TRIM33 prevents pulmonary fibrosis by impairing TGF-β1 signalling

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TRIM33 has a protective role against fibrogenesis, inhibiting the TGF-β1 pathway through a direct association with HSPB5. Interactions between TRIM33, SMAD4 and HSPB5 may represent key targets to prevent the progression of pulmonary fibrosis. http://bit.ly/3aVCuxc

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ABSTRACT

Background: Idiopathic pulmonary fibrosis (IPF) is a devastating disease characterised by myofibroblast proliferation and abnormal extracellular matrix accumulation in the lungs. Transforming growth factor (TGF)-β1 initiates key profibrotic signalling involving the SMAD pathway and the small heat shock protein B5 (HSPB5). Tripartite motif-containing 33 (TRIM33) has been reported to negatively regulate TGF-β/SMAD signalling, but its role in fibrogenesis remains unknown. The objective of this study was to elucidate the role of TRIM33 in IPF.

Methods: TRIM33 expression was assessed in the lungs of IPF patients and rodent fibrosis models. Bone marrow-derived macrophages (BMDM), primary lung fibroblasts and 3D lung tissue slices were isolated from *Trim33*-floxed mice and cultured with TGF-β1 or bleomycin (BLM). *Trim33* expression was then suppressed by adenovirus Cre recombinase (AdCre). Pulmonary fibrosis was evaluated in haematopoietic-specific *Trim33* knockout mice and in *Trim33*-floxed mice that received AdCre and BLM intratracheally.

Results: TRIM33 was overexpressed in alveolar macrophages and fibroblasts in IPF patients and rodent fibrotic lungs. *Trim33* inhibition in BMDM increased TGF-β1 secretion upon BLM treatment. Haematopoietic-specific *Trim33* knockout sensitised mice to BLM-induced fibrosis. In primary lung fibroblasts and 3D lung tissue slices, *Trim33* deficiency increased expression of genes downstream of TGF-β1. In mice, AdCre-*Trim33* inhibition worsened BLM-induced fibrosis. *In vitro*, HSPB5 was able to bind directly to TRIM33, thereby diminishing its protein level and TRIM33/SMAD4 interaction.

Conclusion: Our results demonstrate a key role of TRIM33 as a negative regulator of lung fibrosis. Since TRIM33 directly associates with HSPB5, which impairs its activity, inhibitors of TRIM33/HSPB5 interaction may be of interest in the treatment of IPF.

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a rare, chronic and progressive disease of the lung parenchyma of unknown origin, with a median survival of 3–5 years [1]. With the exception of pirfenidone and nintedanib, which exhibit moderate efficacy on disease progression, no pharmacological treatment is currently available [2]. IPF development associates abnormal alveolar repair with proliferation of activated fibroblasts named myofibroblasts, which leads to abnormal extracellular matrix (ECM) accumulation and tissue remodelling. Transforming growth factor (TGF)-β1 is a key profibrotic growth factor, and the adenoviral vector-mediated gene transfer of active TGF-β1 (by instilling/injecting the recombinant adenovirus, AdTGF-β1) in rodent lungs leads to progressive and severe fibrosis [3]. TGF-β1 is responsible for fibroblast activation into myofibroblasts. Mechanistically, TGF-β1 classically signals *via* the SMAD proteins, a crucial pathway in fibrogenesis [4].

We previously demonstrated that the small heat shock protein B5 (HSPB5; also termed heat shock protein αB-crystallin) is overexpressed in human IPF lungs. HSPB5 interferes with SMAD4 localisation by inhibiting its export from the nucleus. At the molecular level, HSPB5 hampers SMAD4 ubiquitination and, consequently, SMAD4 accumulates in the nucleus and promotes TGF-β1 signalling and fibrogenesis [5]. Tripartite motif-containing 33 (TRIM33) is an E3 ubiquitin ligase responsible for SMAD4 ubiquitination, inducing its export from the nucleus and therefore inhibiting SMAD transcriptional activity [6]. TRIM33 is involved in several processes, such as embryogenesis, haematopoiesis and tumorigenesis [6–8]. Specifically, TRIM33 is involved in the turnover of TGF-β receptor I [8] and in the innate immune response by regulating a subset of genes, including TGF-β1 or interferon-β in macrophages [9, 10]. Anti-TRIM33 antibodies are elevated in humans with myositis, a set of diseases with increased prevalence of cancer and interstitial lung disease (ILD) [11]. However, the impact of TRIM33 in lung fibrosis and IPF remains unstudied.

