Journal of Pathology
J Pathol 2018; **245:** 399–409
Published online 5 June 2018 in Wiley Online Library
(wileyonlinelibrary.com) DOI: 10.1002/path.5092

ORIGINAL PAPER

An ST2-dependent role of bone marrow-derived group 2 innate lymphoid cells in pulmonary fibrosis

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Abstract

Recent evidence supports that bone marrow (BM)-derived hematopoietic progenitor cells play an important role in lung injury and fibrosis. While these cells give rise to multiple cell types, the ST2 (Il1rl1)-expressing group 2 innate lymphoid cells (ILC2s) derived from BM progenitors have been implicated in tissue repair and remodeling, including in lung fibrosis. To further investigate the precise role of BM-derived ILC2s in the pathogenesis of fibrotic lung disease, their importance in the bleomycin-induced lung fibrosis model was evaluated by analyzing the effects of selective ST2 deficiency in the BM compartment. The results showed that while ST2-sufficient control mice exhibited activation of lung IL-33/ST2 signaling, ILC2 recruitment, IL-13 induction, and fibrosis, these responses were significantly diminished in ST2-deficient-BM chimera mice, with selective loss of ST2 expression only in the BM. This diminished response to bleomycin was similar to that seen in ST2 global knockout mice, suggesting the predominant importance of ST2 from the BM compartment. In wild-type mice, ILC2 recruitment to the lung was accompanied by a concomitant decrease in $ST2^+$ BM cells. ST2-deficient BM cells were unresponsive to IL-33-induced ILC2 maturation. Finally, lineage-negative wild-type, but not ST2-deficient BM cells from bleomycin-treated mice stimulated lung fibroblast type I collagen expression, which was associated with elevated TGFβ expression in the BM cells. Taken together, these findings suggested that the BM-derived ILC2s were recruited to fibrotic lung through the IL-33/ST2 pathway, and contributed to fibroblast activation to promote lung fibrosis.
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Keywords: bone marrow; ILC2; IL-33; ST2; Il1rl; fibrosis

Received 15 January 2018; Revised 17 April 2018; Accepted 26 April 2018

No conflicts of interest were declared.

Introduction

Pulmonary fibrosis is a common end result of multiple insults to the lung [1]. Idiopathic pulmonary fibrosis (IPF) is the most common fibrotic interstitial lung disease of uncertain etiology with a poor prognosis [2]. A growing body of evidence suggests the importance of bone marrow (BM)-derived cells in animal model studies [3–6]. BM-derived lung hematopoietic progenitor cells rapidly migrate into the lung after injury, and subsequently contribute to fibrogenesis in a paracrine manner [4]. Moreover, BM $CD11c^+$ cell-derived amphiregulin promotes pulmonary fibrosis [7]. ST2-expressing group 2 innate lymphoid cells (ILC2s) represent another source of amphiregulin [8,9], and they have been implicated in fibrosis by studies analyzing the effects of deficiency of ST2 (Il1rl1) [10–12]. However, these studies cannot rule out the importance of ST2 in other (non-ILC2) cell types.

The ST2 ligand IL-33 is important for innate mucosal immunity in the lung and gut [13,14], and is also implicated in multiple pathogenic processes such as inflammation, tissue repair/fibrosis, and scleroderma [10,15–17]. It is expressed by endothelial and type II alveolar epithelial cells in the lung [13,14,16]. Its receptor ST2 is also widely expressed on multiple cell types including Th2 cells, mast cells, Th1 cells, regulatory T (Treg) cells, $CD8^+$ T cells, natural killer (NK) cells, and group 2 innate lymphoid cells (ILC2s) [18]. *ST2* (*Il1rl1*) pre-mRNA produces four isoforms through alternative splicing: ST2L, soluble ST2 (sST2), ST2V, and ST2LV [19]. ST2L is expressed on the membrane of hematopoietic cells [20], whereas sST2 is predominantly expressed by fibroblasts and epithelial cells functioning as a decoy receptor that exhibits anti-inflammatory properties [21]. ST2-expressing ILC2s represent important effector cells in the host response to infection, wound remodeling, allergic responses, fat biogenesis, and obesity [22–25].

ILC2s accumulate in tissue during type 2 inflammation, and function as a main source of cytokines such as IL-13, IL-4, IL-5, and amphiregulin [8,22,26–28], which are implicated in the process of tissue repair and fibrosis [7,29,30]. Both ST2 and IL-33 levels are induced in murine bleomycin (BLM)-induced pulmonary fibrosis and human IPF, and increased numbers of lung ILC2s are associated with fibrosis in the mouse [10,31,32]. Moreover, adoptive transfer of lung ILC2s enhances fibrosis in recipient mice, whereas anti-IL-33 antibodies or ST2 deficiency diminishes fibrosis [10]. These findings, along with association with alternative activation of macrophages (M2), suggest important roles for both ILC2s and M2 macrophages in fibrosis. However, that previous study relied on systemic antibody treatment or global ST2 knockout mice; thus, the diminished fibrosis cannot be attributed solely to ILC2s in view of the multiplicity of cell types that express ST2 and are IL-33 responsive. Furthermore, while the ILC2 adoptive transfer study indicates the ability of exogenous ILC2s to enhance fibrosis, it does not necessarily reveal a role for endogenous ILC2s in pathogenesis, nor does it give insight into the origin of lung ILC2s in pulmonary fibrosis.

In this study, we hypothesized the BM origin and paracrine role of ILC2 in BLM-induced lung fibrosis. To assess potential BM origin, we used ST2-deficient BM chimera mice lacking ST2 expression only in the BM and resulting in deficiency of BM-derived ILC2 because IL-33/ST2 signaling is essential for ILC2 differentiation/activation [24,33,34]. The results revealed that BLM-induced expansion of the lung ILC2 population and fibrosis was virtually abrogated in ST2-deficient BM chimera mice. Control lung ST2 expression and ILC2s were not affected by BM transplantation with ST2-deficient BM. *In vitro* cell co-culture studies suggested paracrine regulation of myofibroblast differentiation by the BM-derived ILC2s, which if adequately amplified could serve as a potential mechanism by which ILC2s could promote fibrosis. These findings suggested that in response to a distal lung insult, ILC2s were recruited from the BM and activated/differentiated through IL-33/ST2 signaling, and in association with M2 macrophage differentiation contributed to fibroblast activation to promote the development of lung fibrosis in an ST2-dependent manner.

