ORIGINAL

Analysis of the anti-fibrotic potential of a JAK inhibitor in a bleomycin-induced pulmonary fibrosis model

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Abstract : Background : Interstitial lung disease (ILD) associated with connective tissue diseases (CTD-ILD) remains to be a major cause of mortality. Different from idiopathic form, CTD-ILD involves more immune dysregulation along with aberrant fibroblast activation. Therefore, therapy targeting both profibrotic and proinflammatory molecules could be ideal for CTD-ILD. Janus kinase (JAK) is a family of intracellular, non-receptor tyrosine kinases that transduce cytokine-mediated signals. The purpose of this study is to reveal the anti-fibrotic potential of JAK inhibitors (JAKis). Methods : The anti-fibrotic effect of a JAKi with a particular focus on baricitinib was examined using a human lung fibroblast cell line and a bleomycin (BLM)-induced pulmonary fibrosis model in mice. Results : Baricitinib, a selective JAK1, 2 inhibitor suppressed transforming growth factor-β (TGF-β)-induced phosphorylation of JAK2 in human lung fibroblasts. Baricitinib also strongly suppressed the TGF-β-induced collagen1 and α-smooth muscle actin (α-SMA) expression in fibroblasts. Moreover, baricitinib ameliorated lung fibrosis in BLM-treated mice, particularly when administered in the late phase. The number of α-SMA or collagen triple helix repeat containing 1 (CTHRC1) positive fibroblasts in BLM-treated lungs was reduced by administration of baricitinib. Conclusions : Our data suggest that baricitinib may improve pulmonary fibrosis by directly inhibiting fibroblast activation via JAK2 blockade. J. Med. Invest. 72 : 298-307, August, 2025

Keywords **:** *Connective tissue disease, Pulmonary fibrosis, Transforming growth factor-β, Janus kinase Inhibitor, baricitinib*

INTRODUCTION

The respiratory system is one of the most frequently affected organs in connective tissue disease (CTD). Among various lesions formed in the airways, alveoli, interstitium, vascular system, and pleura, interstitial lung disease (ILD) is a particularly serious respiratory manifestation as it has the greatest impact on survival (1-4).

In general, CTD-ILD involves not only aberrant fibrosis but also marked immune dysregulation with autoimmune features (e.g., ectopic lymphoid structure formation and autoantibody production in the lungs (5, 6)). As the disease progresses, the pathological autonomous fibrotic process which is resistant to immunosuppressive treatment becomes obvious. It is common to any other forms of ILD including idiopathic pulmonary fibrosis (IPF). Indeed, a substantial proportion of rheumatoid arthritis (RA)-ILD patients with a usual interstitial pneumonia (UIP) pattern will show a progressive phenotype with the poor outcome like IPF (7-9).

Currently, two anti-fibrotic agents, nintedanib and pirfenidone, showed the effect to delay the decline rate of forced vital capacity (FVC) and are recommended for the treatment of IPF (10, 11). Nintedanib also demonstrated similar effectiveness to progressive fibrosing ILD (PF-ILD), including CTD-ILD (12, 13). However, these drugs can only show partial response and are still inadequate to control progressive pulmonary fibrosis. To develop a novel anti-fibrotic agent, our department has focused on the pathological role of kinases in fibrosis and reported anti-fibrotic effect of various kinase inhibitors using an animal model of pulmonary fibrosis (14-20).

Janus kinase (JAK) is a family of receptor-bound intracellular tyrosine kinase composed of four isoforms (JAK1, JAK2, JAK3, and TYK2) and plays a vital role in transducing the intracellular signals of various cytokines. Upon undergoing phosphorylation, JAKs subsequently phosphorylate the tyrosine residues of intracellular tail of receptors, followed by the phosphorylation of the signal transducer and activator of transcription (STAT) family. STATs become activated and translocate into the nucleus to regulate the expression of target genes (21).

Based on the concept that disruption of the JAK/STAT pathway

Abbreviations :
ILD, interstitial lung disease ; CTD, connective tissue diseases ; FVC, forced vital capacity ; JAK, Janus kinase ; STAT, signal transducer and activator of transcription ; TGF-β, transforming growth factor-β ; JAKis, JAK inhibitors ; BLM, bleomycin ; α-SMA, α-smooth muscle actin ; ECM, extracellular matrix ; IPF, idiopathic pulmonary fibrosis ; RA, rheumatoid arthritis ; UIP, usual interstitial pneumonia ; PF-ILD, progressive fibrosing ILD ; PDGF, platelet-derived growth factor ; VEGF, vascular endothelial growth factor ; IL-4, interleukin-4 ; EMT, epithelial-mesenchymal transition ; tsDMARD, targeted synthetic disease modifying anti-rheumatic drug.

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can inhibit a broad number of proinflammatory cytokine signals, JAKis have already been applied in the clinical practice.(22). On the other hand, several profibrotic mediators (e.g., TGF-β, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), interleukin-4 (IL-4), and IL-13) have also been reported to transmit their signals, particularly through the JAK2/STAT3 pathway (23-28). Considering these recent reports, we speculated that some JAKis in current clinical application may have potential as novel anti-fibrotic drugs with bimodal action targeting both profibrotic immune cells and fibroblasts.

In the present study, we investigated the anti-fibrotic effect of a preexisting selective JAK1, 2 inhibitor —baricitinib—particularly in terms of action on lung fibroblasts to clarify this drug could restore the common pathological fibrotic mechanisms shared by PF-ILD, including CTD-ILD (29).

MATERIALS AND METHODS

Animal and agents

Eight-week-old C57BL/6 male mice were purchased from Nihon Clea Inc. The mice were bred in the specific-pathogen-free environment and maintained in the animal facility of the University of Tokushima according to the guidelines of our university (14). All experimental protocols were approved by the animal research committee of the University of Tokushima, Japan (Approval Number : T2023-46).

