, and H).

IFNγ Induces and Dexamethasone Inhibits ERK1/2 Phosphorylation

Some studies have described a central role for the ERK1/2 pathway in TGFβ-stimulated SSc fibroblast activation and induced the collagen isoforms expression, as well as of contractile proteins involved in improved myofibroblast contraction and migration.[1] So, we investigated the ERK signaling pathway upon IFNγ treatment.

In the fibroblast of the dcSSc patients and the unaffected control, the protein levels of pERK1/2 were higher in IFNγ alone and combination groups, and DEX could decrease the phosphorylation of ERK1/2 compared to the untreated group (Figure 5 I and J).



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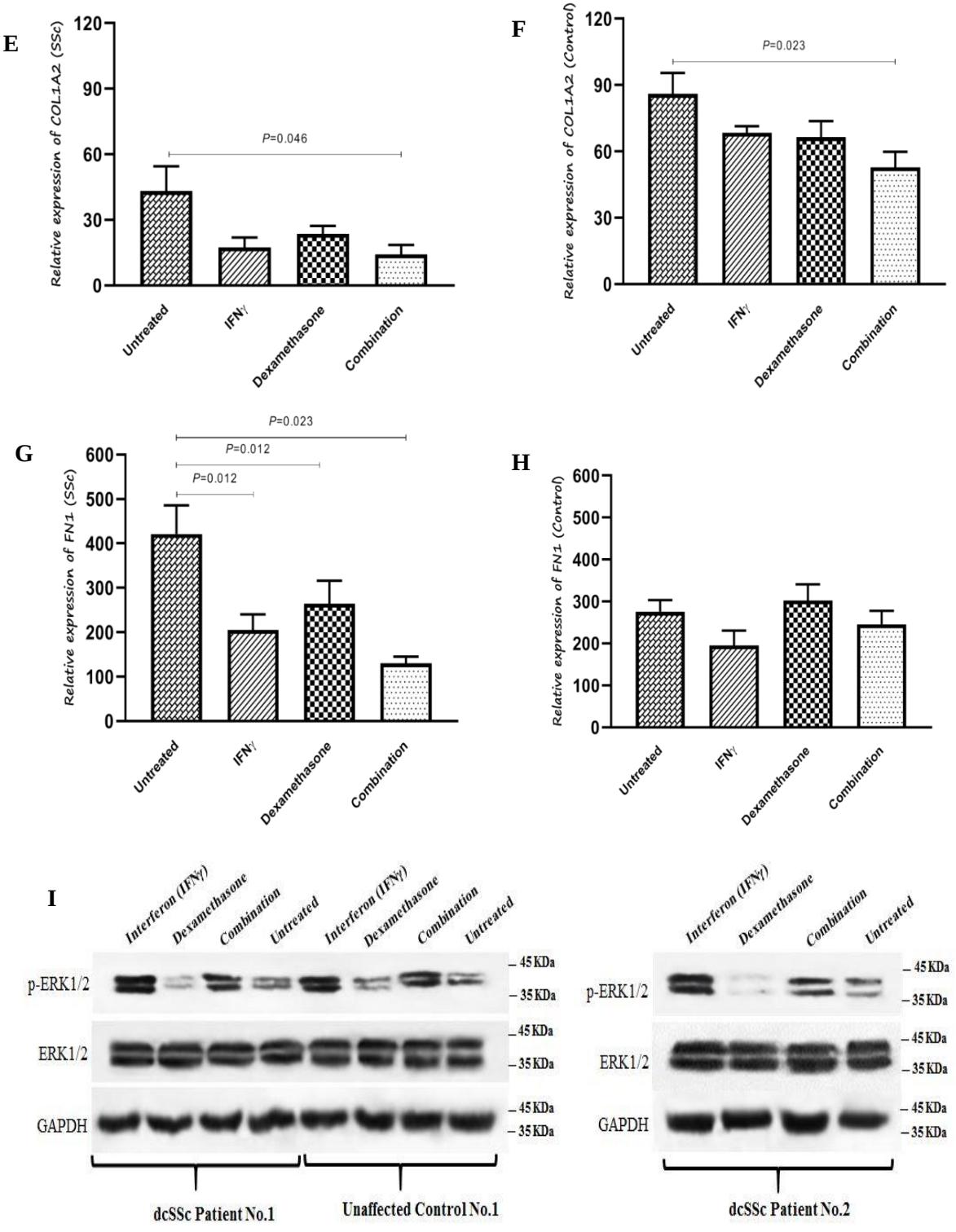
E
Relative expression of COL1A2 (SSc)
120
90
60
30
0
P=0.046
Untreated
IFNγ
Dexamethasone
Combination
F
Relative expression of COL1A2 (Control)
120
90
60
30
0
P=0.023
Untreated
IFNγ
Dexamethasone
Combination
G
Relative expression of FN1 (SSc)
600
500
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100
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P=0.023
P=0.012
P=0.012
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IFNγ
Dexamethasone
Combination
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Relative expression of FN1 (Control)
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Combination
I
Interferon (IFNγ)
Dexamethasone
Combination
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ERK1/2
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Unaffected Control No.1
dcSSc Patient No.2