Materials and methods

Mice and bleomycin model of pulmonary fibrosis

C57BL/6 female mice (6–8 weeks of age) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). ST2 knockout (KO) mice on a C57BL/6 background were from Dr Stefan Wirtz (Friedrich Alexander Universität Erlangen, Erlangen, Germany). All mice were housed in the University Laboratory Animal Facility under animal protocols approved by the Institutional Animal Care and Use Committee at the University of Michigan. The lung fibrosis model was induced by the endotracheal instillation of 2 U/kg BLM (Blenoxane; Mead Johnson, Princeton, NJ, USA) as described previously [3]. Control mice received the same volume of PBS only. *ST2* KO BM chimera mice were generated by transplanting donor *ST2* KO BM cells into recipient WT mice as described previously [3]. Mice receiving WT donor BM cells were used as controls [3]. The chimera mice were maintained on acidified water and autoclaved feed *ad libitum* for 6 weeks before use in the fibrosis model as described above.

Collection of bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) was performed using 0.5 ml of PBS and repeated three times for collection of BAL fluid. BAL fluid was then centrifuged at 500 × *g* for 10 min to pellet cells. Total protein was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

Cell isolation

Single cell suspensions of lung cells for flow cytometry and primary mouse lung fibroblasts were isolated and cultured as described previously [3,35].

Lineage-negative (Lin^-) BM cells were isolated from whole BM cell suspension using a mouse lineage cell cocktail kit (Miltenyi Biotec Inc, San Diego, CA, USA). Where indicated, isolated cells were treated with 30 ng/ml recombinant mouse IL-33 (R&D Systems, Minneapolis, MN, USA) for 48 h. For cell co-culture experiments, normal lung fibroblasts were plated (8 × 10^{4} cells per well) in the bottom chambers of 24-well Transwell plates with 0.4 μm-pore polycarbonate membrane inserts (Thermo Fisher Scientific) for 24 h. The cells were starved in serum-free medium for another 24 h, followed by the addition of 1.5 × 10^{6} Lin^- BM cells from WT or *ST2* KO mice treated with PBS or BLM to the upper inserts. After an additional 24 h of incubation, the fibroblasts were collected for RNA isolation.

Reverse transcription–PCR (RT-PCR) and ELISA

RNA isolation and RT-PCR were performed as described previously [35]. Primers for assessing the mRNA levels for IL-33 (*Il33*), ST2L, type I collagen (*Col1a2*), *Acta2*, TGFβ (*Tgfb1*), IL-13 (*Il13*), amphiregulin (*Areg*), TNFα (*Tnf*), *Arg1*, *Nos2*, and *18S* rRNA were purchased from Thermo Fisher Scientific. Results were expressed as $2^{-\Delta\Delta CT}$ using the indicated control group as calibrator and 18S rRNA as reference 36.

Soluble ST2 (sST2) levels in BAL fluid from control and BLM-treated mice and media from cell co-cultures were detected using an ST2 ELISA kit (R&D Systems) in accordance with the manufacturer's instructions.

Hydroxyproline and histological analysis

Lung collagen content was determined by measuring the hydroxyproline content from lung homogenates as

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described previously [35]. The results were expressed as μg hydroxyproline per lung. The lung sections were stained with H&E or Masson's trichrome for evaluation of histopathology.

Flow cytometry

Single cell suspensions from freshly digested lung tissue or BM were prepared as described above and analyzed by flow cytometry as described previously [37]. Dead cells were excluded from analysis using the Fixable Viability Dye eFluor™ 506 (#65-0866; dilution 1:1000; eBioscience, San Diego, CA, USA). Combinations of the following antibodies (and fluorophores) were used: anti-mouse ST2-PE-Cy7 (#25-9335-80, clone: RMST2-2; dilution 1:100; eBioscience), IL-13-Alexa Fluor 488 (#53-7133-82, clone: eBio13A; 1:100; eBioscience), ICOS-BV421 (#564070, clone: 7E.17G9; 1:100; BD Biosciences, San José, CA, USA), Lineage-APC cocktail of anti-CD3e, CD11b, B220, TER-119, Ly-6C, Ly-6G (#558074; 1:5; BD Biosciences), CD90.2-APC-Cy7 (#105732, clone: 30-H12; 1:100; Biolegend, San Diego, CA, USA), CD45-Pacific Blue (#103126, clone: 30-F11; 1:200; Biolegend), along with their respective isotype controls. The data were acquired using a NovoCyte flow cytometer and analyzed by NovoExpress software (Aeca Biosciences, Inc, San Diego, CA, USA). All other fluorescence conjugated-antibody controls except the target antibody were used for compensation.

Statistical analysis

All data are presented as mean values ±SEM. Differences between any two groups were assessed for statistical significance by ANOVA followed by *post hoc* analysis using Scheffé's test. A *P* value less than 0.05 was considered to be statistically significant.

Results

Effects of selective BM ST2 deficiency on lung ILC2 induction and pulmonary fibrosis

To study the involvement of the IL-33/ST2 signaling pathway and ILC2 in BLM-induced pulmonary fibrosis, we first confirmed that lung *Il33* and *ST2* mRNAs were highly induced at days 7 and 21 after BLM treatment (Figure 1A, B). The protein level of sST2 in BAL fluid was significantly induced at days 7 and 21 after BLM treatment (Figure 1C) but not in plasma (data not shown). Flow cytometry analysis revealed a significant increase in $Lin^-CD45^+CD90.2^+ST2^+$ ILC2s in BLM-injured lung at day 7 (Figure 1D), which was sustained at day 21 after BLM treatment (data not shown).

To exclude the contribution of ST2 in endogenous lung cell types and assess the specific importance of BM-derived ILC2s, we evaluated the *in vivo* effects of selective ST2 deficiency in BM progenitors using *ST2* KO BM chimera mice. Since ILC2s are developmentally derived from the BM [34,38,39] with a dependence on IL-33/ST2 signaling for their differentiation/activation and recruitment to distal organs [24,33,34], this approach has been used as a means to assess the importance of BM-derived ILC2s. Indeed, flow cytometric analysis revealed that lung ILC2s were markedly reduced in *ST2* KO chimera mice due to a lack of ST2 in BM cells (Figure 2A, B), without significantly affecting differentiated ($Lin^+/CD45^+$ cells) myeloid cells (10.2 ±1.9 versus 11.09 ±2.23 as % of total lung cells in control versus *ST2* KO BM chimera mice, respectively) or $CD11c^{hi}/MHCII^{hi}$ cells (0.83 ±0.17 versus 1.15 ±0.01% of total lung cells in WT versus BM *ST2* KO BM chimera mice, respectively) in the lungs of BLM-treated mice. Notably, the BLM-induced increase in lung ILC2s in WT mice was essentially abolished in *ST2* KO BM chimera mice, which was accompanied by a significant reduction in BLM-induced *Il13* levels in *ST2* KO BM chimera mice (Figure 2C). Thus, ST2 expression in BM progenitor cells was essential for recruitment/activation of lung ILC2s and IL-13 induction in pulmonary fibrosis.

