ORIGINAL ARTICLE

Mechanical stress-induced mast cell degranulation activates TGF-β1 signalling pathway in pulmonary fibrosis

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ABSTRACT

Background The role of mast cells accumulating in idiopathic pulmonary fibrosis (IPF) lungs is unknown.

Objectives We investigated the effect of fibrotic extracellular matrix (ECM) on mast cells in experimental and human pulmonary fibrosis.

Results In IPF lungs, mast cell numbers were increased and correlated with disease severity (control vs 60%<FVC<90%, mean difference=-222.7, 95% CI −386.3 to −59.2, p=0.004; control vs FVC<60%, mean difference=−301.7, 95% CI of difference −474.1 to −129.34, p=0.0001; FVC>90% vs 60%<FVC<90%, mean difference=−189.6, 95% CI of difference −353.1 to −26.03, p=0.017; FVC>90% vs FVC<60%, mean difference=−268.6, 95% CI of difference −441.0 to −96.17, p=0.0007). Plasma tryptase levels were increased in IPF and negatively correlated with FVC (control vs FVC<60%, mean difference=−17.12, 95% CI of difference −30.02 to −4.22, p=0.006: correlation curves R=−0.045, p=0.025). In a transforming growth factor (TGF)-β1-induced pulmonary fibrosis model, chymase-positive and tryptase-positive mast cells accumulated in fibrotic lung. Lung tissue was decellularised and reseeded with bone marrow or peritoneum-derived mast cells; cells on fibrotic ECM released more TGF-β1 compared with normal ECM (active TGF-β1: bone marrow-derived mast cell (BMMC)-DL vs BMMC-TGF-β1 p=0.0005, peritoneal mast cell (PMC)-DL vs PMC-TGF-β1 p=0.0003, total TGF-β1: BMMC-DL vs BMMC-TGF-β1 p=0.013, PMC-DL vs PMC-TGF-β1 p=0.001). Mechanical stretch of lungs caused mast cell degranulation; mast cell stabilisers inhibited degranulation (histamine: cont vs doxantrazole p=0.004, β-hexosaminidase: cont vs doxantrazole, mean difference=1.007, 95% CI of difference 0.2700 to 1.744, p=0.007) and TGF-β1 activation (pSmad2/Smad2: cont vs dox p=0.006). Cromoglycate attenuated pulmonary fibrosis in rats (collagen: phosphate-buffered saline (PBS) vs cromoglycate p=0.036, fibrotic area: PBS vs cromoglycate p=0.031).

Conclusion This study suggests that mast cells may contribute to the progression of pulmonary fibrosis.

Key messages

What is the key question?

► How does fibrotic extracellular matrix (ECM) in pulmonary fibrosis affect mast cell function and phenotype, and what is the role of mast cells in the pathophysiology of pulmonary fibrosis?

What is the bottom line?

► This study highlights how the profibrotic ECM environment affects mast cell phenotype and function, mast cell sensitivity to mechanical stress in fibrotic lungs and how mast cells contribute to the creation of a profibrotic environment through transforming growth factor-β1 activation.

Why read on?

► This is the first study to directly show that fibrotic lung ECM can regulate mast cell function and phenotype, therefore suggests that activated mast cells by fibrotic ECM play an important role in persistence and/or progression of pulmonary fibrosis.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF), the most common form of the idiopathic interstitial pneumonias, is a chronic and fatal disease of unknown cause. Nintedanib and pirfenidone have recently been approved in several countries for IPF treatment. While providing an important milestone in the treatment of IPF, these therapies are limited to slowing, but not stopping disease progression.[1] Therefore, more targeted therapies are required, focusing on the cellular and molecular pathogenesis of IPF, which remains poorly understood. IPF is characterised by progressive fibroblast and myofibroblast proliferation and extensive disordered deposition of extracellular matrix (ECM), resulting in destruction of the alveolar architecture. Fibroblasts and myofibroblasts are the matrix-producing cells responsible for the excessive deposition of ECM. This aberrant and disordered ECM deposition destroys the structural integrity of the lung and causes abnormal biomechanical and biochemical characteristics. There is increasing evidence that microenvironments created within the fibrotic ECM affect the behaviour of structural pulmonary cells and form a vicious cycle of fibrosis.[2]

Mast cells originate from CD34-expressing haematopoietic stem cells in the bone marrow.[3] Human mast cells exhibit heterogeneity and are

classified by their content of serine proteases as tryptase-only, chymase-only or both tryptase-positive and chymase-positive mast cells.[3] Tryptase-positive mast cells share some properties with rodent mucosal-type mast cells. Mucosal mast cells are usually found in the mucosal tissues of the lung and intestine and mainly express tryptase.[3] On the other hand, tryptase-positive and chymase-positive mast cells, which share characteristics with rodent connective tissue mast cells, are mainly found in the connective tissues of the intestinal submucosa, the peritoneal cavity, surrounding blood vessels and in the skin.[3] However, tissue distribution is not as clearly demarcated as in rodents and most human tissues have a mixed population of mast cell types.

Although mast cells are best known as major effector cells in allergic and acute inflammatory diseases, mast cells are speculated to associate with fibrosis.[4] Mast cells contain many profibrotic mediators, including tryptase, chymase, histamine, leukotrienes, renin and transforming growth factor (TGF)-β1.[5] Coculturing mast cells with fibroblasts has been shown to induce fibroblast proliferation, migration, collagen production, α-smooth muscle actin (α-SMA) expression, and fibroblast contraction by tryptase, histamine, leukotrienes and renin.[6–9] Furthermore, fibroblasts produce stem cell factors (SCF), which nourish mast cells.[9] These findings highlight a close interaction between mast cells and fibroblasts, which may contribute to form a profibrotic milieu that drives disease progression. Mast cell-derived chymase, which is a chymotrypsin serine protease, can also activate latent TGF-β1.[10 11] TGF-β is one of the most potent profibrotic factors in the development of pulmonary fibrosis through activation of fibroblasts and induction of fibroblast differentiation into myofibroblasts.[12 13] Thus, chymase can induce fibroblast proliferation and collagen synthesis through latent TGF-β1 activation,[11] and the activation and degranulation of mast cells may, directly and/or indirectly, contribute to tissue fibrosis.

The association of mast cells with IPF is both an old and new topic of research. Increased numbers of mast cells have long been known to be present in the fibrotic lung tissue in IPF or experimental pulmonary fibrosis model.[8 14–16] Nevertheless, mast cells have not been investigated as extensively as other cells during fibrogenesis, and their role in the progression of pulmonary fibrosis has not been pursued.

In this study, we assessed the impact of the interaction of mast cells with fibrotic ECM and the pathophysiological role of mast cells in pulmonary fibrosis development. Understanding how matrix microenvironments in fibrotic lungs control the phenotype and function of mast cells may offer new opportunities for targeting that cell as a therapeutic option in IPF.

MATERIALS AND METHODS

Detailed methods are described in the online supplementary materials and methods.

TGF-β1 adenovirus vector-induced pulmonary fibrosis

Female Sprague-Dawley rats (225–250 g; Charles River, Wilmington, MA, USA) were maintained in 12 hours light, 12 hours dark cycles with free access to food and water. Rats were given intratracheal instillations of TGF-β1 adenovirus (Ad-TGF-β1223/225) or control virus (Ad-DL) was prepared and treated to rats as previously described.[17] Rats received 5.0×10^8 plaque-forming unit virus in 300 µL sterile saline intratracheally and were culled on day 7, 14, 21 or 35 by terminal anaesthesia. For bleomycin model, rats received saline or bleomycin intratracheally (0.56 U in 300 µL of saline). Rats were culled on day 7, 14 or 21 by terminal anaesthesia. All work was conducted under the guidelines of the Canadian Council on Animal Care and approved by the Animal Research Ethics Board of McMaster University under protocol No 13.12.48. For details, see online supplementary materials and methods.