In the current study, we demonstrate for the first time that TRIM33 is overexpressed in the lungs of IPF patients and has a protective effect against pulmonary fibrosis *in vivo*. In addition, we highlight a mechanism by which HSPB5 interferes with the antifibrotic properties of TRIM33, paving the way for the development of novel therapeutic strategies targeting the interaction between HSPB5 and TRIM33.

Materials and methods

Human tissue samples

Lung tissue samples (n=5) were obtained by open lung biopsy (INSERM U700, Paris, France). IPF was diagnosed according to the American Thoracic Society/European Respiratory Society consensus criteria [12], including clinical, radiographic and characteristic histopathological features. Control non-IPF lung tissue samples were obtained from smokers who underwent thoracic surgery for localised primary lung carcinoma (n=5). The local ethics committee (Comité de Protection des Personnes, Ile de France 1) approved the study, and patients provided informed consent before lung surgery.

Human microarray data

GSE110147 was performed and analysed by Cecchini *et al.* [13] and GSE49072 was performed and analysed by Shi *et al.* [14].

Animal procedures

SV129 wildtype mice (Charles River, Saint Germain-sur-l'Arbresle, France) and SV129 knockout mice for the HSPB5 gene were housed in pathogen-free conditions. We generated mice selectively deficient for TRIM33 by breeding floxed *Trim33* mice with cFes-Cre transgenic mice. *Trim33*$^{f/f}$-Cre^{cFes} (hs*Trim33*$^{-/-}$) mice thus corresponded to haematopoietic tissue-restricted knockout mice, as previously described [15]. Mouse food and water were provided *ad libitum*. The animals were treated according to the guidelines of the Ministère de l'Enseignement Supérieur et de la Recherche (Ministry of Higher Education and Research, Paris, France). All experiments were approved by the Comité d'Ethique de l'Expérimentation Animale du grand campus Dijon (Animal Experimentation Ethics Committee of the large Dijon campus, Bourgogne, France). Intratracheal instillation of bleomycin (BLM) at 1.5 mg·kg^{-1} (Santa Cruz Biotechnology, Dallas, TX, USA) was performed as previously described [16]. AdTGF-β1 (5×10^8 plaque-forming units (PFU) per mouse) and adenovirus Cre recombinase (AdCre; 5×10^8 PFU per mouse) were instilled following the same protocol. BLM and adenovirus were diluted in 0.9% NaCl. Mice were euthanised by abdominal aortic bleeding at day 21 after administration. Bronchoalveolar lavage fluid (BALF) was gathered as previously described [4].

Collagen quantification

For histomorphometric assay, the amount of collagen in paraffin-embedded tissue sections was quantified by staining with Picro Sirius Red as previously described [17, 18].

For colorimetric assay, the Sircol assay was performed on lung left lobe extracts using the Sircol kit (Biocolor Ltd, Carrickfergus, UK) and following the manufacturer's recommendations.

Precision-cut lung slice preparation

3D lung tissue slices were generated as previously described [19] and cultured for 72 h in DMEM +10% serum, 1% L-glutamine and 1% penicillin/streptomycin. Three slices were pooled for each mRNA extraction.

Statistical analysis

Comparisons between different groups were performed using the nonparametric Mann–Whitney test and Prism 6 software (Graphpad, San Diego, CA, USA). p-values below 0.05 were considered statistically significant. All results are representative of at least three different experiments.