Next, we investigated the impact of BM ST2 deficiency on pulmonary fibrosis. BLM induced extensive fibrosis in WT mouse lungs but it was attenuated and more circumscribed in the lungs of *ST2* KO BM chimera mice (Figure 2D), which was accompanied by reduced deposition of collagen in *ST2* KO BM chimera lungs, as shown by Masson's trichrome staining (supplementary material, Figure S1). This reduced fibrosis was confirmed quantitatively by a significant reduction in lung collagen content (Figure 2E) and *Col1a2* mRNA levels (Figure 2F). Significant inhibition of BLM-induced myofibroblast differentiation was also noted, as assessed by *Acta2* expression (Figure 2F). Additionally, the induction of *Tnfa* and *Tgfb1* was also inhibited in *ST2* KO BM chimera lung after BLM treatment (Figure 2G). Finally, the BLM-induced increase in M2 marker *Arg1* mRNA in WT controls was significantly reduced in *ST2* KO BM chimera mice at day 7 but not at day 21 (Figure 2H). No significant effect was noted in the induction of the M1 marker *Nos2* (data not shown). These findings together suggested that the BM ST2 deficiency prevented ILC2 recruitment/activation and early M2 polarization, which was accompanied by subsequent impairment of BLM-induced pulmonary fibrosis.

Given that the absence of ST2 expression was selectively confined to the BM compartment, this would suggest that the expansion in lung ILC2s in response to BLM-induced lung insult in WT mice might be due to IL-33/ST2-dependent recruitment of ILC2s or ILC2 precursors from the BM, as previously shown [34,38–40]. Flow cytometric analysis revealed that the number of $ST2^+$ cells was increased more than three-fold in the lung after BLM treatment (Figure 3A). In contrast, $ST2^+$ BM cells were significantly decreased after BLM treatment, which would be consistent with recruitment of these cells to the lung, resulting in their depletion in

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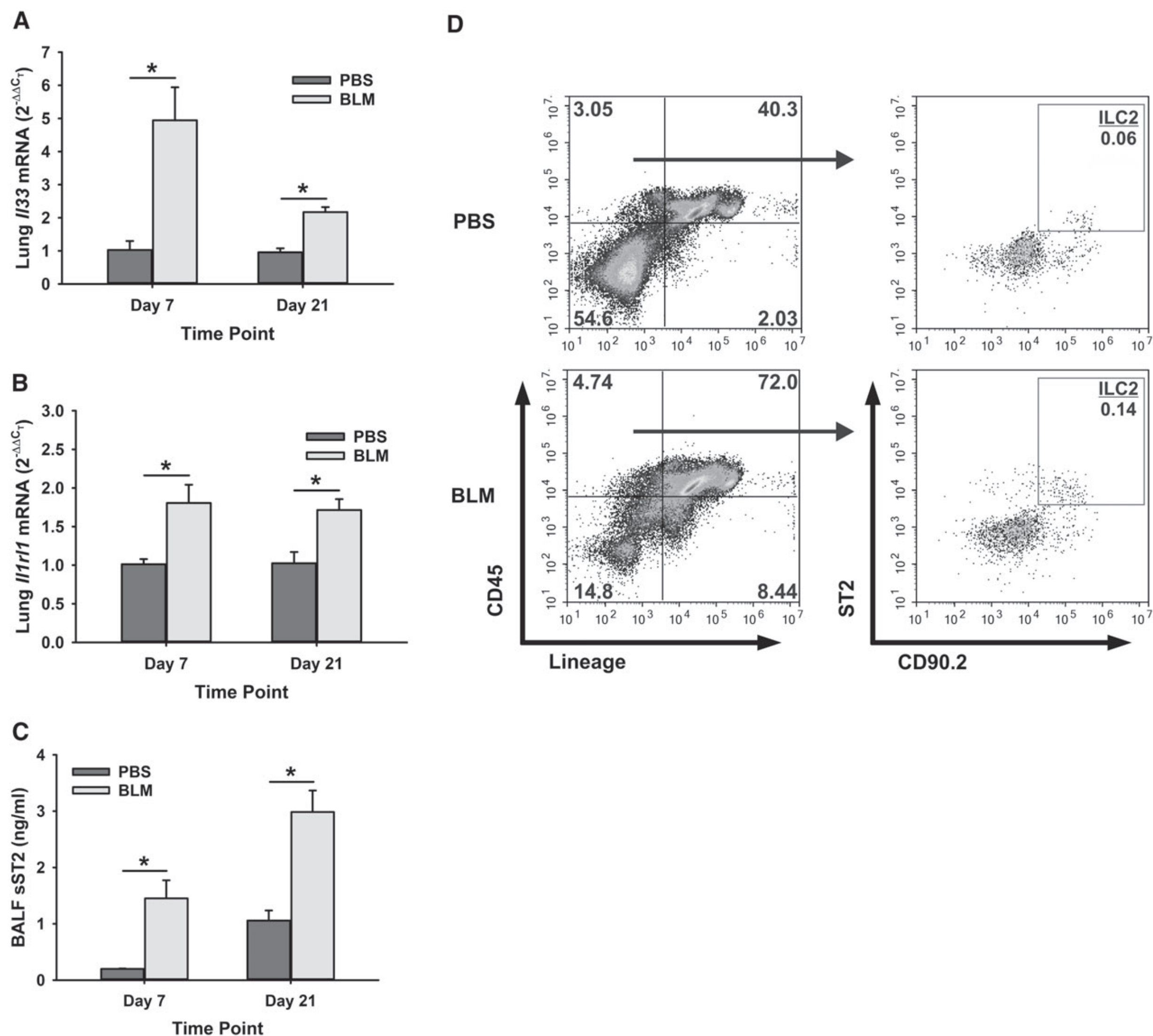