Human plasma and lung tissue

All plasma and tissues were collected with patient consent in compliance with the Research Ethics Board of St Joseph's Healthcare Hamilton. Hamilton Integrated Research Ethics Board (No 00-1839) approval was obtained prior to beginning the study. For details, see online supplementary materials and methods.

Mechanical stretch bath solution model

We modelled the breathing in the tissue bath by setting the resting tissue tension to 15 mN and cyclically stretching, oscillating at a frequency of 2 Hz, to a length of 1.1 times the original resting lung length.[18] For details, see online supplementary materials and methods.

Statistical analysis

All data were expressed as the mean±SD. Data were analysed with GraphPad Prism V.6.0 (GraphPad Software, La Jolla, CA) with either one-way analysis of variance (followed by Tukey's post hoc tests) or non-parametric analysis (Kruskal-Wallis test), followed by post-Dunn's test for multiple comparison. Statistical analysis between two groups was performed using a non-parametric Mann-Whitney test. Shapiro-Wilk normality test was used to check normal distribution. In all cases, a p value less than 0.05 was considered significant.

RESULTS

The number of mast cells is increased in IPF lung and tryptase levels are negatively correlated with lung function

Mast cells were detected by toluidine blue staining in lung tissues from non-fibrotic control and patients with IPF (FVC<60%). Mast cells were significantly increased in the fibrotic areas in IPF compared with control lung in toluidine blue staining and in chymase/tryptase immunofluorescence (figure 1A,B and online supplementary figure 1A). In addition to the increase in the number of mast cells, chymase-positive or tryptase-positive mast cells also expressed TGF-β1 in IPF lung (figure 1C,D and online supplementary figure 1B,C). To assess the correlation of mast cell number and lung function, patients were separated into three groups according to their FVC predicted value: mild IPF (FVC>90%, n=15), moderate IPF (90%<FVC>60%, n=20) and severe IPF (FVC<60%, n=15). The total number of mast cells (chymase-single-positive, tryptase-single-positive, and chymase and tryptase-double-positive cells) was significantly increased in moderate and severe IPF compared with control (control vs 60%<FVC<90%, mean difference=−482.2, 95% CI of difference −725.2 to −239.2, p<0.0001; control vs FVC<60%, mean difference=−467.6, 95% CI of difference −723.8 to −211.4, p<0.0001; FVC>90% vs 60%<FVC<90%, mean difference=−264.2, 95% CI of difference −507.2 to −21.2, p=0.028) (figure 1E). The chymase and tryptase-double-positive mast cells were significantly increased in moderate and severe IPF compared with control (control vs 60%<FVC<90%, mean difference=-222.7, 95% CI −386.3 to −59.2, p=0.004; control vs FVC<60%, mean difference=−301.7, 95% CI of difference −474.1 to −129.34, p=0.0001; FVC>90% vs 60%<FVC<90%, mean difference=−189.6, 95% CI of difference −353.1 to −26.03, p=0.017; FVC>90% vs FVC<60%,