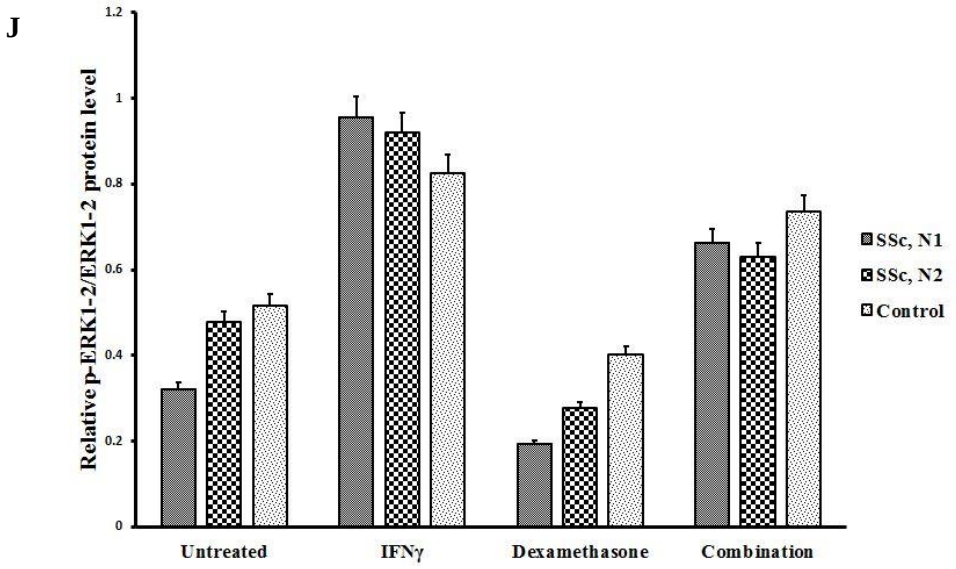

Figure 5. Effect of IFNγ (100 IU) alone and in combination with DEX (1 µM) on the expression of the fibrosis related- genes (*TGFB1*, *COL1A1*, *COL1A2* and *FN1*) in the fibroblast of early dcSSc patients (A, C, E, and G), and unaffected control (B, D, F, and H) in comparison with untreated groups. The effect of IFNγ (100 IU) alone and in combination with DEX (1 µM) on protein levels of ERK1/2 and pERK1/2 in fibroblast cells of early dcSSc and unaffected control (I and J), IFNγ could stimulate and DEX could inhibit the protein levels of the pERK1-2/ERK1-2 in the dermal fibroblast cells when compared to untreated groups. *p<0.05, **p<0.01, ***p<0.001, TGFB1: Transforming growth factor beta 1, FN1: Fibronectin 1, COL1A2: Collagen type I alpha 2 chain, COL1A1: Collagen type I alpha 1 chain, IFNγ: Interferon-gamma, DEX: Dexamethasone, dcSSc: Diffuse cutaneous systemic sclerosis, ERK1/2: Extracellular signal-regulated kinases 1 and 2, pERK1: phosphate Extracellular signal-regulated kinases 1 and 2.

DISCUSSION

SSc is an autoimmune disease highlighted by immune dysregulation and subsequence vasculopathy, inflammation, and tissue remodeling that usually leads to extreme ECM deposition and fibrosis in the dermis and numerous internal organs.[1] Corticosteroids are one of the most widely prescribed medications in SSc. Some reports have proposed the advantage of corticosteroids such as DEX in the therapy of SSc, including decreased inflammatory and fibrotic processes which lead to the recovery of cutaneous thickening.[18-20] Approximately 40 to 75% of SSc patients take corticosteroids, and they are predominantly taken in patients with diffuse form.[20,21] In our investigation, nearly 80% of SSc patients had taken corticosteroids (prednisolone). So, it seems that the lower mRNA levels of the *IL6*, *TGFB1*, *COL1A2*, and *C/EBPB* in SSc patients than the unaffected controls could be caused by corticosteroid treatment of patients or other medications.

