
Spleen tyrosine kinase (Syk) inhibitor fostamatinib limits tissue damage and fibrosis in a bleomycin-induced scleroderma mouse model

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ABSTRACT

Objective. *The pathogenesis of fibrosis in scleroderma (SSc) is unknown. TGF- β and platelet-derived growth factor are important in the development of fibrosis and tyrosine kinases are involved in these pathways. The possible antifibrotic effects of various kinase inhibitors in SSc have been studied before. Spleen tyrosine kinase (Syk) is a protein tyrosine kinase which activates intracellular signal transduction pathways; and has been claimed to be involved in the pathogenesis of systemic autoimmune diseases. Inhibition of Syk suppresses IgE- and IgG-associated FcR signal activation in various cell types; and suppresses experimental arthritis and skin and kidney disease in lupus-prone mice. We investigated the ability of a small drug, the Syk inhibitor, fostamatinib, to protect mice from bleomycin-induced SSc.*

Methods. *Four study groups of BALB/c mice were included into this study: control, bleomycin (administered subcutaneously to BALB/c mice for 21 days), bleomycin and fostamatinib (mice fed with chow containing a Syk inhibitor for 21 days), and fostamatinib alone groups. Skin and lung tissue specimens were obtained and evaluated histologically.*

Results. *Treatment with fostamatinib significantly reduced skin thickness and fibrosis. Mice treated with fostamatinib also displayed less fibrosis and inflammation in the lung tissue. Following fostamatinib treatment, Syk, phospho-Syk, and TGF- β expression decreased in both skin and lung tissues.*

Conclusion. *The Syk inhibitor fostamatinib prevented bleomycin-induced fibrosis and inflammation in the skin and in the lung. The anti-fibrotic effect of fostamatinib is linked to reduced Syk phosphorylation and TGF- β expression. The Syk pathway appears as a potential molecular target for therapeutic intervention in SSc.*

Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterised by fibrosis in several organs like the skin, lungs, gastrointestinal system, kidneys and the heart (1). Microvascular changes like capillary dilatation together with perivascular inflammation occur in early stages of the disease (2) followed by fibrosis (3) and organ injury (4).

The exact mechanisms involved in the pathogenesis of fibrosis in SSc are unknown. Transforming growth factor (TGF)- β and platelet-derived growth factor (PDGF) have been claimed to activate fibroblasts (3, 5-7) and stimulate the secretion of extracellular matrix from fibroblasts. Inhibition of the TGF- β and PDGF pathways results in attenuation of fibrosis in experimental models (8-10). Imatinib, nilotinib and dasatinib are tyrosine kinase (TK) which target TGF- β and PDGF (11), and were shown to cause regression of fibrosis in animal models of SSc (12, 13). Importantly, imatinib proved to be useful in the treatment of patients with nephrogenic and diffuse cutaneous SSc and refractory graft-versus-host disease (14-16). In a recent, open-label, single-arm study the long-term safety and efficacy of imatinib was demonstrated in a substantial proportion of patients with diffuse SSc (17).

The non-receptor spleen tyrosine kinase (Syk) is responsible for signal transduction in various types of cells (18) and it is a key mediator of immune receptor signalling in mast cells, macrophages, B cells and neutrophils (19). Syk-mediated phosphorylation of several adaptor proteins and the formation of signalling complex at the cell surface membrane leads to activation of downstream pathways followed by an effector cell response (20). Syk phosphorylation has been invoked in the pathogenesis of different autoimmune

diseases (21). Given the importance of Syk in immune cell signalling, a number of small drug inhibitors have been developed in an effort to suppress the autoinflammatory response (22). The Syk inhibitor, R406, potently inhibits IgE- and IgG-mediated activation of Fc receptor signalling and reduces immune complex-mediated inflammation (23). The pro-drug of R406, R788, behaves similarly and has been considered in the treatment of allergic and autoimmune diseases, including arthritis and systemic lupus erythematosus (SLE) (24, 25). In a phase II/III clinical trial in patients with rheumatoid arthritis (RA), Syk proved its clinical promise with acceptable side effects and significant clinical improvement (26, 27). We designed this study to evaluate the effect of a Syk inhibitor in a bleomycin-induced mouse SSc model.

We demonstrate that inhibition of Syk activation in mice limits local and remote organ damage and fibrosis in SSc mouse model. Prevention of tissue injury and fibrosis may be associated with limited Syk phosphorylation and TGF- β expression. We propose that Syk inhibitors could be considered for the treatment of SSc.

Materials and methods

Mice

We used adult BALB/c mice (6–8 weeks old) for the study. Prior to the experiment, the mice were subjected to at least a 7-day acclimatisation. The mice used in the study were kept under specific pathogen-free conditions in the Animal Research Facility at Trakya University Medical Faculty.