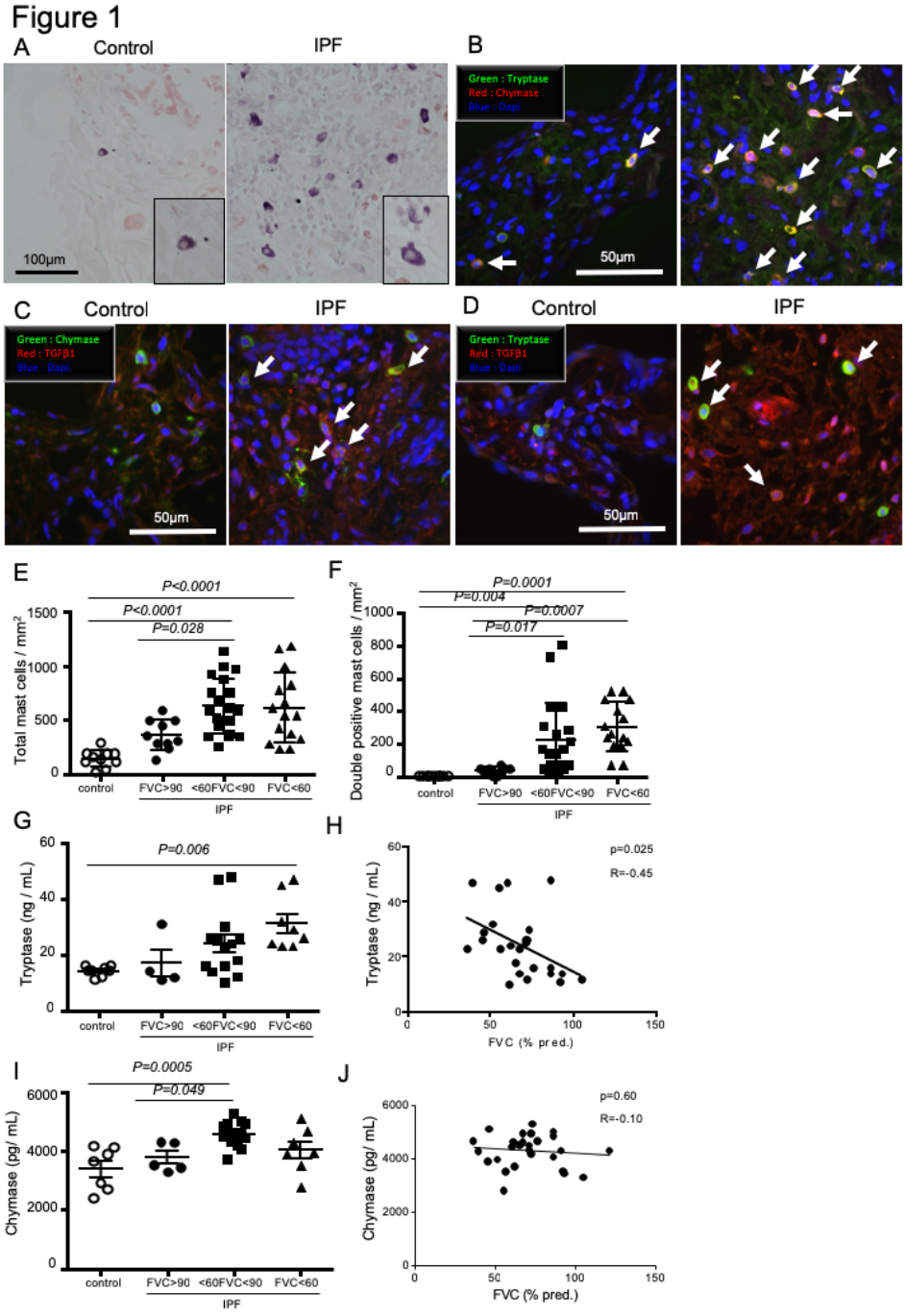

Figure 1 The number of mast cells is increased in idiopathic pulmonary fibrosis (IPF) lung and tryptase levels are negatively correlated with lung function. (A) Mast cells were detected by toluidine blue staining in non-fibrotic control lung and in IPF (FVC<60%) lung. (B) Tryptase-positive and chymase-positive mast cells were detected by immunofluorescence. Green is tryptase, Red is chymase and Blue is Dapi. Tryptase and chymase-double-positive cells are indicated by white arrow. The lung tissues are from non-fibrotic control lung and IPF (FVC<60%) lung. Representative pictures are shown for each group. (C) Transforming growth factor (TGF)-β1-positive cells were detected in chymase-positive mast cells by immunofluorescence. Green is chymase, Red is TGF-β1 and Blue is Dapi. Chymase and TGF-β1-double-positive cells are indicated by white arrow. The lung tissues are from non-fibrotic control lung and IPF (FVC<60%) lung. Representative pictures are shown for each group. (D) TGF-β1-positive cells were detected in tryptase-positive mast cells by immunofluorescence. Green is tryptase, Red is TGF-β1 and Blue is Dapi. Tryptase and TGF-β1-double-positive cells are indicated by white arrow. The lung tissues are from non-fibrotic control lung and IPF (FVC<60%) lung. Representative pictures are shown for each group. (E) The total number of mast cells (including chymase-single-positive cells, tryptase-single-positive cells, and chymase and tryptase-double-positive cells) was counted in control and IPF lung. Patients with IPF were separated into three groups according to their FVC predicted value: mild IPF (FVC>90%), moderate IPF (90%<FVC>60%) and severe IPF (FVC<60%). (F) Chymase and tryptase-double-positive cells were counted in IPF lung. (G) Plasma levels of tryptase measured by ELISA for patients with IPF and respective age-matched controls. (H) Correlation curves between the plasma levels of tryptase in patients with IPF and clinical lung function parameters. (I) Plasma levels of chymase measured by ELISA in patients with IPF and respective age-matched controls. (J) Correlation curves between the plasma levels of chymase in patients with IPF and clinical lung function parameters. Results are presented as mean ± SD. Data were analyzed by one-way ANOVA and Tukey's post-hoc test.

mean difference=−268.6, 95% CI of difference −441.0 to −96.17, p=0.0007) (figure 1B,F, online supplementary figure 1A). Interestingly, chymase and tryptase-double-positive cells were significantly increased about three times in the severe IPF group compared with the mild group (figure 1F). The plasma levels of chymase and tryptase were measured in patients with IPF and age-matched controls. Interestingly, plasma tryptase was significantly higher in severe IPF (control vs FVC<60%, mean difference=−17.12, 95% CI of difference −30.02 to −4.22, p=0.006) (figure 1G) and was negatively correlated with FVC (R=−0.045, p=0.025) (figure 1H). Conversely, plasma chymase was upregulated in only patients with moderate IPF compared with controls and mild IPF (figure 1I), and there was no correlation between chymase plasma level and FVC (figure 1J).

Mast cells are increased during the chronic phase of lung fibrosis in experimental pulmonary fibrosis model

The AdTGF-β1-induced and bleomycin-induced pulmonary fibrosis in rats were employed to investigate mast cell function in experimental pulmonary fibrosis. Toluidine blue staining showed that mast cells accumulated (dark purple) in the fibrotic lesions (blue colour in Masson's trichrome staining) in rat lungs during the chronic phase of the AdTGF-β1 model (after day 21), well after the fibrogenic process was established, but not at earlier time points (DL vs d21, p=0.025; DL vs d35, p=0.047) (figure 2A,B). A similar finding in the number of mast cells was observed in the bleomycin model (phosphate-buffered saline (PBS) vs d14, p=0.011; PBS vs d21, p=0.04) (figure 2C,D). Immunofluorescence staining of chymase and tryptase suggested the recruitment of only a few mast cells to the parenchymal area at day 14 in the AdTGF-β1 pulmonary fibrosis lung (figure 2E). However, a small number of chymase-single-positive mast cells accumulated around the bronchial area (figure 2E). By day 21, a significant amount of chymase and tryptase-double-positive cells was present in the parenchyma (figure 2E) while there was no accumulation of mast cells around the bronchi (figure 2E). Chymase, tryptase, SCF and TGF-β1 mRNA levels were measured in fibrotic lungs after AdTGF-β1 to assess the effect of TGF-β1-induced pulmonary fibrosis on these mast cell-related mRNA expressions (figure 2F). Chymase, tryptase and TGF-β1 mRNA levels tend to be increased in fibrotic lung compared with control (chymase, main effect p=0.019; tryptase, main effect p=0.014; TGF-β1, main effect p=0.006) (figure 2F). Tryptase mRNA level was increased at day 35 compared with control (main effect p=0.014, DL vs day 35, p=0.018) (figure 2F). SCF mRNA was decreased at day 21 compared with control (main effect p=0.011, DL vs day 21, p=0.022) (figure 2F).

Bone marrow- derived mast cells reseeded on decellularised fibrotic ECM release active and total TGF-β1

The effect of the ECM on mast cell phenotype was evaluated on decellularised normal versus fibrotic lungs. Decellularised lungs were prepared from day 21 Ad-DL (normal) or AdTGF-β1 (fibrotic) treated rats. Mouse bone marrow-derived mast cells (BMMC) were reseeded in the decellularised lung and recellularised lungs were cut into strips and cultured for 5 days in cell culture media. H&E, toluidine blue and immunofluorescence staining showed that mast cells successfully recellularised the decellularised normal and fibrotic ECM (figure 3A,B). Immunofluorescence also showed successful recellularisation of tryptase-positive mast cells which exhibited a TGF-β1-positive phenotype (figure 3B). Five days after recellularisation, active and total TGF-β1 content in the culture media was evaluated by ELISA. Non-fibrotic and fibrotic ECMs which were not reseeded with mast cells released no active TGF-β1 in cell culture media (figure 3C,E). This indicates that decellularised fibrotic lung per se did not release any active TGF-β1 in culture media. BMMC recellularised in non-fibrotic ECM did not release any active TGF-β1, however, BMMC recellularised in fibrotic ECM secreted active TGF-β1 (no cells-DL vs BMMC-TGF-β1 p=0.0005, no cells-TGF-β1 vs BMMC-TGF-β1 p=0.0008, BMMC-DL vs BMMC-TGF-β1 p=0.0005) (figure 3C). On the other hand, minimal amount of total TGF-β1 was released from decellularised no-fibrotic and fibrotic lung with no cells (figure 3D,F). Importantly, BMMC recellularisation of the fibrotic ECM induced a marked increase in total TGF-β1 while no increase in total TGF-β1 release in BMMC recellularised on normal ECM (no cells-DL vs BMMC-TGF-β1 p=0.0003, BMMC-DL vs BMMC-TGF-β1 p=0.013) (figure 3D). Rat peritoneal mast cells (PMC) also showed similar result as BMMC, such as rat PMCs recellularised in fibrotic ECM secreted active (no cells-DL vs PMC-TGF-β1 p=0.0003, PMC-DL vs PMC-TGF-β1 p=0.0003) (figure 3E) and total TGF-β1 (no cells-DL vs PMC-TGF-β1 p=0.0005, no cells-TGF-β1 vs PMC-TGF-β1 p=0.045, PMC-DL vs PMC-TGF-β1 p=0.001) (figure 3F). These results demonstrate that BMMC and PMC recellularised in the fibrotic ECM secrete an increased amount of profibrotic active and total TGF-β1.