Figure 1. Effects of BLM on lung IL-33 and ST2 expression, and ILC2 cell number. Lung tissue RNA samples from BLM- or PBS-treated mice at days 7 and 21 after treatment were analyzed for *Il33* (A) or *ST2* (*Il1rl1*) (B) mRNA levels by RT-qPCR. $N = 3$ mice per group. In C, soluble ST2 protein levels in BAL fluid from BLM- or PBS-treated mice were quantified by ELISA ($N = 3$–8). In D, whole lung single cell suspensions were prepared 7 days after BLM treatment. ILC2s were gated as lineage-negative (Lin^-) $CD45^+ST2^+CD90.2^+$. The cells were first gated on Lin^-CD45^+ and then analyzed for ST2 and CD90.2 expression for enumeration of ILC2s shown as a percentage of total cells. Data are shown as mean ±SE. $^*p < 0.05$ between the two groups indicated in A–C. The experiments were independently repeated twice, and a representative data set is shown.

the originating BM. Since IL-33 is the cognate ligand for ST2, the response of ST2-deficient BM cells to IL-33 was evaluated to confirm its importance in ILC2 activation/differentiation. The results showed that while ILC2s were highly induced by IL-33 in WT Lin^- BM cells, *ST2*-deficient cells failed to respond (Figure 3B). Thus, BM $ST2^+$ ILC2s or their progenitors were recruited to the lung in an IL-33/ST2-dependent manner and accounted for the expansion of lung ILC2s in BLM-induced pulmonary fibrosis.

Comparative role of BM versus lung ST2 expression in BLM-induced pulmonary fibrosis

To evaluate the relative importance of BM versus lung IL-33/ST2 signaling *in vivo*, BLM-induced lung fibrosis was also evaluated in WT and global *ST2* KO mice. Compared with WT mice, *ST2* KO mice showed reduced BLM-induced lung fibrosis morphologically, with a smaller and less diffuse distribution of fibrotic lesions (Figure 4A), which was accompanied by a significant reduction in the BLM-induced increase of lung *Col1a2* (Figure 4B) and *Acta2*, a myofibroblast differentiation marker (Figure 4C). This reduction in BLM-induced pulmonary fibrosis in globally ST2-deficient mice was comparable to that seen in mice deficient in ST2 expression restricted to the BM compartment (Figure 2D, E).

Flow cytometric analysis of the lung cells revealed that the significant increase in lung ILC2s after BLM treatment also markedly reduced in global *ST2* KO mice (Figure 5A), which was also comparable to that seen in mice with ST2 deficiency restricted to the BM (Figure 2B). This was accompanied by significantly

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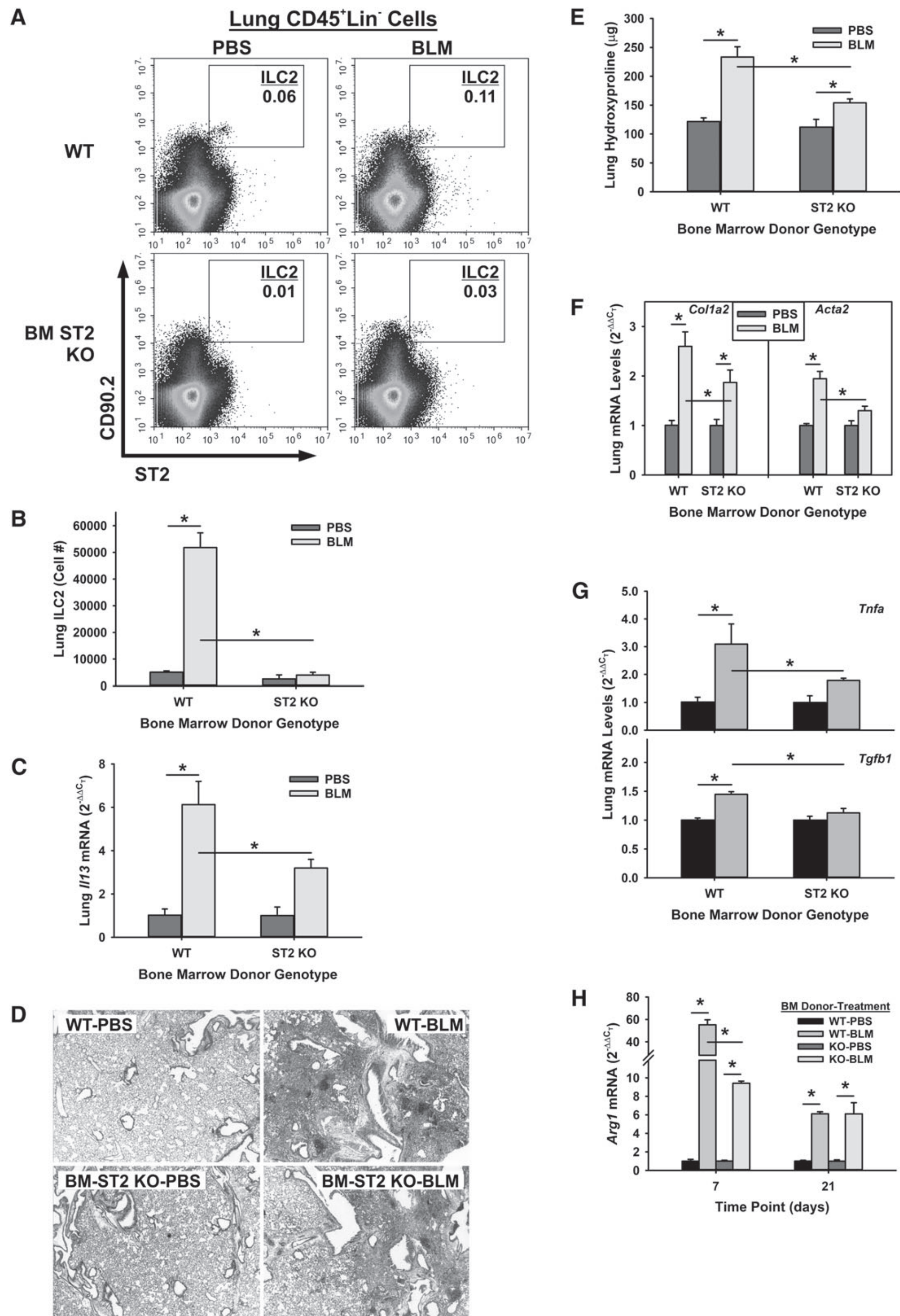