Administration of bleomycin and Syk inhibitor to mice

In order to induce SSc, mice were injected bleomycin (1 mg per ml, Nippon Kayaku, Tokyo, Japan) subcutaneously (300 μ g in 0.9% NaCl) for 21 days. The Syk inhibitor, R788, was provided kindly by Astra Zeneca, UK. There were 4 study groups: control group, bleomycin group, bleomycin+Syk inhibitor (R788, fostamatinib) group, and Syk inhibitor group. Each study group included 5 mice. Mice in control and bleomycin groups were fed with regular mouse chow and water. The third group was fed with Syk inhibitor-containing chow (R788, fostamatinib, Astra Zeneca, 3 g/kg) for 21 days and bleomycin as stated. The fourth group was given only Syk inhibitor-containing chow for 21 days.

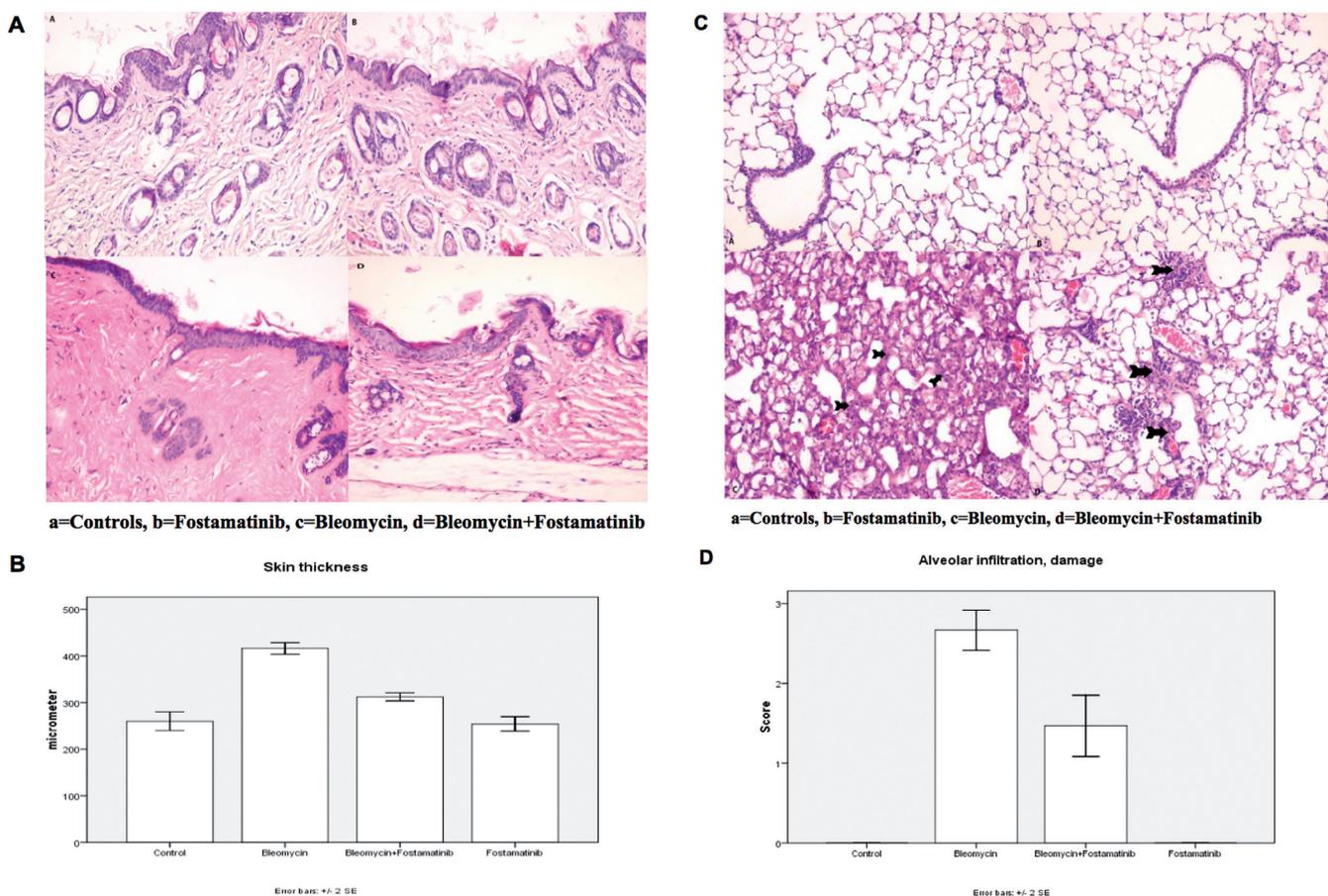


Fig. 1. Syk inhibition attenuates skin thickness and lung damage.

A. Representative H&E stained slides from the skin of mice exposed to bleomycin and treated with fostamatinib (3 g/kg for 21 days) were visualised and captured under a light microscopy. The ocular micrometer was used to measure the dermal thickness at a x400 magnification under the Olympus CX-31 microscope (Tokyo, Japan). **B.** Fostamatinib therapy significantly decreased dermal thickness. **C.** Representative H&E stained slides from the lung tissue of mice subjected to the same treatment as in Fig. 1. Photomicrographs represent x200 magnification. **D.** Lung injury scores from each group were determined based on the criteria outlined in the *Methods* section. Each bar is the mean \pm SEM with 5 mice per group. Data were compared using ANOVA followed by the Tukey's multiple comparison test.