Mechanical stretch induces mast cell degranulation and activates TGF-β1 in fibrotic lung

To investigate whether mechanical stretch can activate TGF-β1 in the whole lung, we employed an ex vivo lung ventilation system. Fibrotic rat lungs were hooked on a flexivent and were ventilated at 5 cmH_2O pressure (control) or at total lung capacity (30 cmH_2O) every 20 s for 20 min. The ventilation challenge induced an increase of mast cell degranulation compared with control ventilation in AdTGF-β1-treated lung (95%CI −2.00 to 43.00, p=0.048) (figure 4A). Then, fibrotic lung slices (AdTGF-β1) were stimulated with cyclic mechanical stretch showing an increase in mast cell degranulation (Non-stretched-cont vs Stretched-cont p=0.029) (figure 4B). Doxantrazole, a mast cell stabiliser, attenuated mechanical stretch-induced mast cell degranulation detected by toluidine blue staining (Stretched-cont vs Stretched-dox p=0.045) (figure 4B). Secreted histamine and active TGF-β1 were measured before and after mechanical cyclic stimulation of the fibrotic lung slices. Mechanical stretch induced the release of histamine and active TGF-β1 from fibrotic lungs (figure 4C: Before-cont vs After-cont p=0.042, figure 4D: Before-cont vs After-cont p=0.002, Before-crom vs After-cont p=0.012, figure 4E: Before-cont vs After-cont p=0.029, figure 4F: Before-cont vs After-cont p=0.0009) (figure 4C–F). Cromoglycate appeared to reduce mechanical stretch-induced histamine and active TGF-β1 release (figure 4C,D). Doxantrazole significantly inhibited stretch-induced histamine and tend to attenuate active TGF-β1 release in the bath solution (After-cont vs After-dox p=0.004) (figure 4E,F). Furthermore, doxantrazole markedly attenuated mechanical stretch-induced β-hexosaminidase in the bath solution (Before-cont vs After-cont, mean difference=−0.8550, 95% CI of difference −1.550 to −0.1602, p=0.014; Before-dox vs After-cont, mean difference=−1.169, 95% CI of difference −1.906 to −0.4323, p=0.002; After-cont vs After-dox, mean difference=1.007, 95% CI of difference 0.2700 to 1.744, p=0.007) (figure 4G). Additionally, protein extracts from lung strips were collected 6 hours after mechanical stimulation to assess Smad2 phosphorylation, as an indication of downstream activation by the released TGF-β1. There was

Figure 2

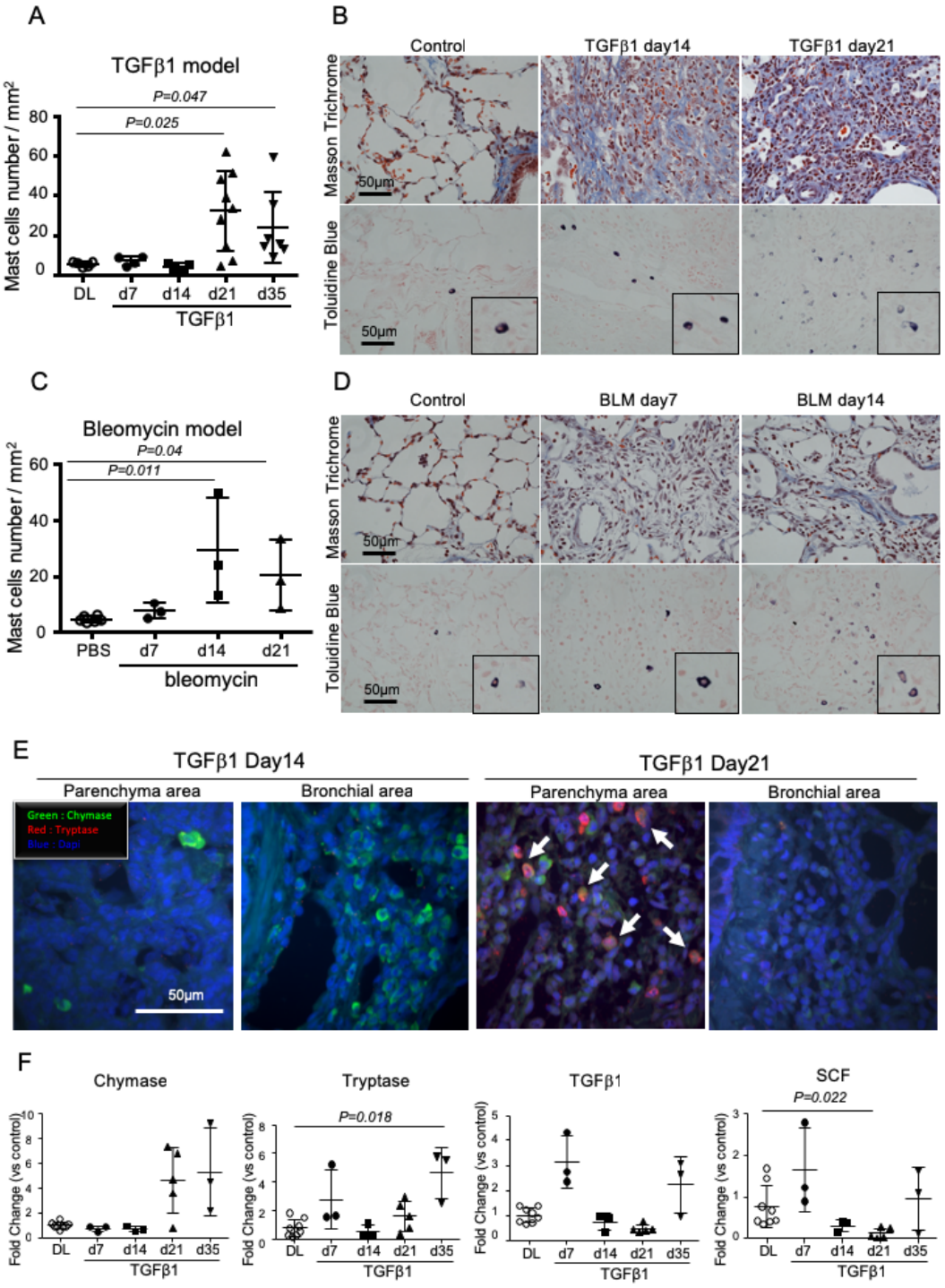

Figure 2 Mast cells are increased during the chronic phase in experimental pulmonary fibrosis. (A) Mast cells were detected by toluidine blue staining in the lungs of AdTGF-β1 pulmonary fibrosis model at days 7, 14, 21 and 35, and Ad-DL treated control lung . (B) The representative pictures of toluidine blue and Masson's trichrome staining in non-fibrotic control lung and AdTGF-β1-induced pulmonary fibrosis at days 14 and 21 are shown. (C) Mast cells were detected by toluidine blue staining in bleomycin-induced pulmonary fibrosis at days 7, 14 and 21, and phosphate-buffered saline (PBS) treated control lung . (D) The representative pictures of toluidine blue and Masson's trichrome staining in non-fibrotic control lung and bleomycin-induced pulmonary fibrosis at days 7 and 14 are shown. (E) Immunofluorescence shows chymase-positive cells and tryptase-positive cells in fibrotic lung parenchymal area and bronchial area in AdTGF-β1 pulmonary fibrosis at days 14 and 21. Green is chymase, Red is tryptase and Blue is Dapi. Tryptase and chymase-double-positive cells are indicated by white arrow. Representative pictures are shown for each group. (F) Chymase, tryptase, stem cell factor (SCF) and TGF-β1 mRNA levels were detected by real-time (RT) PCR in the lung tissue of non-fibrotic control and AdTGF-β1-induced pulmonary fibrosis at days 7, 14, 21 and 35. All results are presented as mean±SD. Data were analyzed with Kruskal-Wallis non-parametric test followed by Dunn's post-hoc test. TGF, transforming growth factor; BLM, bleomycin.