Figure 2. BLM-induced lung ILC2 recruitment and pulmonary fibrosis were abrogated in *ST2* KO BM chimera mice. Lung cell suspensions from BLM- or PBS-treated WT (receiving WT BM) or *ST2* KO BM chimera (receiving ST2 KO BM) mice at day 21 after treatment were analyzed for ILC2s as in Figure 1. A representative plot set is shown in A, and a summary from three separate experiments is shown in B. Data are shown as mean ± SE. $N = 3$. $^*p < 0.05$ between the two groups indicated. (C) Lung *Il13* mRNA was analyzed from BLM- or PBS-treated *ST2* KO BM chimera lung at day 21 after treatment. Data are shown as mean ± SE. $N = 3$ mice per group. $^*p < 0.05$ between the two groups indicated. (D) Representative lung tissue sections from WT or *ST2* KO BM chimera mice treated as described in A were stained with H&E (original magnification×40). (E) Lung tissue samples were evaluated for collagen content by hydroxyproline assay. (F) Lung tissue mRNA levels of type I collagen (*Col1a2*) and α-SMA (*Acta2*). *Tnfa* and *Tgfb1* (G), as well as the M2 macrophage marker *Arg1* (H), were analyzed by RT-qPCR. Data are shown as mean ± SE. $N = 5$ mice per group. $^*p < 0.05$ between the two groups indicated.

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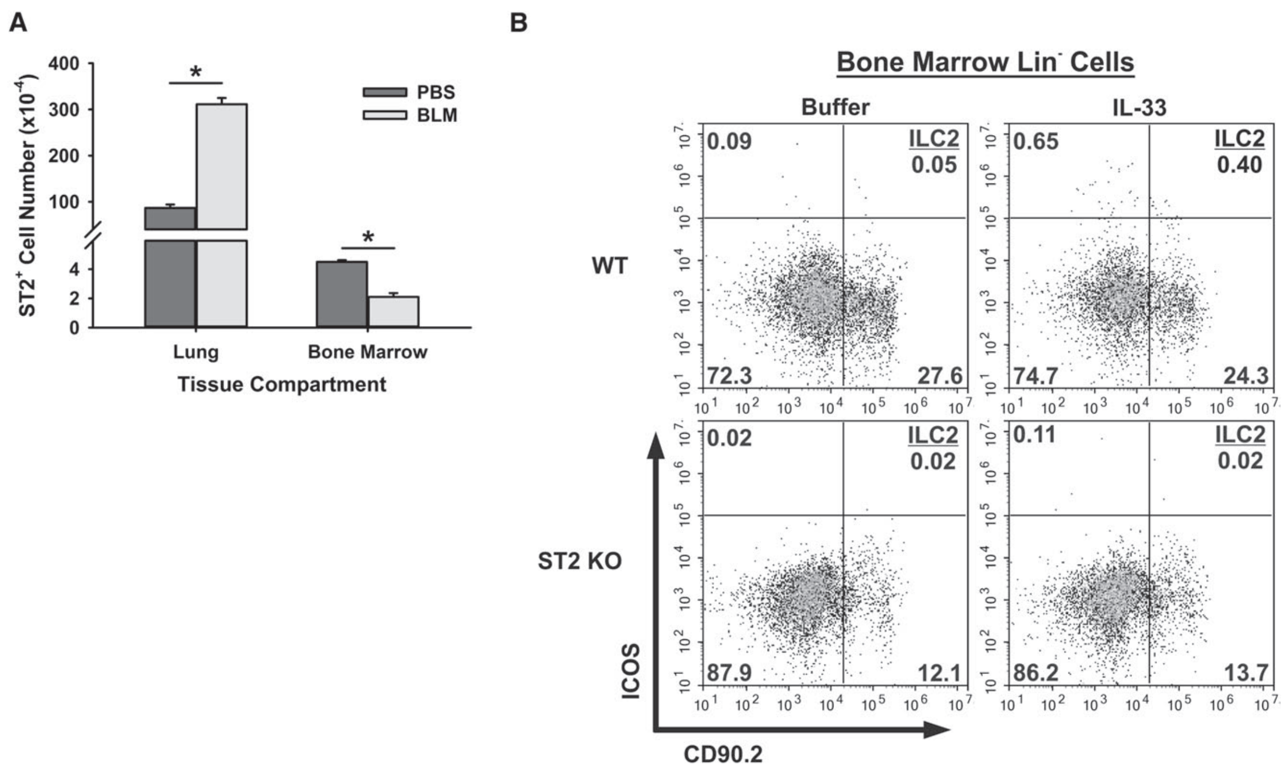

Figure 3. Effects of BLM on lung and BM ST2+ cells and ILC2 response to IL-33. (A) Lung and BM cells from BLM- or PBS-treated mice were analyzed for ST2 expression by flow cytometry, and the absolute numbers of ST2+ cells are shown. Data are shown as mean ± SE. $N = 3$. *$p < 0.05$ between the two groups indicated. (B) Lin− BM cells isolated from WT or *ST2* KO mice were treated with 30 ng/ml IL-33 *in vitro* and then analyzed for ILC2 by flow cytometry. A representative scatter plot is shown. $N = 3$ per group.

decreased numbers of IL-13-expressing ILC2s in BLM-treated ST2-deficient mice (Figure 5A). BLM induction of both lung IL-13 and amphiregulin expression was also reduced in ST2-deficient mice (Figure 5B, C). The reduction in lung *Il13* levels in global *ST2* KO mice (Figure 5B) was also similar to that in mice lacking ST2 only in the BM (Figure 2C). These findings indicated that ST2 deficiency impaired the recruitment/activation and function of ILC2s in BLM-induced lung fibrosis, and that ST2 expression in the BM compartment was of predominant importance in the BLM-induced expansion of lung ILC2s and fibrosis.