Bleomycin exposure and treatment intervention

Mice were anesthetized with the inhalation of 4% isoflurane, and underwent a single transbronchial instillation of 4.0 mg/kg BLM (Nippon Kayaku Co.) or saline on day 0. Baricitinib (AdooQ BioScience) or vehicle (0.5% carboxymethyl cellulose) were administered daily by oral gavage at 10 mg/kg/day from day 1 to 10 (early baricitinib group) or day 11 to 20 (late baricitinib group). The experimental design was set up based on previous reports (30-34). Mice were sacrificed on day 21. Each experiment was performed in at least four mice per group.

Bronchoalveolar lavage

Mice were anesthetized and underwent tracheal cannulation (17). Bronchoalveolar lavage (BAL) was performed by instillation of 1 ml of sterile saline from both lungs on day 17 after BLM exposure. The total cell count was determined by counting Trypan Blue (Thermo Fisher Scientific Inc.)-stained cells on a hemocytometer. The corresponding amount of BAL fluid for 10^3 cells was centrifuged onto a microscope slide using a Cellspin I (Tharmac), at 500 rpm for 5 minutes at room temperature. The slide was air-dried and stained with Diff-Quick staining solution (Baxter). Differential cell counts—including the percentages of neutrophils, lymphocytes, macrophages, and eosinophils—were determined.

ELISA

BAL fluid (BALF) were analyzed for mouse TGF-β1 (R&D Systems) levels by sandwich ELISA.

Hydroxyproline colorimetric assay

Mice were sacrificed by cervical dislocation on day 21 after BLM instillation. The left lung was isolated and homogenized in distilled water. The hydroxyproline content was analyzed using a Bio-vision hydroxyproline colorimetric assay kit (Bio Vision).

Histopathology

The right lung was isolated and fixed in 10% formalin, then embedded in paraffin. Three- micrometer-thick paraffin sections were deparaffinized and hematoxylin and eosin (HE) and Azan Mallory staining were performed. In the quantitative analysis, a numeric fibrotic scale was used (Ashcroft score). In short, under 100×magnification, each successive field was given a score ranging from 0 (normal lung) to 8 (total fibrous obliteration of the field) (35). All scores from five sections were averaged.

Immunohistochemistry

Paraffin-embedded lung sections were stained with mouse anti α-smooth muscle actin (α-SMA) antibody (1A4) (R&D Systems) and rabbit anti CTHRC1 antibody (Proteintech, No. 16534-1-AP) at 4℃ overnight and subsequently stained with Alexa 594-conjugated anti-mouse IgG antibody (Thermo Fisher Scientific Inc., No. A-11062), Alexa 488-conjugated anti-rabbit IgG antibody (Thermo Fisher Scientific Inc., No. A-11008) and 4',6-Diamidino-2-Phenylindole (DAPI, Thermo Fisher Scientific Inc.) at room temperature for 1 hour for detection. Fluorescence images were captured with a confocal laser scanning microscope at 20×magnification. The area stained with Alexa 594 was measured using a software for biomedical image analysis (ImageJ).

Immunoblot analysis

To quantify the level of JAK phosphorylation, and the expression α-SMA, MRC-5 cells, a human lung fibroblast cell line (DS Pharma Biomedical) were cultured in DMEM containing 0.1% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (50 μg/ml) with TGF-β (R&D Systems, 5 or 10 ng/ml) and various concentration of baricitinib for 24 or 48 hours. Whole-cell extracts from these cells were prepared at various time points using M-PER reagents (Thermo Fisher Scientific Inc.) containing phosphatase and protease inhibitor cocktails (Roche) (18). The same amount of protein from each cell extract was used for immunoblotting with a Simple Western™ System (ProteinSimple) as described previously. According to the manufacturer's instructions, we analyzed the protein amounts based on the signal intensity.

The first antibodies used were as follows :

anti-phospho-JAK1 (Tyr 1034/1035) antibody (D7N4Z), anti-phospho-JAK2 (Tyr 1007/1008) antibody (C80C3), anti-phospho-JAK3 (Tyr 980/981) (D44E3) antibody, anti- JAK1 antibody (6G4), anti-JAK2 antibody (D2E12), anti-JAK3 antibody (D7B12), anti-α-SMA antibody (1A4), anti-STAT3 antibody (124H6), anti-phospho-STAT3 (Tyr705) antibody (D3A7). All were purchased from Cell Signaling Technology with the exception of anti-α-SMA antibody, which was purchased from R&D Systems.

qRT-PCR

Total RNA was extracted from MRC-5 using an RNeasy Mini Kit (Qiagen), and was reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. RT-PCR was performed using a CFX96 real-time PCR system (Bio-Rad) and SYBR Premix Ex Taq (TAKARA). Human *GAPDH* mRNA was used as a housekeeping gene, and quantification was performed using the ΔΔCt method (19). MRC-5 cells were incubated with or without TGF-β (5 ng/ml) at various concentrations of baricitinib. The mRNA expression of *COL1A1* and *ACTA2* in MRC-5 cells was analyzed by qRT-PCR after 24 hours of incubation.

The sequences of primers were as follows :

COL1A1 forward, 5'-TCTGCGACAACGGCAAGGTG-3',
COL1A1 reverse, 5'-GACGCCGGTGGTTTCTTGGT-3',
ACTA2 forward, 5'-GAGCGTGGCTATTCCTTCGT-3',
ACTA2 reverse, 5'-GCCCATCAGGCAACTCGTAA-3',
GAPDH forward, 5'-GAAGGTGAAGGTCGGAGTC-3',
GAPDH reverse, 5'-GAAGATGGTGATGGGATTTC-3'.

Statistical analyses

The significance of differences was analyzed using the Kruskal-Wallis test or a one-way ANOVA, followed by Tukey's multiple-comparison post-hoc test. P values of less than 0.05 were considered statistically significant. Statistical analyses were performed using the GraphPad Prism software program (Ver. 6.01, GraphPad Software Inc.).

RESULTS

TGF-β induces phosphorylation of JAK in lung fibroblasts.