Ketamine (10 mg/kg), xylazine (20 mg/kg), and acepromazine (3 mg/kg) were all given intraperitoneally (*i.p.*) to anaesthetise the mice. When necessary, ketamine (5 mg/kg) and xylazine (3 mg/kg) were given intramuscularly (*i.m.*) during the experiment. All procedures were performed on anaesthetised, spontaneously breathing animals; and the body temperature of mice was maintained at 37°C by means of a controlled heating pad. The study protocol was approved by the Institutional Animal Care and Use Committee of Trakya University. Mice were euthanised by carbon dioxide asphyxiation following the guidelines of Trakya University.

Back skin of mice was shaved and harvested. The lungs were removed after expansion of the bronchial tree with 200–300 μ L of 10% phosphate-buffered formalin, fixed in 10% phosphate-buffered overnight, and then intact extraction of the bronchial tree. Skin and lung tissues fixed in formalin were washed extensively in PBS; processed and embedded in paraffin for histological and immunohistochemical (IHC) analysis as described below.

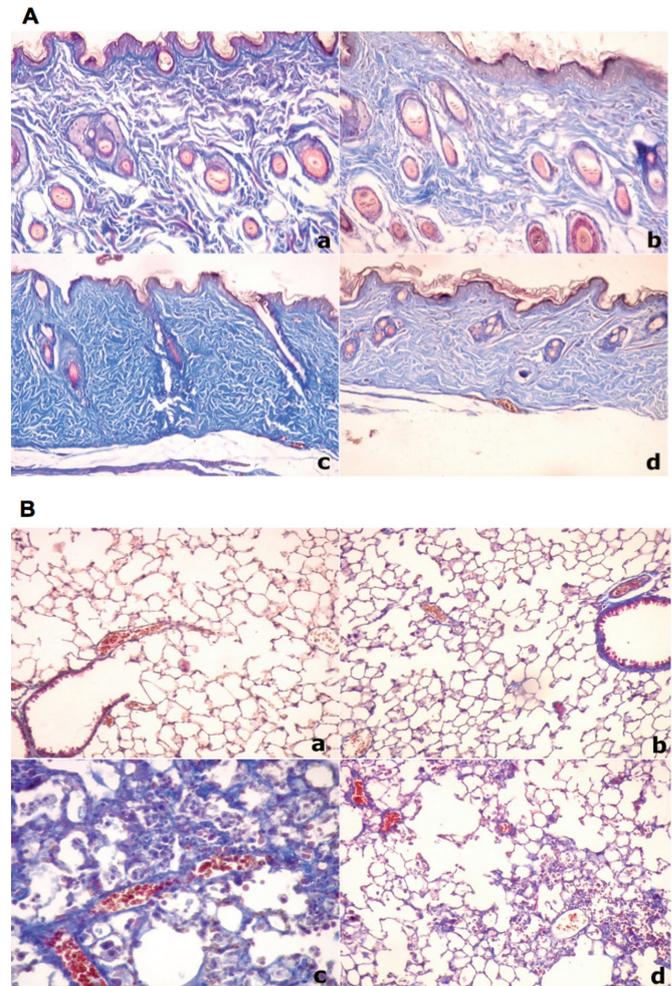
Histological examination

Immediately after removal, skin tissue samples were fixed in 10% neutral-buffered formalin, dehydrated in graded concentrations of ethanol, cleared in xylene and embedded in paraffin. Paraffin blocks were cut into 5 μ m-thick sections and stained with haematoxylin & eosin (H&E). Slides were examined by two independent histologists (T.K. and S.D.) under Olympus CX31 light microscope and photographed. The general histologic appearance of the tissue was examined by H&E.

The lung tissue samples were similarly fixed in 10% neutral-buffered formalin immediately after removal, dehydrated in graded concentrations of ethanol, cleared in xylene, and embedded in paraffin. The paraffin blocks were cut into 5 μ m-thick sections and stained with H&E. Olympus CX31 light microscope was used to examine and photograph the slides. Histopathological changes including intra-alveolar haemorrhage, thickening of alveolar wall/hyaline membrane formation, alveolar

Fig. 2. Syk inhibitor suppresses skin and lung tissue fibrosis in bleomycin-induced SSc mouse model.

Fibrosis score in skin (A) and lung (B) were assessed by Mallory's trichrome staining. Magnification is x400.



a=Controls, b=Fostamatinib, c=Bleomycin, d=Bleomycin+Fostamatinib

oedema, disruption and congestion, and leukocyte infiltration were documented in each lung tissue. A scale from 0 to 3 was used to score pathological changes which can be summarised as: 0=absence of pathology (<5% of maximum pathology), 1= mild changes (<10%), 2= moderate changes (15–20%), and 3= severe changes (20–25%). The leukocyte infiltration secondary to bleomycin usage was evaluated to determine the severity of inflammation. Each section was divided into 15 subsections and leukocyte infiltration in each subsection was examined at a x400 magnification by using the following scale, 0: no leukocytes in extravascular area; 1: <10 leukocytes; 2: 10–45 leukocytes; and 3: >45 leukocytes. For comparison, average numbers were used.