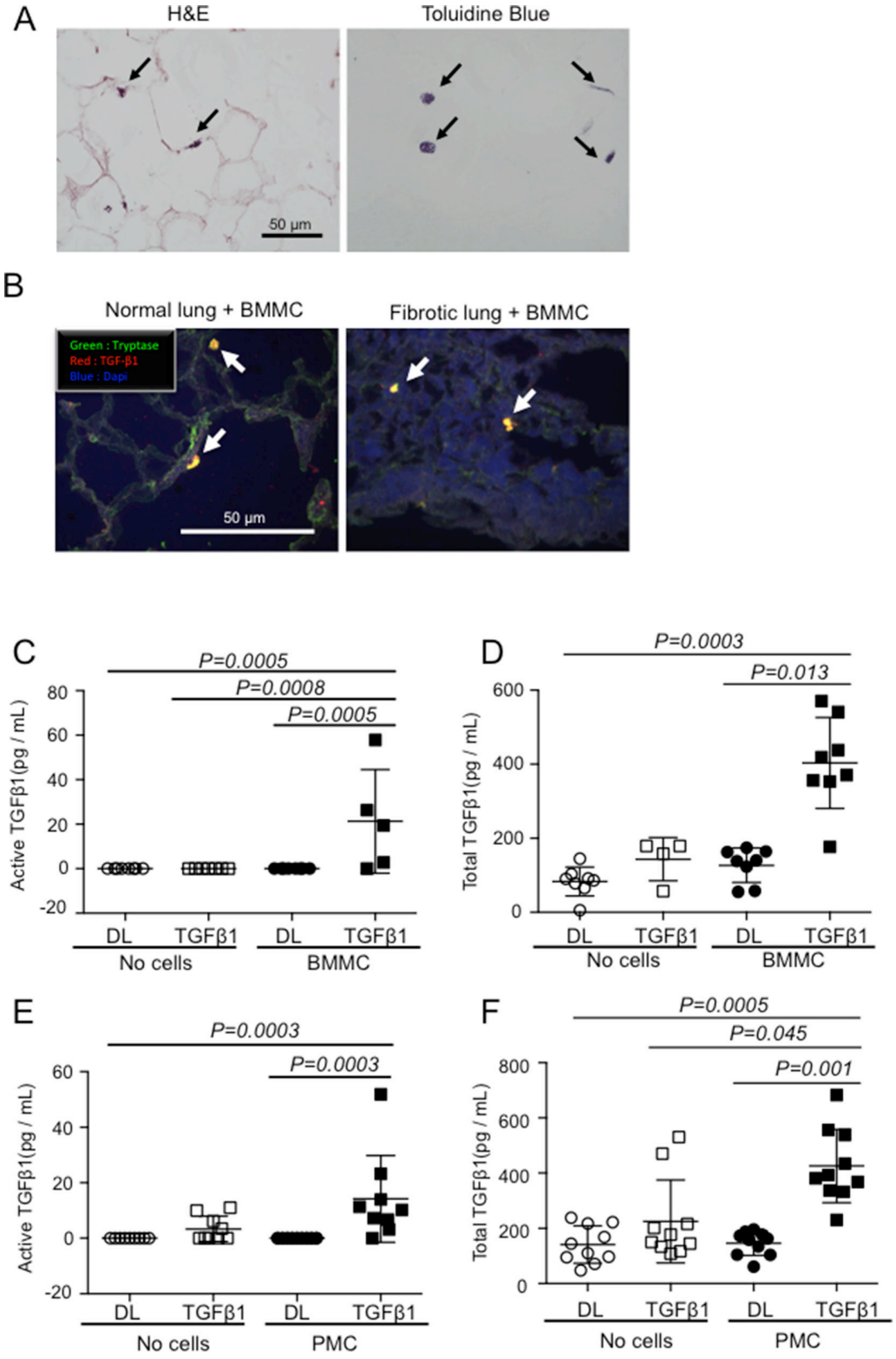

Figure 3 Bone marrow-derived mast cells reseeded on decellularised fibrotic extracellular matrix (ECM) release active and total TGF-β1. Decellularised rat lungs were prepared in day 21 Ad-DL or AdTGF-β1 treated rat lung. Mouse bone marrow-derived mast cells (BMMC) were reseeded in the decellularised lung. Recellularised lungs were sliced and cultured for 5 days in cell culture media. (A) Representative pictures of H&E and toluidine blue staining of BMMC reseeded decellularised lung. Positive cells are indicated by black arrow. (B) Tryptase and TGF-β1-positive cells were detected by immunofluorescence for BMMC recellularised in non-fibrotic and fibrotic lungs. Green is tryptase, Red is TGF-β1 and Blue is Dapi. Double-positive cells are indicated by white arrow. (C and D) BMMCs were reseeded in the decellularised non-fibrotic or fibrotic lung. Active and total TGF-β1 levels in cell culture media were measured by ELISA. Non-fibrotic lung with no cells (no cells-DL), fibrotic lung with no cells (no cells-TGF-β1), non-fibrotic lung with BMMC (BMMC-DL), fibrotic lung with BMMC (BMMC-TGF-β1). (E and F) Rat peritoneal mast cells (PMC) were reseeded in the decellularised non-fibrotic or fibrotic lung. Active and total TGF-β1 levels in cell culture media were measured by ELISA. Results are presented as mean±SD. Data were analyzed with Kruskal-Wallis non-parametric test followed by Dunn's post-hoc test. TGF, transforming growth factor.