In order to elucidate whether the TGF-β signal passes through JAK, we first investigated the effect of TGF-β on the phosphorylation of JAK1, 2, and 3 in MRC-5 cells (a human fibroblasts cell line). JAK1 and 2 were expressed in steady state MRC-5 cells (Figure 1A and 1B). JAK3 was not expressed in MRC-5 cells (data not shown). Upon stimulation with TGF-β, phosphorylated JAK1 (p-JAK1) was weakly induced in MRC-5 cells from 1 to 24 hours later (Figure 1A), while p-JAK2 was strongly induced from 1 to 24 hours with a peak at 4 hours ($P<0.05$) (Figure 1B). JAK3 and p-JAK3 were not detected (data not shown). These results suggest that JAK1 and 2, particularly JAK2, are activated in a TGF-β- dependent manner in lung fibroblasts.

JAKi is involved in TGF-β-induced phosphorylation of JAK2 in lung fibroblasts.

As JAK2 was clearly activated in lung fibroblasts after stimulation by TGF-β, we focused the further study on JAK2 among JAK. Based on the previous study (36), we next examined the effects of a preexisting JAKi, baricitinib, on the phosphorylation of JAK2 in lung fibroblasts. The phosphorylation of JAK2 in TGF-β stimulated fibroblasts was significantly inhibited upon treatment with 150 nM baricitinib ($P<0.05$) (Figure 2A). Furthermore, baricitinib actually inhibited STAT3 activation in fibroblasts (Figure 2B). Thus, we found that JAKi treatment worked on TGF-β-stimulated lung fibroblasts via the inhibition of JAK-STAT signaling and that the results were in line with the pharmacology of baricitinib, with the selectivity for JAK2 inhibition (29).

JAKi exerts functional inhibition in lung fibroblasts.

The vigorous production of ECM in response to TGF-β stimulation is the key pathogenetic function of fibroblasts in pulmonary fibrosis. To reveal whether TGF-β/JAK signal in lung fibroblasts is related to profibrotic function, we evaluated the effect of JAKi on TGF-β- induced ECM expression in lung fibroblasts.

TGF-β increased the α-SMA expression in lung fibroblasts at the gene (*ACTA2*) (Figure 3B) and protein levels (Figure 3A), which was suppressed significantly when treated with baricitinib. Moreover, baricitinib, significantly inhibited TGF-β-induced upregulation of the *COL1A1* gene expression ($P<0.05$) (Figure 3B). Considering the more prominent induction of phosphorylated JAK2 by TGF-β and a lower IC_{50} value of baricitinib for phosphorylating JAK2 (29), these results suggest that—above all—TGF-β/JAK2 signaling contributes to the profibrotic function of lung fibroblasts.

JAKi has anti-fibrotic potential in mice with BLM-induced pulmonary fibrosis.

To examine the anti-fibrotic effect of JAKi in vivo, we used a mouse model of BLM-induced pulmonary fibrosis. After a single transbronchial instillation of BLM at 4.0 mg/kg, the mice were

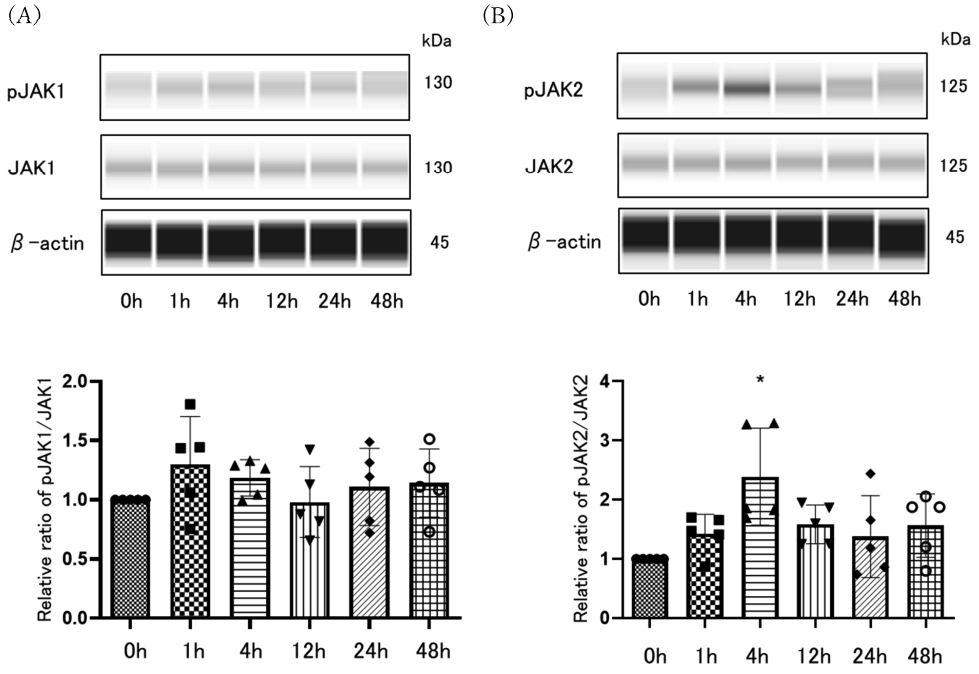

Figure 1. TGF-β induces JAK phosphorylation in lung fibroblasts.
MRC-5 cells (a human lung fibroblasts cell line) were cultured with or without TGF-β at 5 ng/ml. A : The expression levels of JAK1 and p-JAK1 were examined over time by Western blotting. B : The expression levels of JAK2/p-JAK2 were examined in the same way as in Figure 1A. Data were analyzed using the the Kruskal-Wallis test. Bars show the mean ± SD. * = $P<0.05$, ** = $P<0.005$, versus non-treated MRC-5 cells. Representative blots from a total of five repeats are shown.