Several studies revealed that IFNγ expression could up-regulate in the dermis of SSc patients.[6] In addition, IRF1 is the initial recognized interferon regulatory factor. Collecting documents has indicated that IRF1 functions as an essential transcriptional factor in multiple biological functions, such as inflammation and fibrosis. Previous studies noted that IRF1 was relevant to SSc and fibrotic disorders. IRF1 has been stated to be slightly expressed in healthy tissues, although its expression is increased in $CD4^+$ T and fibroblast cells of SSc patients and could induce inflammation and irregular immune responses.[9,10,12] IRF1 is involved in fibrosis through the up-regulation of α-SMA and FN1 expression.[10] Besides, IRF1 up-regulation leads to the decreased expression of Klotho protein which results in the enhancement of fibrosis, and the C/EBP-β transcription factor is a main mediator for IRF1 modulating Klotho expression. Up-regulation of C/EBP-β expression notably diminished fibronectin (FN1) expression and contributed to ameliorating fibrosis. C/EBP-β operates as transcriptional activators of

inflammation-related genes such as IL-6.[10,22] Our results revealed that IRF1 expression is induced by IFNγ and suppressed by DEX. Therefore, IRF1 stimulus by IFNγ might describe a positive feedback mechanism under inflammatory situations. Conversely, IRF1 inhibition by DEX might describe an inhibited feedback mechanism under anti-inflammatory and anti-fibrosis situations. However, many studies have described the IFNγ or DEX effect on IRF1 in challenged fibroblast cells (and non-fibroblast cells) similarly.[23-25]

This study showed that IFNγ and DEX did not affect the *C/EBPB* expression in early dcSSc and unaffected control fibroblasts, despite IRF1 overexpression upon IFNγ treatment. Contradictorily, Li et al., have reported that IRF1 overexpression leads to the reduction of Klotho levels through down-regulation of *C/EBPB* which results in the enhancement of fibrosis.[10] This discrepancy may be related to the differences between cells and diseases.

IL-6 has been implicated in the initiation and dissemination of chronic inflammation through the induction of its production by fibroblast cells in positive autocrine feedback. Therefore, it is not surprising that IL-6 has a function in propagating chronic inflammation, including that observed in SSc.[26] On the other hand, IL-6 can act as either a pro-fibrogenic or anti-fibrogenic cytokine, depending on the different specific receptors involved[26,27] The IL-6 and IL-6 soluble receptor (sIL-6R) complex could decrease both the Sp1/Sp3 ratio and DNA-binding activities, thus inhibiting COL2A1 transcription.[27] In the current investigation, we indicated that IFNγ could up-regulate and DEX could down-regulated the *IL6* gene in dcSSc fibroblasts compared to the untreated group. In addition, our result revealed that IFNγ or DEX therapy could not affect the *IL6* gene expression in the unaffected control fibroblasts.

IL-6 could induce the mRNA and protein expression of IRF1 via palindromic IFN-response element (pIRE), which was observed to respond to IL-6 with higher efficiency when compared with IFNγ. Also, IL-6 not only induces IRF1 expression but also transactivates its pathway.[28,29] On the other hand, both IFNγ and IL-6 were needed for enhanced IRF1 expression which controls IFN-stimulated genes. Thus IRF1 may be at the nexus of the interplay between IFNγ and IL-6 in aggravating inflammatory response, regulation of IFN-stimulated genes, and autoimmune diseases.[30] The absence of IL-6 or IRF1 (knockout or knockdown) affected the expression of genes controlled by either IFNγ or IFN I.[31] According to IRF1 is a target for IL-6 signaling, so, in our study, overexpression of IRF1 may be directly related to IFNγ treatment or may be related to an increase IL-6 upon IFNγ treatment.

In the dermis of SSc patients, the existence of myofibroblasts α-SMA$^+$ relates to the amount of collagen type I and dermis parameters associated with fibrosis including tightness and hardness, and does so more greatly than inflammation, supporting the myofibroblasts α-SMA$^+$ role in the pathogenesis of these clinical symptoms.[32] Our results verified that IFNγ could induce fibroblast cell differentiation into myofibroblast α-SMA$^+$ cells, which is inhibited by DEX. A study conducted by Chrobak et al. indicated that IFNγ increases α-SMA$^+$ mRNA and protein levels in endothelial cells which results in endothelial-to-mesenchymal transition (EndoMT).[33] Besides, it has been revealed that IFNγ can induce epithelial-to-mesenchymal transition (EMT) through up-regulation of α-SMA.[34] However, some studies demonstrated that IFNγ could inhibit fibroblast-myofibroblast differentiation in a Smad proteins-dependent manner, and DEX has a synergistic effect with IFNγ.[35,36] This inconsistency may be related to the different cell types. Furthermore, it has been reported that IL-6 induces myofibroblast differentiation and expression of α-SMA[13] and inhibition of IL-6 signaling is involved in decreased myofibroblast differentiation in skin fibroblasts in the scleroderma mice model.[14] With regards to our results, myofibroblast differentiation in SSc fibroblasts which is induced by IFNγ may be enhanced through increased expression of IL-6, which induces myofibroblast differentiation by IRF1.[37] The *IL6* gene of humans includes various cis-regulatory elements, such as nuclear factor kappa B (NF-κB), C/EBPβ, and IRF1, proposing that a wide variety of stimuli can induce IL-6. The rise in IL-6 production affected the expansion, migration, and differentiation of myofibroblast and enhanced the α-SMA expression via the MAPK-ERK1/2 activity.[38]