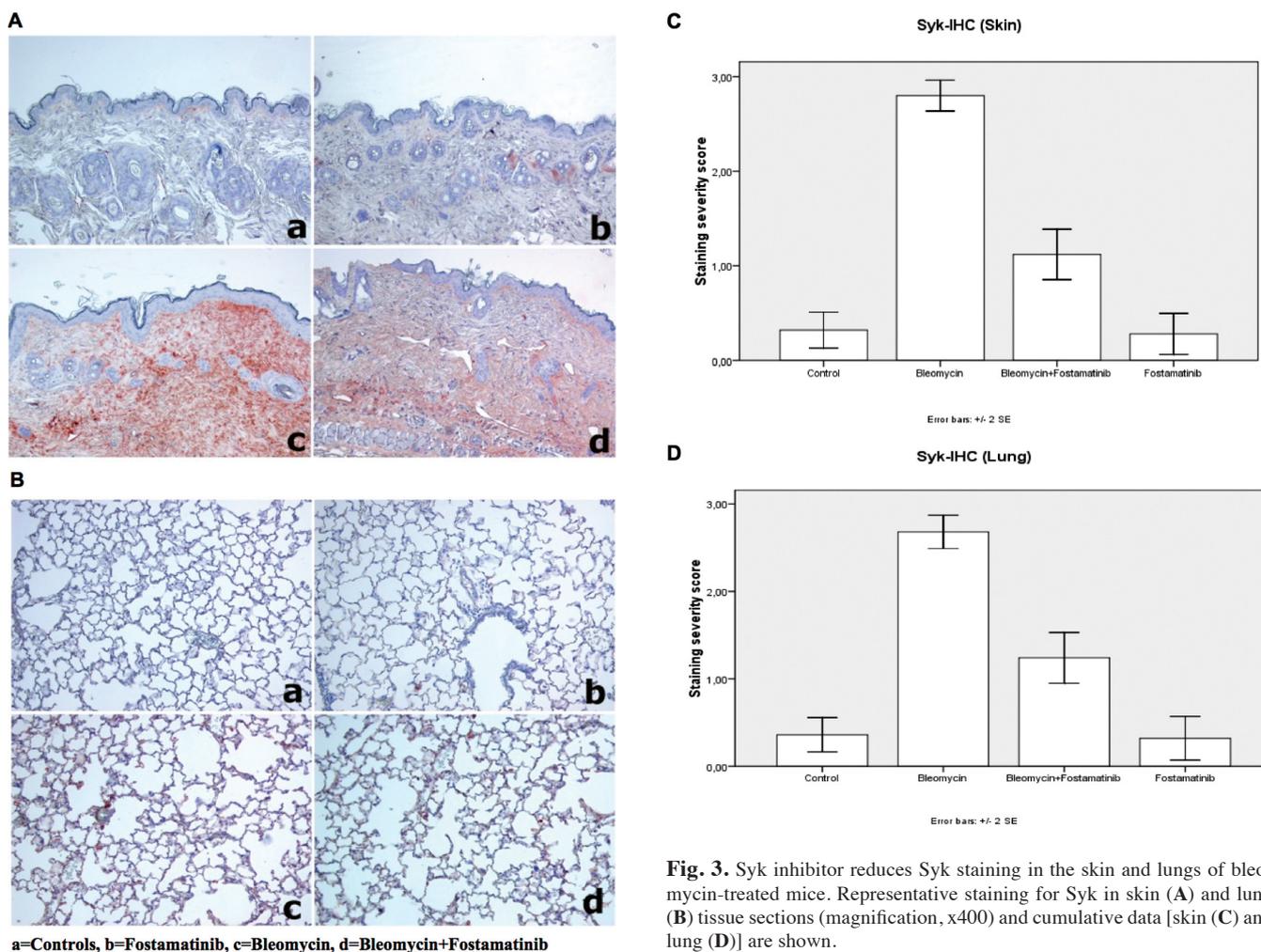
Measurement of dermal thickness and evaluation of fibrosis

The dermal thickness was measured at

a x400 magnification under the Olympus CX-31 microscope (Tokyo, Japan) by using the ocular micrometer. For each animal, the distance between epidermal-dermal junction and dermal-subcutaneous fat junction were measured in 4 consecutive skin sections. In each group of mice, dermal thickness was expressed in micrometers. For the evaluation of fibrosis, tissue samples were stained with Mallory's trichrome stain.

Immunohistochemistry (IHC) staining

Five-micrometer paraffin tissue sections were mounted on poly-L-lysine slides. The slides were air-dried and the tissue deparaffinised. The avidin biotin complex method was used for immunohistochemical staining, according to the manufacturer's instructions. The following reagents were used for IHC studies: rabbit anti-mouse Syk (N-19) (1:100; Santa Cruz, CA, USA), rab-



bit anti-mouse p-Syk (phospho Y323) (1:100; Abcam Inc.), rabbit anti-mouse TGF- β (B-9) (1:100; Santa Cruz, CA, USA). 3-amino-9-ethylcarbazole (AEC) was used as a chromogen, and the sections were counterstained with haematoxylin and mounted in Faramount aqueous mounting medium. In each sample, Syk, p-Syk and TGF- β positive-stained cells were counted. One hundred microscopic areas were counted in each group, and the percentage of positive cells in the tissues was determined.