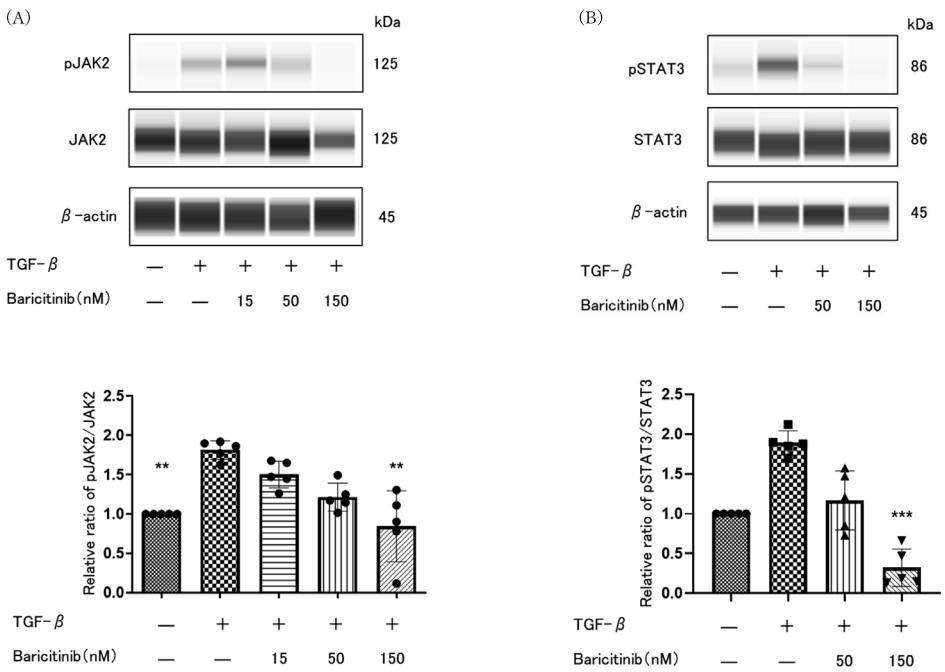

Figure 2. JAKi treatment inhibits JAK2-STAT3 phosphorylation in TGF-β stimulated lung fibroblasts.
A : MRC-5 cells were cultured with TGF-β (10 ng/ml) for 24 h under treatment with baricitinib at concentrations of 15 to 150 nM. The p-JAK2 protein levels in MRC-5 cells were evaluated by Western blotting (upper panel). The quantification of the p-JAK2 expression from the same experiments is shown (lower panel) (n = 5). B : In the same culture condition for 4 h under treatment with baricitinib at concentrations of 50 and 150 nM, the levels of STAT3 and p-STAT3 were evaluated by Western blotting (upper panel). The quantification of the p-STAT3 expression from the same experiments is shown (lower panel) (n = 5). Data were analyzed using the the Kruskal-Wallis test. Bars show the mean ± SD. * = $P<0.05$, *** = $P<0.001$ versus TGF-β-stimulated MRC-5 cells. Representative blots from a total of five repeats are shown.

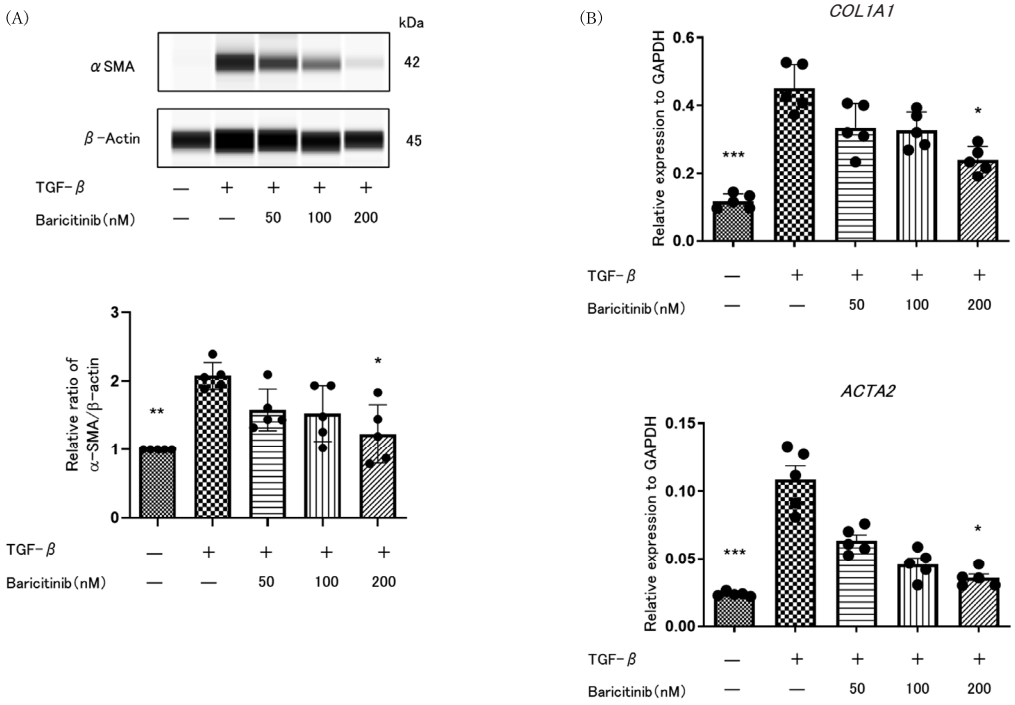

Figure 3. JAKi treatment inhibits the profibrotic function of lung fibroblasts.
A : MRC-5 cells were cultured with TGF-β (5 ng/ml) for 48 h under treatment with baricitinib at concentrations of 50-200 nM. The levels of α-SMA protein in MRC-5 cells were evaluated by Western blotting (upper panel). The quantification of the α-SMA expression from the same experiments is shown (lower panel). B : In the same culture condition for 24 h, the expression levels of *COL1A1* and *ACTA2* in MRC-5 cells were examined by real-time quantitative polymerase chain reaction. Data were analyzed using the the Kruskal-Wallis test. Bars show the mean ± SD. * = $P<0.05$, ** = $P<0.005$, *** = $P<0.001$ versus TGF-β-stimulated MRC-5 cells. One representative experiment from a total of five repeats is shown.