BM-derived ILC2s activated lung fibroblasts by increasing collagen I expression

To elucidate the possible mechanism by which BM-derived ILC2s promoted lung fibrosis, we evaluated the effect of isolated BM-derived ILC2s on lung fibroblast activation. As only very few ILC2s could be isolated from mouse lungs, we used the more abundant Lin− BM cells to assess possible effects on lung fibroblast activation. These Lin− BM cells consisted of progenitor cells as well as ILC2s, and were used in co-cultures with normal lung fibroblasts. The results showed that Lin− BM cells isolated from BLM-treated WT mice caused a significant increase in *Col1a2* levels in lung fibroblasts, relative to BM cells from control PBS-treated mice (Figure 6A). However, BM cells from BLM-treated *ST2* KO mice fibroblasts, compared with PBS-treated controls, failed to stimulate *Col1a2* levels. Interestingly, the Lin− BM cells isolated from BLM-treated WT mice expressed significantly higher levels of *Tgfb1* relative to cells from WT PBS controls (Figure 6B), which was abrogated in BM cells from *ST2* KO mice. This suggested that induction of TGFβ in BM progenitors and/or ILC2s could have caused the noted effects on fibroblast activation. This induction of TGFβ was likely due to BLM-induced IL-33, since it was dependent on ST2, and thus supported the identification of the Lin− BM cell as a likely ILC2 progenitor. Taken together, these findings would be consistent with BLM-induced lung IL-33 as an important signal for BM progenitor/ILC2 activation to enhance TGFβ expression leading to the activation of lung fibroblasts after BM-derived ILC2 recruitment to the lung.

Discussion

Innate lymphoid cells (ILCs) are recently discovered immune cells that reside at epithelial barriers such as the lung, skin, and gut, and contribute to immunity, inflammation, tissue homeostasis, and repair processes following exposure to allergens, pathogens or chemical irritants [41–43]. Three subsets of ILCs produce different type of cytokines. ILC2s produce type 2-associated cytokines, including IL-4, IL-5, and IL-13, as well as amphiregulin, thus playing important roles in parasite

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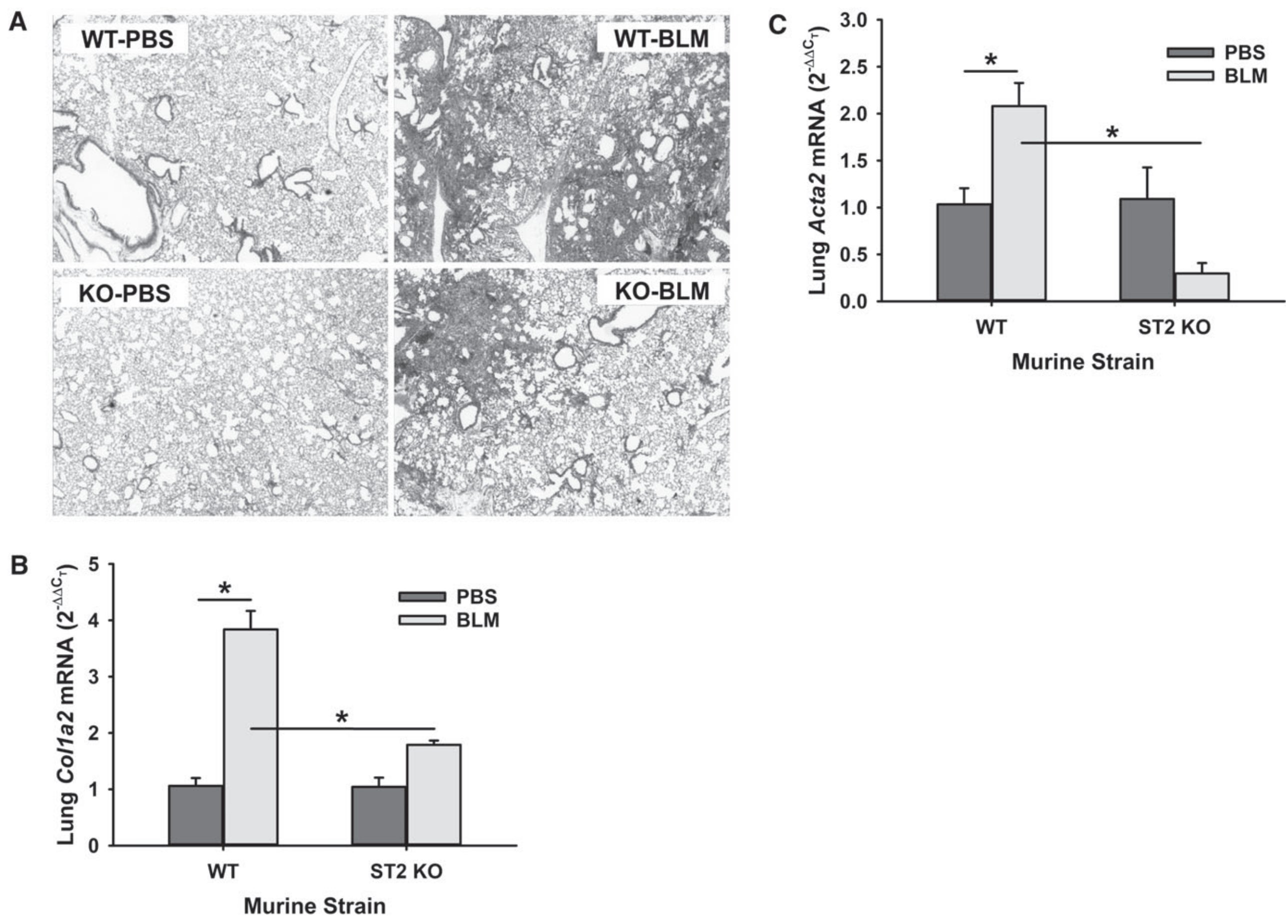

Figure 4. BLM-induced pulmonary fibrosis was attenuated in *ST2* KO mice. WT or *ST2* KO mice were treated with BLM or PBS endotracheally. Pulmonary fibrosis was evaluated at day 21 after treatment by histopathology (A; representative tissue section shown; ×40 objective magnification). *Col1a2* (B) and *Acta2* (C) mRNA levels. Data are shown as mean ± SE. $n = 3–5$. $^*p < 0.05$ between the two groups indicated. The experiments were independently repeated twice, and a representative data set is shown.