Mast cell analysis

The lung and skin tissues fixed in 10% formalin solution for 24 h were embedded in paraffin. Sections of 5 mm thickness were cut and stained with 1% toluidine blue (Gurr, England) for 30 min. Tissue sections were examined under light microscopy (x400) and the number of mast cells per square millimeter

were counted in random high-power fields using an Olympus CX31 (Japan) light microscope incorporating a square graticule in the eyepiece (eyepiece x10; objective x40). Intact and partially degranulated mast cells were counted in 100 high-power fields in lung and skin preparations of each group. The mast cell density at each site was calculated and recorded as MC numbers mm^{-2} .

Statistical analysis

Data are presented as mean \pm SEM. One-way ANOVA with posthoc analysis using Tukey test was used for comparison of data. The difference was considered significant when p -value was <0.05 .

Results

1. Syk inhibition reduced skin thickness and fibrosis

To evaluate the effect of Syk inhibition on bleomycin-induced skin fibrosis,

we measured dermal thickness in mice treated with bleomycin while they were fed with fostamatinib-containing chow. Mice treated with bleomycin alone had significantly thicker skin (416.1 ± 6.1) compared to controls (260.1 ± 10.1) and fostamatinib-treated mice (254.3 ± 7.9) ($p < 0.001$). Mice subjected to bleomycin and fed with fostamatinib-containing chow had more (312.3 ± 4.4) dermal thickness than controls and fostamatinib-treated mice (p -values < 0.001); but, significantly less when compared to mice treated with bleomycin alone ($p < 0.001$) (Fig. 1 A-B).

2. Syk inhibition limited lung inflammation and fibrosis

Alveolar haemorrhage, oedema, damage and leukocyte scores in the lungs of mice treated with bleomycin were significantly higher when compared to control or fostamatinib alone-treated mice (p -values < 0.001). Mice exposed