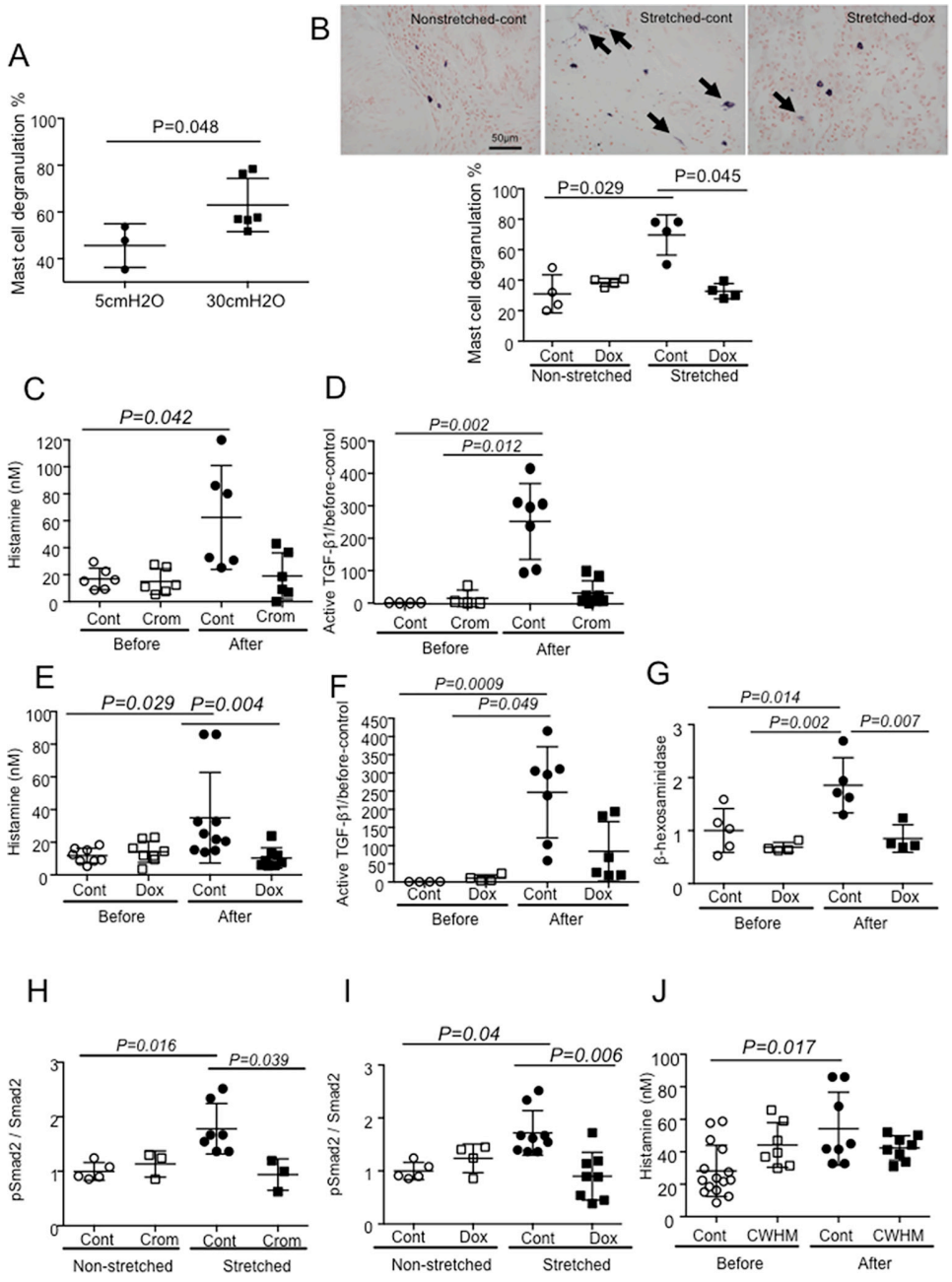

Figure 4 Mechanical stretch induces mast cell degranulation and activates TGF-β1 in fibrotic lung. (A) In the ventilation study, the control group received 5 cm H_2O ventilation and challenge group received 30 cm H_2O ventilation in fibrotic rat lungs (AdTGF-β1, day 21). Lung tissue was stained with toluidine blue. Mast cell degranulation was defined as cells showing the release of 10% cellular granules. (B) Fibrotic lung slices (AdTGF-β1, day 21) were submitted to cyclic mechanical stretch and stained with toluidine blue to evaluate mast cell degranulation. Degranulated mast cells are indicated by black arrow. Non-stretched-Cont group refers to a fibrotic lung strip without mechanical stretch, Non-stretched-Dox group refers to doxantrazole (Dox) soaked fibrotic lung strip without mechanical stretch, Stretched-Cont group fibrotic lung strip with mechanical stretch, and Stretched-Dox group Dox soaked fibrotic lung strip with mechanical stretch. (C and D) The effect of cromoglycate (Crom) on mechanical stretch-induced mast cell degranulation and active TGF-β1 release. Histamine (C) and active TGF-β1 levels (D) in bath solution were measured by ELISA and PAI/l uciferase reporter assay. Bath solution was taken before (Before) and after mechanical stimulation (After) from each lung strip without or with Crom. (E and F) The effect of doxantrazole (Dox) on mechanical stretch-induced mast cell degranulation. Histamine (E) and active TGF-β1 levels (F) in bath solution were measured by ELISA and PAI/ l uciferase reporter assay. Bath solution was taken before and after mechanical stimulation from each lung strip without or with Dox. (G) β-hexosaminidase was measured in bath solution before and after mechanical stimulation from each lung strip without or with Dox. (H and I) Phosphorylated Smad2 expression in fibrotic rat lung tissues soaked with Crom (H) or Dox (I) was measured by western blotting. Smad2 represents loading control. (J) The effect of αv-integrin inhibitor (CWHM-000012-8) on mechanical stretch-induced mast cell degranulation. Histamine level in bath solution was measured by ELISA. Bath solution was taken before and after mechanical stimulation from each lung strip without or with CWHM. All results are presented as mean±SD. Data were analyzed with Mann Whitney test (A), Kruskal-Wallis nonparametric test followed by Dunn's post-hoc test (B-F, H-J) or one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test (G). TGF, transforming growth factor.

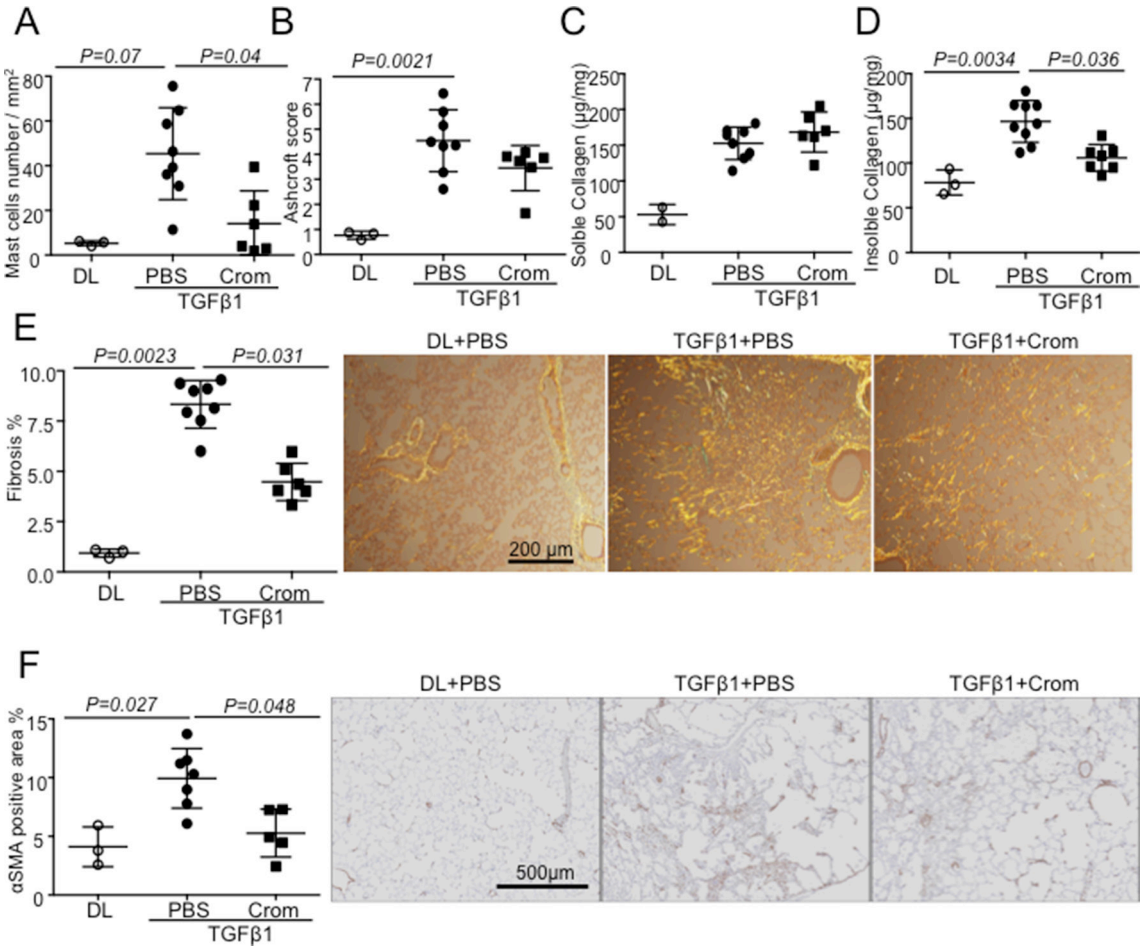

Figure 5 The effect of mast cell stabilisers in TGF-β1-induced pulmonary fibrosis rats. Therapeutic effect of cromoglycate on TGF-β1-induced pulmonary fibrosis. Fourteen days after the first vector (AdTGF-β1), drug cromoglycate (Crom) or PBS treatment was started. Rats were examined 21 days after first vector exposure. Control rats were treated PBS (DL). (A) Mast cell number was counted in toluidine blue stained lung tissue. (B) Ashcroft scoring following Masson's trichrome staining was performed by 5 independent individuals. (C) Soluble collagen content in the lung tissue. (D) Insoluble collagen content in the lung tissue. (E) Fibrotic area was calculated from Picrosirius Red (PSR) staining. Bright colour shows collagen. (F) α-SMA-positive area was calculated. The image was taken by automatic slide scanner microscope. All results are presented as mean±SD. Data were analyzed with Kruskal-Wallis non-parametric test followed by Dunn's post-hoc test. PBS, phosphate-buffered saline; SMA, smooth muscle actin; TGF, transforming growth factor.

a significant increase in Smad2 phosphorylation in fibrotic lung tissues after stretch compared with the unstretched lung. The mast cell stabilisers used inhibited mechanical stretch-induced TGF-β1 signalling pathway in lung tissues, as indicated by reduced levels of pSmad2 (figure 4H: Non-stretched-cont vs Stretched-cont p=0.016, Stretched-cont vs Stretched-crom p=0.039, figure 4I: Non-stretched-cont vs Stretched-cont p=0.04, Stretched-cont vs Stretched-dox p=0.006) (figure 4H,I). An αv-integrin inhibitor, CWHM-000012-8, had no effect on the release of histamine induced by mechanical stretch (figure 4J).