treated daily with baricitinib (10 mg/kg/day) or vehicle from day 0-10 (early group) or day 11-21 (late group). Baricitinib tended to suppress body weight loss and significantly improved survival of late group mice after BLM treatment ($P<0.05$) (Figure 4A). On day 21, BLM instillation clearly induced inflammatory and fibrotic changes in the lungs, accompanied with an increase of histological score and hydroxyproline content. Only when baricitinib was administered in the late phase, both the Ashcroft score and hydroxyproline content improved significantly ($P<0.05$ and $P<0.001$ respectively) (Figure 4B and 4C). As the late group of our study could be considered more appropriate for assessing the utility of JAKi in the treatment of pulmonary fibrosis, these results suggest that baricitinib has anti-fibrotic potential in our BLM model.

Anti-fibrotic effect of late baricitinib treatment in the BLM model is less dependent on the repression of immune cells.

Originally, many JAKis were developed based on the concept of inhibiting a broad number of pro-inflammatory cytokine signals, which led us to investigate the effects of JAKi on immune cells in the lungs. Focusing on the late baricitinib treatment group, we examined the cell counts, cell fractions and the level of TGF-β in BALF collected on day 17 after BLM instillation. Both cell counts and cell fractions in BALF did not change to a

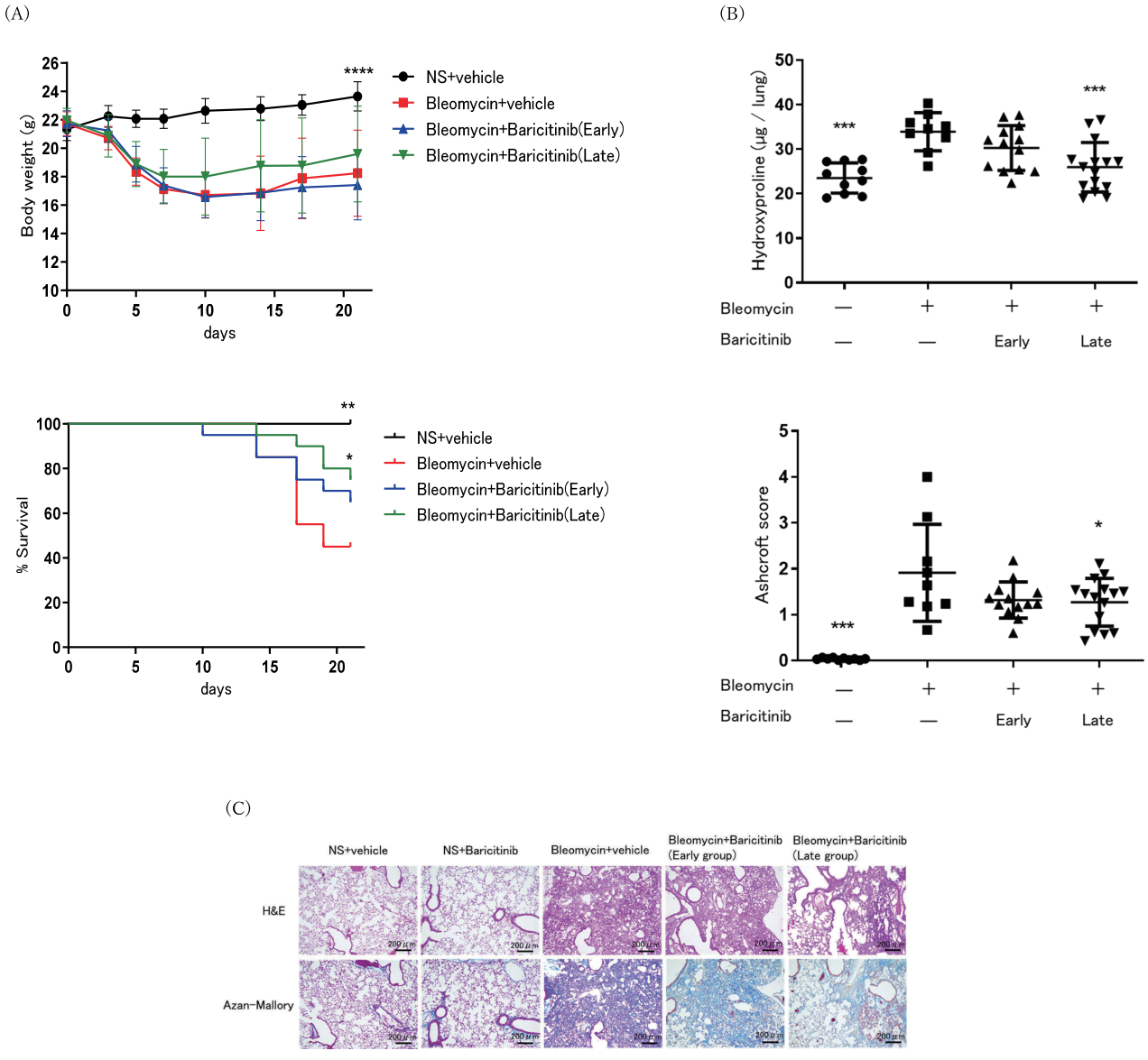

Figure 4. JAKis have anti-fibrotic potential in mice with BLM-induced pulmonary fibrosis.
A : Serial changes in the body weight and proportion of surviving mice after BLM instillation are shown (n = 4-15/group). B : The Ashcroft score and hydroxyproline content were used to quantify fibrosis in the lungs. Comparison of each parameter among vehicle and early or late baricitinib treatment groups (n = 9-15/group). C : Representative images of lung sections from each treatment group in (B), stained with hematoxylin and eosin (HE) or Azan-Mallory. The body weight change was analyzed by a two-way ANOVA followed by Tukey's multiple comparison test. Values are expressed as the mean ± SD. The proportion of surviving mice was analyzed by the Kaplan-Meier method followed by a log-rank (Mantel-Cox) test. Scale bars indicate 200 μm. The comparison of the Ashcroft score and hydroxyproline assay results was performed by a one-way ANOVA followed by Tukey's multiple comparison test. Values are expressed as the mean ± SD. * = $P<0.05$, ** = $P<0.005$, *** = $P<0.001$ versus BLM and vehicle treatment group.

significant extent, despite baricitinib treatment (Figure 5A and 5B). Moreover, the level of TGF-β1 in BALF from BLM treated mice did not show significant decrease after the late treatment of baricitinib (Figure 5C). These results suggest—in part—that baricitinib may directly act on fibroblasts via the inhibition of TGF-β mediated JAK signaling rather than inflammatory cells in the lungs of the BLM model during the late phase.