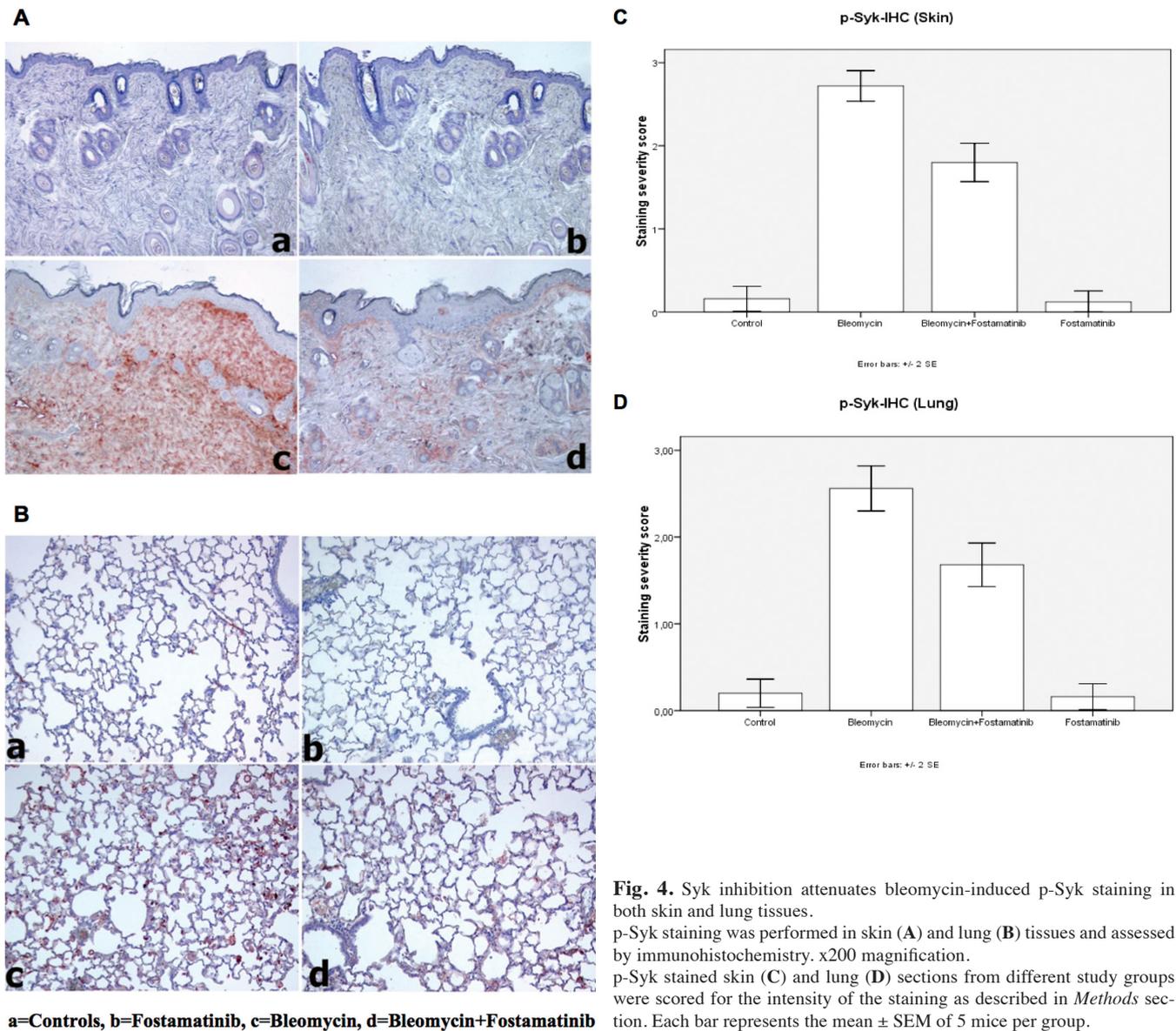


Fig. 4. Syk inhibition attenuates bleomycin-induced p-Syk staining in both skin and lung tissues. p-Syk staining was performed in skin (A) and lung (B) tissues and assessed by immunohistochemistry. x200 magnification. p-Syk stained skin (C) and lung (D) sections from different study groups were scored for the intensity of the staining as described in *Methods* section. Each bar represents the mean \pm SEM of 5 mice per group.

to bleomycin and treated with fostamatinib had significantly less alveolar damage and leucocyte infiltration compared to mice exposed to bleomycin and fed with regular chow (Fig. 1 C-D).

3. Syk inhibition reduced skin fibrosis score

In order to determine whether fostamatinib prevented bleomycin-induced skin and pulmonary fibrosis, tissue samples were stained with Mallory's trichrome stain to examine collagen deposition histologically. At the end of the 21-day bleomycin administration, there was prominent fibrosis and it reduced significantly in the group of mice which received in parallel fostamatinib (Fig. 2 A-B).

4. Skin and lung tissue Syk and p-Syk expression

Syk expressions in the skin and lung tissues were evaluated immunohistochemically. Syk expression in both the lung and the skin were noted to be more intense in the bleomycin-treated mice compared to control mice. Interestingly, treatment of mice with the Syk inhibitor fostamatinib decreased Syk expression in both skin and lung tissues significantly (Fig. 3).

The active form of Syk is phospho-Syk, and its expression is suppressed by Syk inhibitor therapy. Accordingly, we evaluated phospho-Syk expression by IHC. As can be seen in Figure 4, bleomycin induced the expression of phospho-Syk;

but, in the presence of the Syk inhibitor its expression was limited significantly. Control mice and mice treated with fostamatinib alone had minimal, if any, expression of phospho-Syk. (Fig. 4).

5. TGF- β staining

The finding of increased deposition of TGF- β in involved skin of SSc patients implies its involvement in the pathologic fibrotic process (28). Therefore, we evaluated the expression of TGF- β by IHC in skin and lung tissues. Mice treated with bleomycin displayed more prominent TGF- β staining of skin and lung tissues. The administration of Syk inhibitor to the bleomycin-treated mice abolished TGF- β staining (Fig. 5).