The effect of mast cell stabilisers on TGF-β1-induced pulmonary fibrosis in rat

The therapeutic effect of the mast cell stabiliser, cromoglycate, was investigated in TGF-β1-induced pulmonary fibrosis. Cromoglycate treatment effect, in a therapeutic protocol after fibrogenesis was established, from day 14 to day 21, on the number of mast cells in the lung parenchyma in the TGF-β1-induced fibrosis model (PBS vs Crom p=0.04) (figure 5A). Cromoglycate treatment also appeared to attenuate the Ashcroft score (histological fibrosis scoring system) (DL vs PBS p=0.002) (figure 5B). Although cromoglycate did not show any effect on soluble collagen content of the lungs (figure 5C, total effect p=0.042), in contrast, cromoglycate treatment significantly attenuated insoluble collagen content in the lungs (DL vs PBS p=0.0034; PBS vs Crom p=0.036) (figure 5D). Fibrotic areas as shown by Picrosirius Red staining in AdTGF-β1-induced pulmonary fibrosis were reduced by cromoglycate treatment (DL vs PBS p=0.0023, PBS vs Crom p=0.031) (figure 5E). Furthermore, α-SMA-positive area in the lung parenchyma was significantly attenuated by cromoglycate (DL vs PBS p=0.027, PBS vs Crom p=0.048) (figure 5F). These data suggest that therapeutic administration of cromoglycate has an effect on mature collagen accumulation in TGF-β1-induced pulmonary fibrosis with potential impact on disease progression.

DISCUSSION

Our study showed that the number of mast cells is increased in the fibrotic lung parenchyma of patients with IPF, with most of them being TGF-β1 positive. This result is consistent with previous studies that also found increased mast cells[14–16] and their TGF-β1-positive phenotype in IPF lungs.[15] Our decellularised

lung experiments also showed that mouse BMMC or rat PMCs were activated while reseeded in fibrotic matrix as shown by an increase in the secretion of active and total TGF-β1. This is the first study to directly show that the fibrotic lung ECM itself can have an effect on mast cell function and phenotype. A recent study demonstrated that TGF-β1-positive mast cells were increased in skin fibrosis in patients with secondary lymphoedema.[19] These findings suggest that the profibrotic ECM induces a profibrotic phenotype in the mast cells, increasing TGF-β1 content and possible release.

Interestingly, mast cell phenotypes may also indicate the disease severity. Our data showed that the number of chymase and tryptase-double-positive mast cells was negatively correlated with lung function in IPF. Furthermore, our current data also showed that plasma chymase level was increased in patients with moderate IPF (FVC 60%–90%), and tryptase level was increased in patients with advanced IPF (FVC<60%), and plasma tryptase levels showed a negative correlation with lung function. It is suggested that chymase-positive mast cells may be increased in moderate IPF and tryptase-positive mast cells may be increased in advanced IPF. Our experimental fibrosis model study also supports this hypothesis. In rat AdTGF-β1 pulmonary fibrosis lung, small numbers of chymase-positive mast cells accumulated at day 14 around bronchi and chymase and tryptase-double-positive cells were increased in the fibrotic parenchyma after day 21. These findings demonstrate that mature mast cells accumulate in fibrotic lesions in the chronic phase of pulmonary fibrosis. Previous in vitro studies reported that fibroblasts induce mast cell phenotype changes from tryptase-single-positive to chymase and tryptase-double-positive mast cells in a coculturing system.[20] Moreover, mast cells from human pulmonary fibrosis develop a tryptase and chymase-positive phenotype and their numbers correlate with the accumulation of myofibroblasts expressing α-SMA.[21] Furthermore, Andersson *et al* also showed that tryptase and chymase-double-positive mast cells were increased in IPF lung and the number of double-positive mast cells was correlated with the degree of fibrosis and parenchymal collagen density.[15] In contrast, a study showed that the increased chymase-positive mast cell density in IPF predicted slower disease progression.[16] Mast cell phenotype diversity depending on the disease severity may explain the discrepancy of the mast cell number or related mediators and disease severity.

The biophysical properties of the matrix regulate a variety of cellular mechanosensory pathways and profibrotic responses. These biomechanical changes deliver important spatial and contextual cues to drive normal and abnormal cell behaviours in fibroblasts and epithelial cells.[2] Moreover, previous studies including our study demonstrate that mechanical stretch induces latent TGF-β1 activation in stiff substrates and fibrotic lung.[18 22] TGF-β1 is secreted in a latent inactive form[10] and is secreted as a large latent complex, consisting of TGF-β1, latency associated protein (LAP) and latent TGF-β-binding protein 1. Several cellular mechanisms have been described that activate latent TGF-β1 by promoting its dissociation from LAP as active TGF-β.[13] In the lungs, TGF-β1 is produced by a wide range of cell types, including epithelial cells, fibroblasts, myofibroblast, endothelial cells, platelets, macrophages, neutrophils and mast cells.[5 23 24]

Previous experiments have shown that chymase and TGF-β1 can be released by mast cells, and chymase immediately activates latent TGF-β1 complexes by disrupting the non-covalent interaction between the LAP TGF-β1 and mature TGF-β1.[10 11] A recent study found that both active TGF-β1 and LAP TGF-β1 were overexpressed in fibrotic skin.[19] A recent in vitro study also showed that mechanical loading stimulates activation and degranulation of RBL-2H3 cells, a rat basophilic leukaemia cell line through RGD integrin.[25] Our ex vivo experiments showed that mechanical stretch activates mast cell degranulation and TGF-β1 signalling pathway in fibrotic rat lungs. Mast cell stabilisers attenuated the mechanical stress-induced mast cell degranulation and TGF-β1 signalling pathway activation. These data suggest that mast cells are sensitive to mechanical stress in fibrotic lungs and mechanical stretch-induced mast cell degranulation also induces TGF-β1 activation. Therefore, mechanical stiffness is a crucial factor for mast cell activation, and mast cells may play an important role to form a vicious cycle for fibrosis persistence and/or progression. Although we have previously reported that αv-integrin inhibitors affect mechanical stretch-induced TGF-β1 signalling pathway,[18] αv-integrins may not be involved in the stretch-induced mast cell degranulation in this model. Although further investigation is required to identify the specific mechanosensor for the mast cell, these data indicate RGD integrins other than αv-integrin, such as α4β1 and α4β7 integrins, may contribute to this mechanism.[25 26]

Booth *et al* have developed an elegant model of a decellularised human lung, on which primary fibroblasts are seeded.[27] They showed that fibrotic ECM promotes TGF-β-independent myofibroblast differentiation compared with normal ECM.[27] A recent study showed that aged mouse acellular lung matrix induced ECM components (laminin, elastin, fibronectin and collagen) in human bronchial epithelial cells and lung fibroblasts compared with young acellular lung matrix.[28] The current study also showed that fibrotic acellular murine lung induces further TGF-β1 release from murine BMMC and PMC. Only mast cell reseeded decellularised fibrotic lung release significant amount of active and latent TGF-β1 in the cell culture media in this study, therefore, mast cells are one of the source of active/latent TGF-β1 in fibrotic lung. Interestingly, while no active TGF-β1 was released in decellularised no-fibrotic and fibrotic lung without reseeding cells, limited amount of latent TGF-β1 was released from decellularised no-fibrotic and fibrotic lung. It is known that latent TGF-β1 is stored in ECM, and latent TGF-β1 was detected in decellularised non-fibrotic and IPF human lung in previous study by proteomics.[27] These results indicate that the presence of a profibrotic microenvironment can have a major effect on the characteristics of these cells and mast cell would be a source of active/latent TGF-β1 in fibrotic lung.