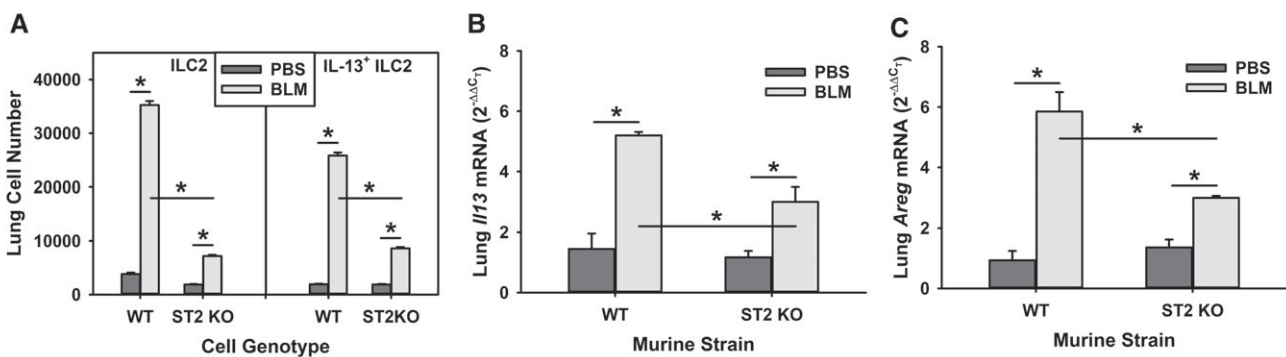

Figure 5. Lung ILC2 recruitment and activation were ablated in *ST2* KO mice. Whole lung single cell suspension were prepared from day 7 PBS- or BLM-treated WT or *ST2* KO lungs, and analyzed for ILC2s by flow cytometry and *Il13* expression in ILC2s. (A) The absolute cell numbers per lung are shown for ILC2s and IL-13$^+$ ILC2s. $N = 3$ mice per group. Lung RNA samples from BLM- or PBS-treated mice were analyzed for IL-13 (B) or amphiregulin (*Areg*) (C) mRNA levels by RT-qPCR. $N = 3$ mice per group. Data are shown as mean ± SE. $^*p < 0.05$ between the two groups indicated.

immunity, allergic inflammation, and tissue homeostasis and fibrosis [23,24,26,44,45]. The ST2 ligand, IL-33, is a potent inducer of ILC2 activation [8,16]. The present study provided evidence that the IL-33/ST2 signaling pathway was induced in a model of lung injury and fibrosis through which the ILC2s were recruited from the BM and activated in the injured lung to promote fibrosis, perhaps by activating lung fibroblasts via TGFβ.

IL-33 induction was also found in the BAL fluid in patients with IPF [46]. The elevated sST2 level in the BAL fluid in BLM-induced lung fibrosis was also consistent with activation of the IL-33/ST2 signaling pathway in lung fibrosis. The basis for this activation of ST2 signaling in pulmonary fibrosis may be due to the induction of IL-33 in injured epithelial cells serving as an alarm signal. Elevated sST2 in BAL fluid, but

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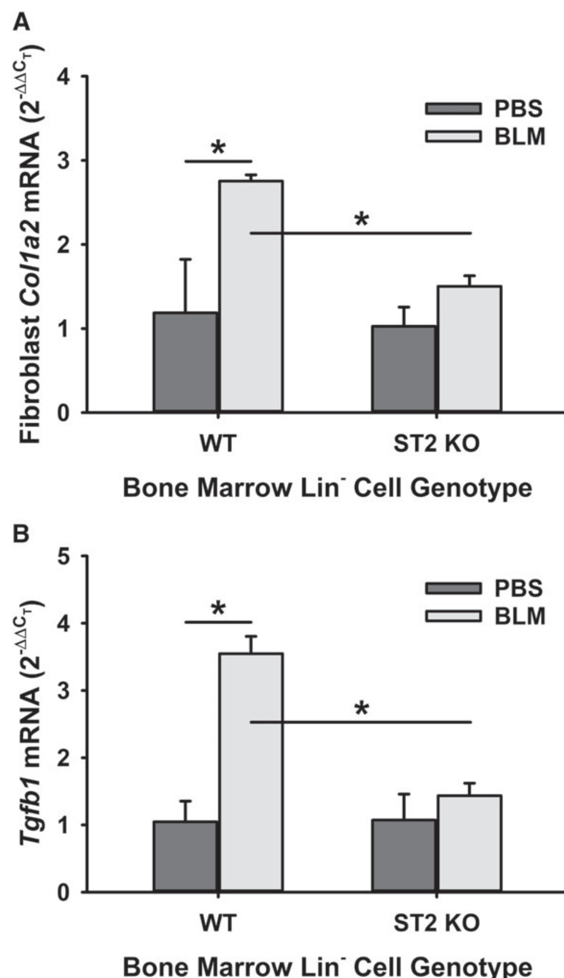

Figure 6. Lung fibroblasts were activated by BM-derived progenitor cells from BLM-treated mice. (A) Normal lung fibroblasts were co-cultured with Lin$^-$ BM cells isolated from BLM- or PBS-treated WT or *ST2* KO mice 21 days after treatment. Fibroblast *Col1a2* was analyzed by RT-qPCR. In B, similarly isolated Lin$^-$ BM cells from the same groups of mice were analyzed for *Tgfb1* mRNA by RT-qPCR. Data are shown as mean ± SE. $N = 5$ mice per group. $^*p < 0.05$ between the two groups indicated.

not in plasma, during lung fibrosis was sustained until the late stage of fibrosis at day 21. As an endogenous decoy receptor of IL-33, sST2 also antagonizes IL-33 and inhibits the IL-33/ST2L-induced Th2 response, and exogenous sST2 administration reduces BLM-induced fibrosis [47–49]. The significance of this induction of sST2 is unclear, given its paradoxical inhibitory effect on IL-33–ST2 signaling. It may be that the site of sST2 secretion occurs at a different location to where IL-33 is released from damaged cells. Further studies are needed to investigate the precise role of sST2.

The increase in IL-33-responsive ILC2s is not unique to animal models of pulmonary fibrosis as they are also increased in systemic sclerosis, correlating with the extent of fibrosis [50,51]. They are also present in the BAL samples and lung tissue of patients with IPF, wherein they are associated with upregulated expression of lung IL-33 and IL-25 [23,52]. The IL-33/ST2-induced increase in ILC2 number in BLM-induced lung fibrosis was ST2-dependent, as the ILC2 number and fibrosis were reduced in *ST2* KO mice after BLM treatment. This is an expected finding given the dependence of ILC2 development on IL-33/ST2 signaling [24,33,34]. These findings are in agreement with another study where BLM-induced fibrosis was attenuated in global *ST2* KO or by neutralizing IL-33 systemically through possible inhibition of M2 macrophage polarization [10]. Moreover, fibrosis is enhanced by adoptive transfer of ILC2s, although this does not indicate a requirement for endogenous ILC2s in fibrosis. Although BM ST2 deficiency also reduced M2 polarization, IL-33 alone cannot induce polarization [10]. While IL-13 alone can induce M2 polarization even in the absence of ST2, the addition of IL-33 magnifies this response [10,53]. This essential requirement for IL-13 to enable IL-33-induced polarization is due to the induction of ST2 by IL-13 [53]. Thus, it is likely that the induction of IL-13 in recruited ILC2s precedes and is essential for the subsequent enhanced M2 polarization by IL-33 [10,54]. IL-33-induced ILC2s are also an important source of IL-13 in liver fibrosis via the ST2-dependent signaling pathway [12], which would be consistent with the reduction in IL-13 expression in ST2-deficient mice in the BLM model of pulmonary fibrosis, thus impacting negatively on M2 polarization as noted.