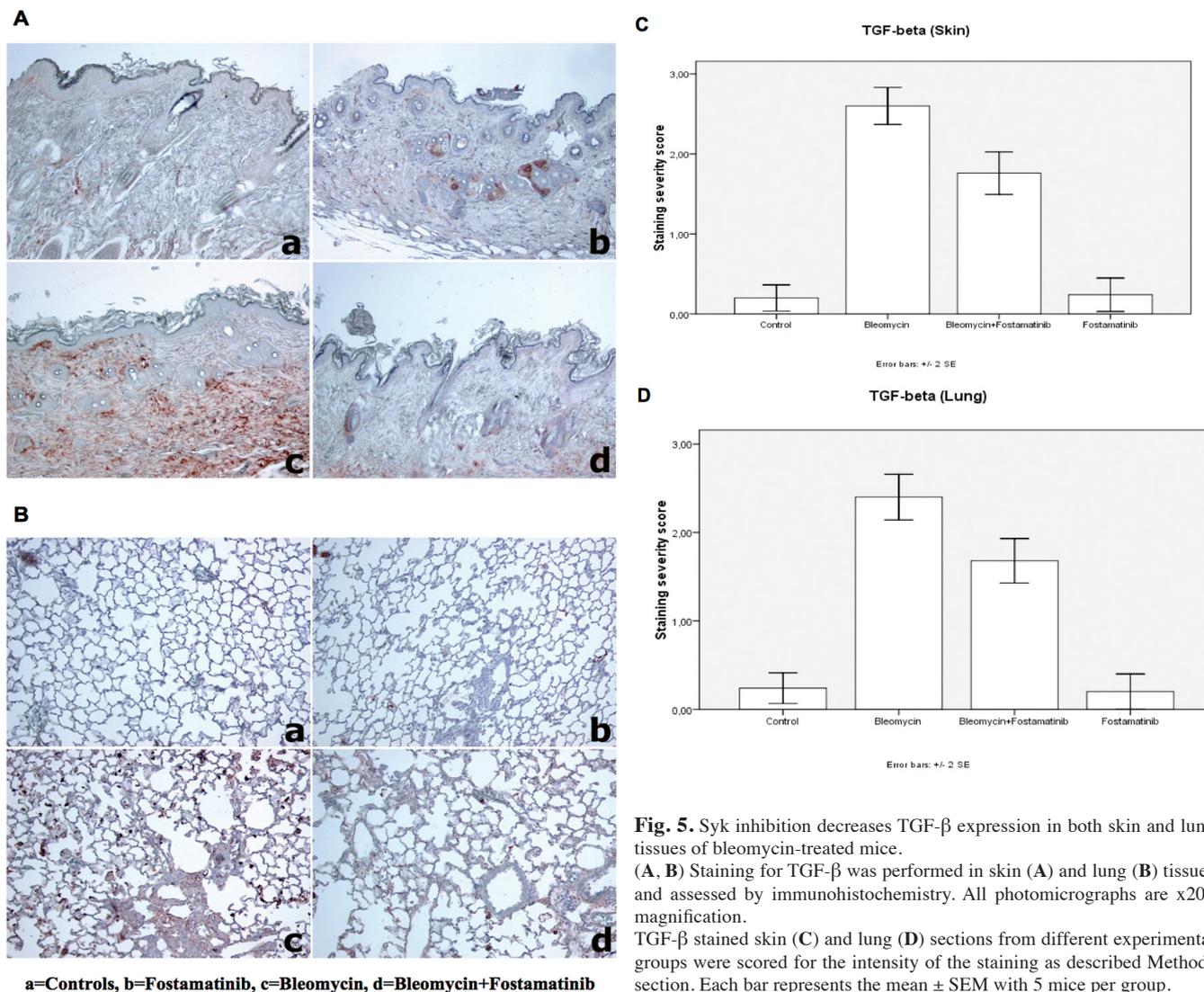


Fig. 5. Syk inhibition decreases TGF- β expression in both skin and lung tissues of bleomycin-treated mice.

(A, B) Staining for TGF- β was performed in skin (A) and lung (B) tissues and assessed by immunohistochemistry. All photomicrographs are x200 magnification.

TGF- β stained skin (C) and lung (D) sections from different experimental groups were scored for the intensity of the staining as described Methods section. Each bar represents the mean \pm SEM with 5 mice per group.

6. Mast cell staining

It is well known that mast cells are active in SSc and that they represent a main source of TGF- β which is stored in their granules and is released after degranulation (29). The number of mast cells was higher in the group of mice that were treated with bleomycin alone ($p < 0.001$) and the administration of fostamatinib reduced significantly the number mast cells (Fig. 6).

Discussion

We present the first evidence that treatment of mice with a small molecule, Syk inhibitor, prevents the development of bleomycin-induced SSc-like lesions in the skin and lung tissues. Our finding that Syk inhibition protected mice against bleomycin-induced SSc-like skin and lung pathology supports

the idea that the Syk signalling pathway is active in the fibrotic process which typifies SSc. It has been reported that Src kinases are important in the synthesis of extracellular matrix and that they become active in the presence of profibrotic cytokines, like TGF- β and PDGF (30), and Syk inhibition has been reported to inhibit the differentiation of human fibrocytes (31). Fostamatinib has been reported to mitigate scleromatous skin changes in mice developing graft-versus-host disease (32), and the Src kinase inhibitor SU6656 decreased dermal thickness and collagen content in an experimental bleomycin-induced skin fibrosis (31).

Syk phosphorylates itself and downstream proteins at tyrosine residues and in some instances at serine and threonine residues (18). Fostamatinib

has been known to inhibit all phosphorylation events downstream of Syk signalling (22, 33). We found that both Syk and p-Syk expression were significantly increased in the bleomycin-treated mice; however, the presence of fostamatinib suppressed it. In human studies and in experimental models, Syk inhibition was found to be effective for various autoimmune and allergic diseases (26, 27, 34, 35).

TGF- β has been shown amply to serve as the principal cytokine causing fibrosis in SSc (3, 6). Fibroblasts from SSc patients have increased and active TGF- β signalling, directly involved in the synthesis of the extracellular matrix (36); and TGF- β is present in increased amounts in skin biopsies from patients with SSc (28). In addition, TGF- β decreases degradation of extracellular