Previous pharmacological studies demonstrated that mast stabiliser attenuated bleomycin-induced pulmonary fibrosis in rodents.[29 30] Other studies employed mast cell deficient animals, however, these results have shown controversial results regarding the role of mast cell in experimental pulmonary fibrosis.[8 31–33] All these pharmacological and genetically modified mice studies evaluated preventive effects of drugs, and not the therapeutic effects. To better apply these findings to a more clinically relevant setting, it might be necessary to also assess the efficacy of the drug therapeutically, after the establishment of the disease.[34] Our animal model data show that mast cell accumulation in the fibrotic lesions in rat lung occurred only during the chronic phase of both the AdTGF-β1-induced (after day 21) and bleomycin-induced fibrosis (after day 14). Day 21 in TGF-β1 model and day 14 in bleomycin model are considered as the chronic periods with robust deposition of fibrosis in lung parenchyma. Mature granulated chymase and tryptase-double-positive mast cells are increased in the late phase of fibrosis in the AdTGF-β1 model, day 21. At day 14, a few chymase-positive cells were observed around the bronchial area but not in fibrotic lung parenchymal regions. It suggests that matured mast cells

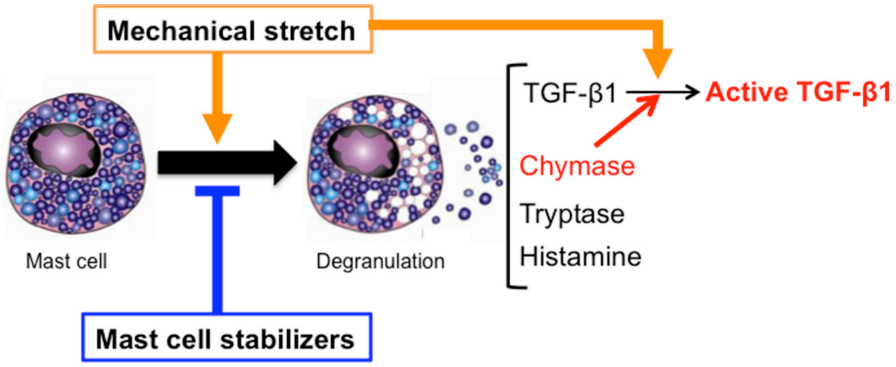

Figure 6 Mechanical stretch-induced mast cell and TGF-β1 activation. Our data indicate that mechanical stress activates mast cell degranulation and TGF-β1 signalling pathway in fibrotic lung. Mast cell stabilisers attenuated the mechanical stress-induced mast cell degranulation and TGF-β1 signalling pathway activation. TGF, transforming growth factor.

accumulate in advanced IPF and chronic phase of experimental fibrosis, therefore, mast cells may contribute to the pulmonary fibrosis progression rather than initiation. Our therapeutic study showed that cromoglycate had a limited effect on AdTGF-β1-induced pulmonary fibrosis development.

Our study has some limitations. First one is the relatively small sample size in each arm. However, we were committed to follow the 3R ethical principles in animal research[35] and used the smallest possible number of animals. Second, while the current study showed the effect of fibrotic ECM on mast cell function and phenotype, further studies are warranted to delineate detailed mechanisms. Third, there is no available drug to inhibit mast cells specifically. The use of mast cell stabilisers is the most basic pharmacological approach. However, these drugs are not specific and stabilisers just suppress mast cells but do not delete them. In the fibrotic microenvironment, ECM and surrounding cells provide profibrotic growth factors and induce stiffness. Therefore, mast cells are continuously exposed to profibrotic stimuli, counteracting the action of stabilisers which, additionally, may have reduced access to mast cells in the fibrotic lung. Furthermore, as we showed in the stretch study, mast cells are sensitive to mechanical stress in fibrotic lung, therefore, mast cells may be stimulated and activated while breathing within stiff fibrotic lungs. Alternative avenues may be required to inhibit mast cell function in pulmonary fibrosis. First, SCF is an important mast cell survival and proliferation factor[4] although we could not find any increase in SCF mRNA levels in TGF-β1-induced pulmonary fibrosis rat lung. SCF deficient mice are protected from bleomycin-induced pulmonary fibrosis and therapeutic anti-SCF treatment also inhibits bleomycin-induced lung fibrosis in mice.[36] Although the participation of mast cells seems limited in their study and mast cells are not the only cells responsive to SCF, SCF-cKit axis may be an interesting target for pulmonary fibrosis. A second target of mast cell inhibitors is the activity of specific mast cell mediators, such as tryptase. A third potential target is the recruitment of mast cells to the lung tissue. The mechanism of mast cell infiltration is not fully understood, but it is suggested that mast cell infiltration is regulated by α4β1 and α4β7 integrins.[26] A fourth target, adjusting the profibrotic ECM microenvironment, would provide an important avenue for fibrosis therapy. Attenuating lung stiffness by inhibiting cross-linking enzymes[37–39] or mechnotranductions[40 41] may also be an interesting target in addition to mast cells.

In conclusion, the present study has demonstrated that the profibrotic ECM environment affects mast cell phenotype and function and that mast cells are sensitive to mechanical stress in fibrotic lungs and contribute to mechanical stretch-induced TGF-β1 activation (figure 6, table 1). This study indicates that mast cells may have an important role in pulmonary fibrosis progression and are a potential treatment target for fibrotic diseases. Studying the relationship between mast cells and ECM microenvironments sheds light on the mechanisms for pulmonary fibrosis progression and may lead to the development of novel therapeutic strategies for fibrotic diseases, particularly IPF.

Table 1 Summary of the study

Species	Experiment	Result
Human	Lung biopsy	Total mast cell number was increased in IPF lung.
		The number of tryptase and chymase-double-positive mast cells was increased and correlated with the disease severity as measured by FVC.
	Plasma	Plasma tryptase, but not chymase levels were markedly increased in patients with severe IPF and negatively correlated with FVC.
Rat	Ex vivo	Mast cells reseeded on fibrotic lung matrix released more active and total TGF-β1 in the culture medium compared with mast cells reseeded on non-fibrotic lung matrix.
		Mechanical stretch-induced mast cell degranulation, TGF-β1 release and its signalling pathway activation in fibrotic lung. Mast cell stabilisers attenuated it.
	In vivo	Mast cell was increased at well after the fibrogenic process was established, but not at earlier time points.
		At day 14, chymase and tryptase-double-positive mast cells are few at parenchymal area, however, a small number of chymase-single-positive mast cells accumulated around the bronchial area. By day 21, a significant amount of chymase and tryptase-double-positive mast cells was present in the lung parenchyma.
		Cromoglycate attenuated the development of TGF-β1-induced pulmonary fibrosis.

IPF, idiopathic pulmonary fibrosis; TGF, transforming growth factor.

Correction notice This article has been corrected since it was published Online First. A system error meant that information from the system was erroneously included in the Abstract. This has now been fixed.

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Contributors CS conducted experiments and data analysis, prepared figures, advised on study design, and wrote and submitted the final manuscript approved by all authors. CU performed some of the experimental work and edited the manuscript.

PSB, EAA, SS and TY contributed to data analysis and interpretation, and editing the manuscript. QZ and AO performed some experiment, data analysis and manuscript editing. KA, JG and PF advised on study design and edited the manuscript. MRJK designed the study and supervised the project, and edited the manuscript.

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