In addition, it has been documented that DEX reduces human α-SMA expression in part by reduction of mRNA translation and increment of protein degradation[39] which is consistent with our results.

It has been reported that DEX[18] and IFNs[40,41] can decrease fibrotic genes (collagen and FN1), so, DEX plays a synergistic effect with IFNγ. Our result showed that IFNγ and DEX cannot change the expression of

genes involved in fibrosis including *COL1A2*, *FN1*, and *TGFB1* in unaffected controls. IFNγ and DEX treatment of unaffected control fibroblasts decreased only *COL1A1* expression. In dcSSc fibroblasts, IFNγ could decrease *COL1A1* and *FN1* expression, while DEX repressed *TGFB1, COL1A1,* and *FN1* expression. So, IFNγ and DEX have more effects on genes that contribute to fibrosis in dcSSc fibroblasts. The mechanism of down-regulation of fibrotic genes upon IFNγ is not clear but with regards to inductive effects of IFNγ on IL-6 and due to IL-6 effects on collagen expression which can decrease collagen (interaction between IL-6 with sIL-6R),[27] it seems that IFNγ may suppress fibrotic genes through IL-6 overexpression. On the other hand, TGF-β and IL-6 signaling can enhance the expression of collagen type I and this effect is dependent on IL-11.[1] So, with regards to our results that TGF-β could not change upon IFNγ treatment, it seems that increased IL-6 in combination with decreased TGF-β (compared to our normal fibroblasts) may decrease the collagen expression.

ERK1/2 has been noted to be involved mainly in the proliferation of fibroblasts.[1] Phosphorylated ERK1/2 also facilitated the differentiation of fibroblast cells into myofibroblasts via improved α-SMA expression.[42] We have reported that the phosphorylations of ERK1/2 were increased upon IFNγ treatment and DEX could decrease the phosphorylation of ERK1/2. Zhou et al. reported that IL-6 is overexpressed in human lung fibroblasts by phosphorylation of the mitogen-activated protein kinases (MAPKs), ERK1/2, and phosphoinositide-3-kinase-protein kinase B (PI3K-Akt) activity.[43] So, it seems that IFNγ acts through ERKs activation to increase IL-6 expression.

Furthermore, DEX can inhibit ERK1/2 activation and reverse TGF-β effects on cell differentiation into myofibroblast α-SMA^{+} and migration.[19] Our result is consistent with this study on the inhibitory effect of DEX on ERK1/2 phosphorylation and differentiation into myofibroblast.

Overall, inflammatory reactions could be mediated by many inflammation-related cytokine genes (such as IFNγ and IL-6) and dysregulation in each signaling pathway by exogenous stress signals (such as infection) could disturb the homeostasis control system in autoimmune diseases. On the other hand, the fibrosis process is related to chronic inflammation, homeostasis, and TGF-β1 signaling. So, with regards to our report, combination therapy with DEX and IFNγ as an effective treatment could inhibit inflammation- and fibrosis-related genes (such as *IFNG*, *IL6*, and *TGFB1*) in SSc patients in comparison with unaffected control.

Given the inductive effect of IFNγ on IL-6 expression and myofibroblast differentiation and its inhibitory effect on collagen and fibronectin expression, IFNγ may play a dual role in SSc. Besides, based on IL-6 roles in myofibroblast differentiation and suppression of collagen expression, it seems that IFNγ acts through IL-6 signaling. Furthermore, IFNγ as well as IL-6 increase IRF1 expression which can induce myofibroblast differentiation, so, it may IFNγ and IL-6 execute myofibroblast differentiation through IRF1 up-regulation.

STATEMENT OF ETHICS

All procedures performed in studies involving human participants were by the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study protocol was approved by the ethics committee of Tehran University of Medical Sciences (Ethical code: IR.TUMS.MEDICINE.REC.1399.1099), and written consent was taken from all subjects.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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