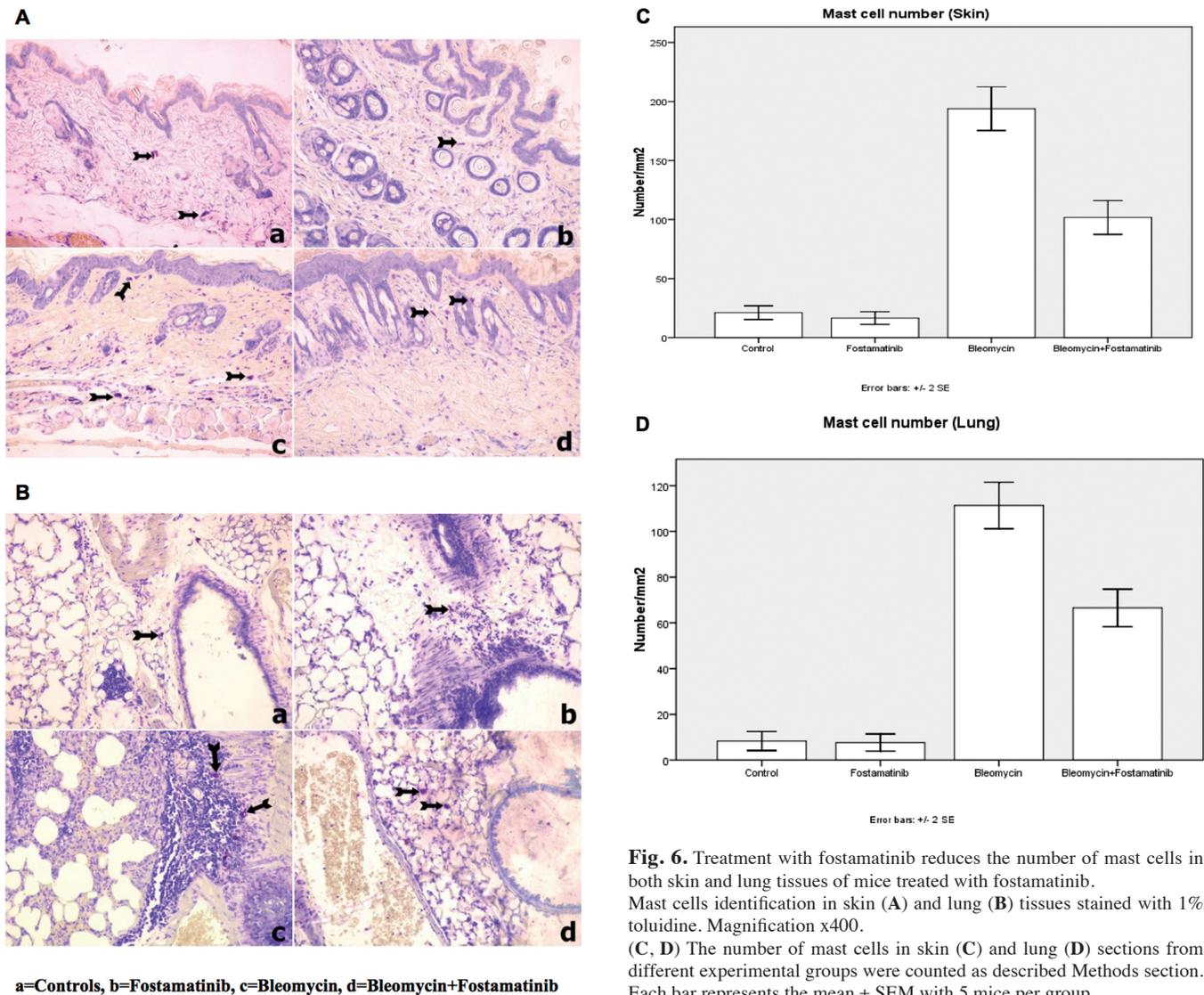


Fig. 6. Treatment with fostamatinib reduces the number of mast cells in both skin and lung tissues of mice treated with fostamatinib. Mast cells identification in skin (A) and lung (B) tissues stained with 1% toluidine. Magnification x400. (C, D) The number of mast cells in skin (C) and lung (D) sections from different experimental groups were counted as described Methods section. Each bar represents the mean \pm SEM with 5 mice per group.

matrix by decreasing the synthesis of matrix metalloproteases and increasing the expression of protease inhibitor (37). TGF- β receptor phosphorylates Smad2 and Smad3, forming a complex with Smad4 and the complex stimulates type 1 collagen and fibronectin synthesis (37). We observed that Syk inhibition significantly decreased the expression of TGF- β in skin and lung tissues. Syk inhibition resulted in decreased TGF- β expression (38, 39) and a Syk inhibitor has been shown to decrease the expression of TGF- β in the skin of mice subjected to graft-versus-host disease (32). Our data suggest that fostamatinib suppresses TGF- β expression by inhibiting Syk phosphorylation. Another possible mechanism whereby Syk inhibition suppresses skin and

lung pathology in response to bleomycin may involve mast cells. Syk is well known to be directly involved in the IgE-dependent mast cell activation; and Syk inhibitors, including fostamatinib, suppress mast cell function and are effective in the treatment of allergic diseases (34, 35). Therefore, since the Syk inhibitor decreased the number of mast cells in the lung and skin tissues of bleomycin-treated mice, we consider it possible that at least part of its effect may involve the suppression of mast cell degranulation. In conclusion, we have presented evidence that the Syk inhibitor fostamatinib can avert bleomycin-induced skin and lung pathology by decreasing phospho-Syk and TGF- β expression along with limiting the presence of mast cells.

Our data provide rational evidence for the use of Syk inhibitors in the treatment of SSc.

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