Baricitinib suppressed lung fibroblasts in the BLM model.

To examine the impact of baricitinib on lung fibroblasts in vivo, we performed immunofluorescent staining of α-SMA and CTHRC1 proteins on lung tissue sections from each baricitinib treatment group in the BLM model. CTHRC1 has recently reported as a specific marker of a subset of lung fibroblasts (37). Both the number of α-SMA positive and CTHRC1 positive cells significantly decreased with scarce colocalization in both the early and late treatment groups, demonstrating that baricitinib suppressed lung fibroblasts activation and differentiation to myofibroblasts in BLM-instilled mice.

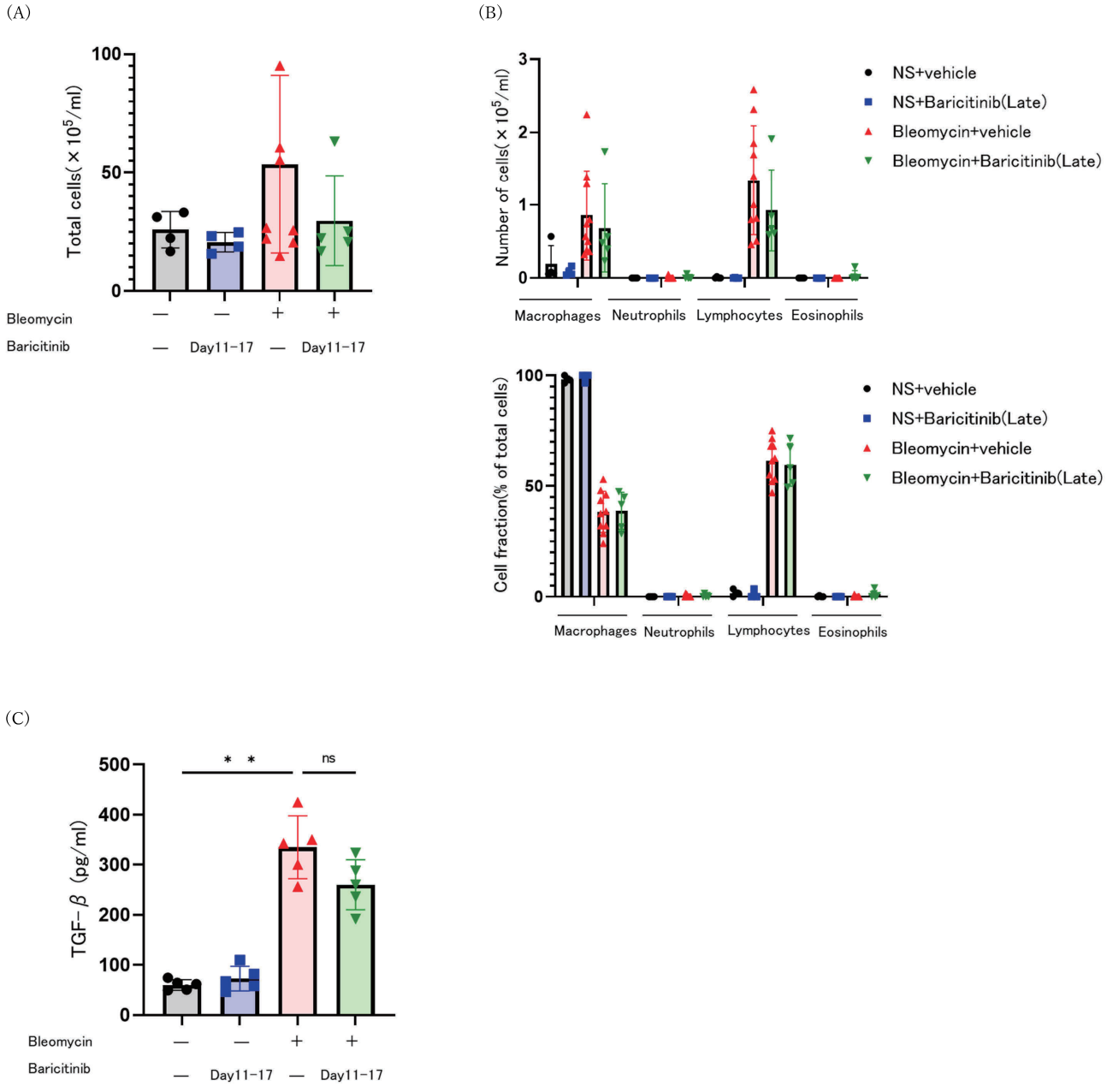

Figure 5. Baricitinib had a limited effect on immune cells in the BLM model.
A : Total cell numbers in BALF on day 17 after BLM instillation were compared among each baricitinib treatment group (n = 5/group). B : Cell numbers and the proportions of each immune cell fraction in BALF were compared as in (A). C : TGF-β1 level in BALF was determined by ELISA. The results were analyzed by a one-way ANOVA followed by Tukey's multiple comparison test. ** = P<0.005 versus group treated without BLM with vehicle.