However, studies using *ST2* KO mice or antibodies to IL-33 cannot exclude impairment of IL-33/ST2 signaling in cells other than ILC2s as ST2 is expressed by diverse cell types [19]. To analyze more specifically the contribution of ILC2s in pulmonary fibrosis, we restricted the ST2 deficiency to the BM compartment by transplanting donor *ST2* KO BM into WT recipients. Given that ILC2s originate developmentally from the BM and given their dependence on IL-33 (via ST2) for their development, this approach has been used as a means of depleting ILC2s [12,34,55]. Previously, ILC2s arising from BM progenitors were shown to migrate into lung in response to parasitic infection or allergic stimulation [33,34,56]. Using the *ST2* KO BM chimera mice in this study, we provided evidence that lung ILC2 development, recruitment, and activation were dependent on ST2 expression on the BM progenitor, and Lin$^-$ BM cells lacking ST2 expression failed to respond to IL-33 stimulation. Although this study did not completely exclude the potential impact of *ST2* deficiency on other BM ST2-expressing cell types and their role(s) in fibrosis, there is evidence that ST2 deficiency has no significant impact on T-cell, mast cell, or lung macrophage development/function, or host defense against mycobacterial infection [10,57,58], consistent with a unique dependence of ILC2 differentiation on IL-33/ST2 signaling. Moreover, IL-33 overexpression in the BM has no significant impact on the number of eosinophils, neutrophils, basophils or M2 macrophages in the BM [59]. Other studies have used similar BM chimera strategies but using RORα-deficient BM donor cells to deplete ILC2s, which showed a similar lack of a significant effect on T cells [60].

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Our results also revealed that *ST2* KO BM chimera mice developed significantly decreased lung fibrosis and myofibroblast differentiation. These findings suggested that IL-33, as a signal, released from injured lung epithelial cells [16] initiated BM-derived ILC2 recruitment, development, and activation through the IL-33/ST2 signaling pathway with subsequent promotion of pulmonary fibrosis. Indeed, a recent study showed that ST2-dependent IL-33 signaling is a critical component in promoting egress of ILC2s from the BM in response to exogenous IL-33 administration, distal tissue depletion of ILC2s or fungal infection [34]. In our current study, the source of IL-33 was from the BLM-injured lung, and similar to this other recent study [34], deficiency of ST2 in the BM cells abrogated the response to the IL-33 from the lung. Other studies also provide support to the BM origin of ILC2s, especially under prolonged injury or disruption of homeostasis in tissues [34,40,61–64]. In our current study, the irradiation and BM transplantation procedure did not cause a significant change in the endogenous lung ILC2 population after stable engraftment was achieved (Figures 2B and 5C), suggesting that only the BLM insult-induced expansion in lung ILC2s was recruited from the BM, likely in response to the associated induction of lung IL-33 expression. Another recent study suggests the small intestine as an alternative site of origin for a subset of lung inflammatory ILC2s (iILC2s) [40]. Small intestine iILC2 precursors, not endogenous lung cells, migrate to the lung and give rise to proliferating iILC2s induced by IL-25 or helminth infection. However, it is noteworthy that the BM ST2-dependent recruited ILC2s in fibrotic lung with IL-33 induction in our study exhibited a $Lin^{-}ST2^{+}CD90.2^{+}$ phenotype, which differed from the IL-25 or helminth infection-induced lung $Lin^{-}KLRG1^{hi}ST2^{-}CD90.2^{lo}$ iILC2s [40], suggesting that pulmonary fibrosis and acute helminth infection-induced inflammation may induce/recruit different ILC2 subsets potentially from different distal organ sites of origin. The mechanism of ILC2 recruitment appears to be IL-33/ST2-dependent in these and our current studies, but may be subject to regulation by additional agents, such as TGFβ, which has been reported to enhance ILC2 chemoattractant activity [65].

The induction of IL-13 from recruited and activated ILC2s could account for their role in fibrogenesis [12,29,30]; however, direct effects of ILC2s on fibroblast activation have not been fully investigated. In our current study, an alternative paracrine role for ILC2s in fibrosis was suggested by the studies using co-culture of Lin^{-} BM cells with lung fibroblasts. While ILC2s or their precursors comprise only a fraction of the Lin^{-} BM cells used in this study, the dependence of the effects on ST2 expression makes it likely that any observed effects are due primarily to ILC2s and/or their precursors. The findings revealed induction of ST2-dependent TGFβ expression in BM cells *in vivo* in response to distal lung insult by BLM, perhaps caused by induction of lung IL-33 with consequent effects on ILC2 differentiation. This induction of TGFβ was associated with activation of the co-cultured lung fibroblasts, as manifested by increased collagen I expression. Such potential crosstalk between activated BM-derived ILC2s and lung fibroblasts via TGFβ could represent an additional novel mechanism by which ILC2s could promote lung fibrosis. While the relative number of lung ILC2s is small, it could have a disproportionately larger effect by amplification of paracrine effects in adjacent cells, including M2 polarization and potentially other BM-derived cell types known to play a role in fibrosis [3,4,7,37]. Moreover, TGFβ is known to be auto-inducible [66], such that the effect of the initial release by activated ILC2s can be amplified by auto-induction of greater levels of this cytokine in the initial target and other adjacent cells. Further studies are needed to fully explore the *in vivo* relevance of these *in vitro* findings and put them in the context of pulmonary fibrosis.

Acknowledgements

We thank Lisa R Riggs for excellent technical assistance in the tissue section preparation and the H&E and Masson's trichrome staining. This study was supported by NIH grants HL052285 and HL112880 (to SHP) and a fellowship from The China Scholarship Council (to YZ).

Author contributions statement

YZ, TL, and SHP conceived experiments. YZ, FG, ZW, and TL carried out experiments and collected data. YZ, TL, and SHP analyzed data and wrote the manuscript. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. BLM-induced pulmonary fibrosis was abrogated in *ST2* KO BM chimera mice

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