DISCUSSION

In the present study, we demonstrated the anti-fibrotic potential of baricitinib, a preexisting JAKi in a bleomycin-induced pulmonary fibrosis model. Baricitinib directly suppressed the TGF-β mediated activation of fibroblasts in vitro and attenuated the development of pulmonary fibrosis in vivo, particularly through JAK2 inhibition.

Recent reports indicate the pathogenetic role of JAKs in ILD (38-40). Profibrotic cytokines overexpressed in ILD, such as IL-4, IL-6, IL-11, and IL-13 activate JAK/STAT molecular pathway (22, 26). Similarly, profibrotic growth factors have been reported to be involved in the JAK/STAT axis. Among them, TGF-β is the major pro-fibrotic factor that promotes fibroblast-to-myofibroblast differentiation and deeply contributes to the pathogenesis of fibrosis, including ILD (41). In addition to a canonical TGF-β signal transduced by the phosphorylation of SMAD2/3, an alternative pathway has been revealed passing through JAK2/STAT3 independently of SMAD (42). Several reports, mainly focused on IPF, have analyzed and described the expression and distribution of primarily JAK1 and JAK2, and STAT1 and STAT3 in animal models and patients (38-40). Zhang *et al*. described the activation of JAK2 by TGF-β in human fibroblasts and mouse models of systemic sclerosis, which in turn phosphorylates STAT3 (36). In IPF, Milara *et al*. demonstrated that TGF-β activates the pathway by JAK2 phosphorylation and that p-JAK2 was found in the nucleus of fibrotic areas (27). More recently, Gu *et al*. demonstrated baricitinib, a JAK1/2 inhibitor, impedes murine myofibroblast activation and epithelial injury via targeting the TGF-β1 signaling pathway and reduces

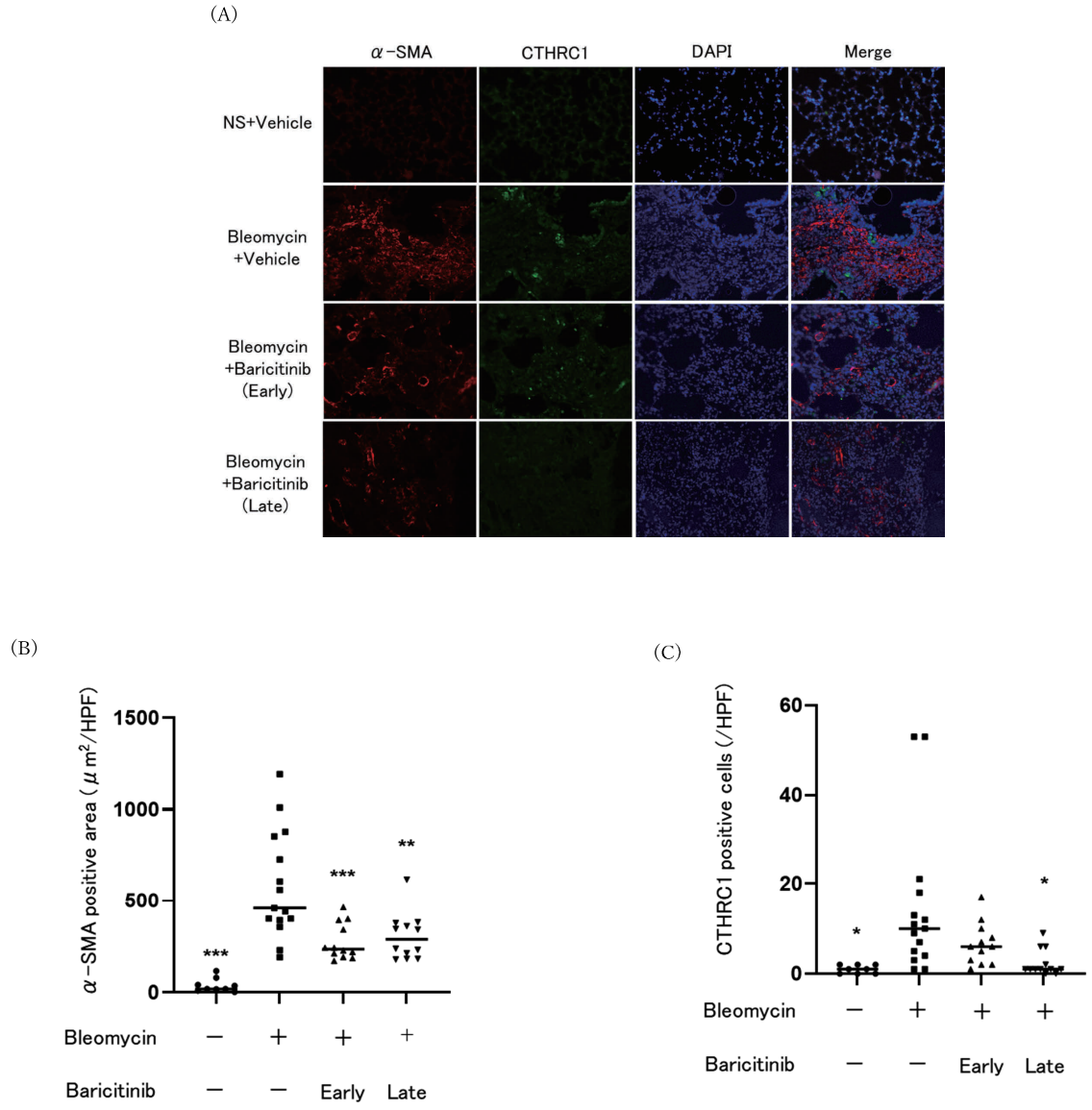

Figure 6. Baricitinib inhibits lung fibroblasts in vivo.
A : Representative images of lung sections on day 21 after BLM instillation from each baricitinib treatment group stained with anti-α-SMA antibody (red), anti-CTHRC1 antibody (green) and DAPI (blue). Scale bars, 100 μm. B : The area of α-SMA positive cells was measured using ImageJ in at least 4 random non-overlapping fields of view (magnification, 20×) per lung section. C : The number of CTHRC1 positive cells was counted manually in at least 4 random non-overlapping fields of view per lung section. The comparisons among each baricitinib treatment group are shown (n = 3/group). Results were compared by a one-way ANOVA followed by Tukey's multiple comparison test. * = $P<0.05$, ** = $P<0.005$, *** = $P<0.001$ versus BLM and vehicle treatment group.

BLM-induced pulmonary fibrosis in mice (43).

Our study confirmed that TGF-β clearly induced the phosphorylation of JAK2 in a human lung fibroblast cell line, and that baricitinib suppressed this reaction. Baricitinib, a selective JAK1, 2 inhibitor, has lower IC_{50} for JAK2 phosphorylation (5.7 nM) than JAK1 (5.9 nM) and JAK3 (>400 nM) (29). Reflecting this selectivity, baricitinib clearly showed the inhibition of JAK2 phosphorylation. Moreover, baricitinib suppressed TGF-β induced collagen1 gene expression and α-SMA expression at both gene and protein levels in fibroblasts strongly, suggesting a pro-fibrotic function of TGF-β actually involves phosphorylation of JAKs, especially JAK2.

We also showed the anti-fibrotic effect of baricitinib in BLM-induced pulmonary fibrosis. Unlike the report from Gu *et al*, we treated mice separately from day 0-10 (early treatment) or day 11-21 (late treatment) (43). The BLM model works by inducing an early inflammatory phase, which transitions into fibrosis after 5-7 days (33). To accurately assess the pure anti-fibrotic efficacy, the intervention should be designed to inhibit fibrosis without impacting early inflammation. While the early treatment group is suitable for assessing the anti-inflammatory effects of JAKi to prevent fibrosis, the late group was more appropriate for assessing the anti-fibrotic effect in the treatment of fibrosis. Indeed, baricitinib, only when treated in the late phase, improved the hydroxyproline content and the extent of fibrosis assessed by scoring the histological findings in BLM-treated lung, without affecting the profile of inflammatory cells and the level of TGF-β1 in BALF. TGF-β1 is the most pivotal pro-fibrotic molecule, widely expressed in the immune system and primarily produced by macrophage in an inflammatory phase of BLM model (44, 45). These data support that the anti-fibrotic effect of baricitinib in our BLM model was less likely to rely on suppressing immune cells, but—at least in part—on the direct inhibition of the fibroblast function by blocking TGF-β/JAK2 signaling. In order to confirm that in vivo, we performed immunohistochemistry for co-staining α-SMA with CTHRC1. Both $CTHRC1^+$ cells and $\alpha\text{-SMA}^+$ cells significantly decreased on lung tissue sections in each baricitinib treatment group. Consistent with the previous report that $CTHRC1^+$ pathologic fibroblasts belong to a different cluster from $\alpha\text{-SMA}^+$ cluster which contains myofibroblasts, smooth muscle cells and pericytes, $CTHRC1^+$ cells and $\alpha\text{-SMA}^+$ cells scarcely showed colocalization in our study. Given the role for TGF-β1 in increasing the expression of CTHRC1 in fibroblasts (46) and inducing the differentiation of fibroblasts into myofibroblasts, the results support the effect of baricitinib on TGF-β-mediated fibroblast activation, shown in vitro study.

The present study was associated with some limitations. Although we set up the animal experiment design for baricitinib to inhibit the phosphorylation of its target JAKs in vivo, we cannot assert the alveolar concentration of the drug exceeded the IC_{50} for JAK2 phosphorylation due to lack of precise pharmacokinetic data in this study. In addition to JAK2, baricitinib acts on JAK1, and the inhibition of both JAK1 and JAK2 by this agent may synergistically contribute to its anti-fibrotic potential. While we could not approach that concern, Wang *et al*. recently reported upadacitinib, a selective JAK1 inhibitor did not affect the BLM-induced pulmonary fibrosis significantly (47). Further investigation to reveal the anti-fibrotic effect of a selective JAK2 inhibitor and the precise molecular mechanism through which TGF-β phosphorylates JAK2 remains to be needed. Besides, our study did not cover the specific function of JAK2 in fibroblasts in vivo and our BALF data was not sufficient to conclude that the anti-inflammatory effects of late baricitinib treatment did not contributed to the improvement of lung fibrosis. Various cell populations in the lungs interact intricately to form pulmonary fibrosis (41). For example, the epithelial-mesenchymal transition (EMT) is considered to be a source of myofibroblasts in the lung interstitium. Milara *et al*. reported that the TGF-β-induced EMT in alveolar type Ⅱ cells was attenuated by dual p-JAK2/p-STAT inhibition (27). Profibrotic mechanisms of macrophages are associated with M2 polarization (48). M2-promoting cytokines, IL-4 and IL-13, can also be targeted by baricitinib (22). Moreover, there have been multiple studies on the role of Th17 cells in fibrosis (49, 50). Baricitinib can suppress the differentiation of Th17 cells via blocking IL-6 and IL-23 signals (22). Conditional and fibroblast-specific deletion of JAK2 in a BLM model or additional in vivo experiment using TGF-β1-induced lung fibrosis should be performed to further determine the profibrotic role of JAK2 in fibroblasts.

CONCLUSIONS

In summary, our findings suggest that a preexisting, ready-to-apply JAKi, baricitinib attenuated pulmonary fibrosis in BLM-treated mice partly via the suppression of TGF-β/JAK2 axis in fibroblasts. Baricitinib has been used as a targeted synthetic disease modifying anti-rheumatic drug (tsDMARD) for RA. ILD is a lethal condition associated with CTDs including RA. We speculate that the inhibition of JAK2 signaling will be a novel target of anti-fibrotic therapy for not only CTD-ILD but also IPF and non-IPF PF-ILD.

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CONFLICT OF INTEREST DISCLOSURE

The authors have no conflicts of interest.

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