Results

TRIM33 is upregulated during pulmonary fibrosis

Histological examination of IPF lungs showed overexpression of TRIM33 when compared to non-IPF control lungs, in which TRIM33 was barely detectable (figure 1a–c). TRIM33 was overexpressed preferentially in myofibroblasts expressing smooth muscle actin (α-SMA; figure 1d–f) and in macrophages expressing macrosialin (CD68; figure 1g–i). TRIM33 overexpression could also be observed in ATII cells expressing pulmonary surfactant-associated protein C (SP-C; figure 1j–l). Analysis of publicly available microarray datasets showed that the *TRIM33* mRNA level was upregulated in total lung tissue from IPF patients compared to control lung tissue (dataset GSE110147; figure 1m) but also specifically in alveolar macrophages from IPF patients (dataset GSE49072; figure 1n). The analysis of our patient cohort confirmed the increase of *TRIM33* expression in the whole lung tissue of IPF patients compared to control tissue (supplementary figure S1).

Similar to the results in humans, TRIM33 was upregulated in fibrotic areas of mice receiving repeated intravenous injections of BLM (figure 2a–c). This increase was confirmed by immunoblotting and reverse transcription quantitative PCR (figure 2d and e). TRIM33 upregulation was also observed in fibrotic areas in other models of lung and pleural fibrosis, such as in mice receiving a single intratracheal instillation of BLM, or in rats and mice receiving an intrapleural injection of AdTGF-β1 compared to control rodents (supplementary figure S2a). In the BLM model, TRIM33 was expressed in fibroblasts (vimentin) and macrophages (CD68) (supplementary figure S2b).

Trim33 *inhibition impairs TGF-β1 secretion in macrophages*

As *TRIM33* is overexpressed in alveolar macrophages of IPF patients (figure 1), we investigated the effects of TRIM33 on bone marrow-derived macrophages (BMDM). BMDM isolated from *Trim33*-floxed (*Trim33*$^{f/f}$) mice were depleted for *Trim33* by *ex vivo* exposure to an adenovirus encoding the Cre recombinase (AdCre; figure 3a). *Trim33* depletion was confirmed using quantitative PCR (supplementary figure S3a). BLM stimulation of BMDM expressing TRIM33 led to an increase in TGF-β1, tumour necrosis factor (TNF)-α and interleukin (IL)-6 production (figure 3b–d), with no impact on cellular viability (supplementary figure S3c). The depletion of *Trim33* did not affect the level of TGF-β1 without stimulation, while it resulted in a three-fold increase in TGF-β1 secretion upon BLM stimulation (figure 3b). In parallel, we did not observe any significant difference in *Tgfb1* gene expression (supplementary figure S3b), or in secretion of pro-inflammatory cytokines (TNF-α and IL-6; figure 3c and d) or other cytokines (supplementary figure S3d) between BMDM expressing TRIM33 (exposed to the control adenovirus vector, AdDL) and BMDM depleted for TRIM33 (AdCre), both at basal level or upon BLM exposure.

Next, we investigated the functional effect of *Trim33* deficiency in the crosstalk between macrophages and lung fibroblasts (figure 3e–h). Conditioned media were collected from BMDM cultured in four distinct conditions: 1) unstimulated BMDM expressing *Trim33* (AdDL NaCl); 2) BMDM expressing *Trim33* stimulated with BLM (AdDL BLM); 3) unstimulated BMDM depleted for *Trim33* (AdCre NaCl); and 4) BMDM depleted for *Trim33* stimulated with BLM (AdCre BLM). The expression of mesenchymal markers was then investigated in mouse primary pulmonary fibroblasts incubated with these conditioned media. Conditioned media from unstimulated BMDM, expressing *Trim33* or not (AdDL NaCl and AdCre NaCl), had no impact on the expression of mesenchymal markers in fibroblasts (figure 3e–h). However, conditioned media from BLM-stimulated BMDM expressing *Trim33* (AdDL BLM) induced an increase in mesenchymal markers, which was further enhanced when fibroblasts were stimulated by conditioned media from BLM-stimulated BMDM depleted for *Trim33* (comparison of AdDL BLM *versus* AdCre BLM: actin alpha 2, smooth muscle *(Acta2)* 1.87-fold increase; serpin family E member 1 (*Serpine1)* 1.46-fold increase; lymphoid enhancer-binding factor 1 (*Lef1)* 1.66-fold increase; vimentin (*Vim)* 1.2-fold increase; figure 3e–h).

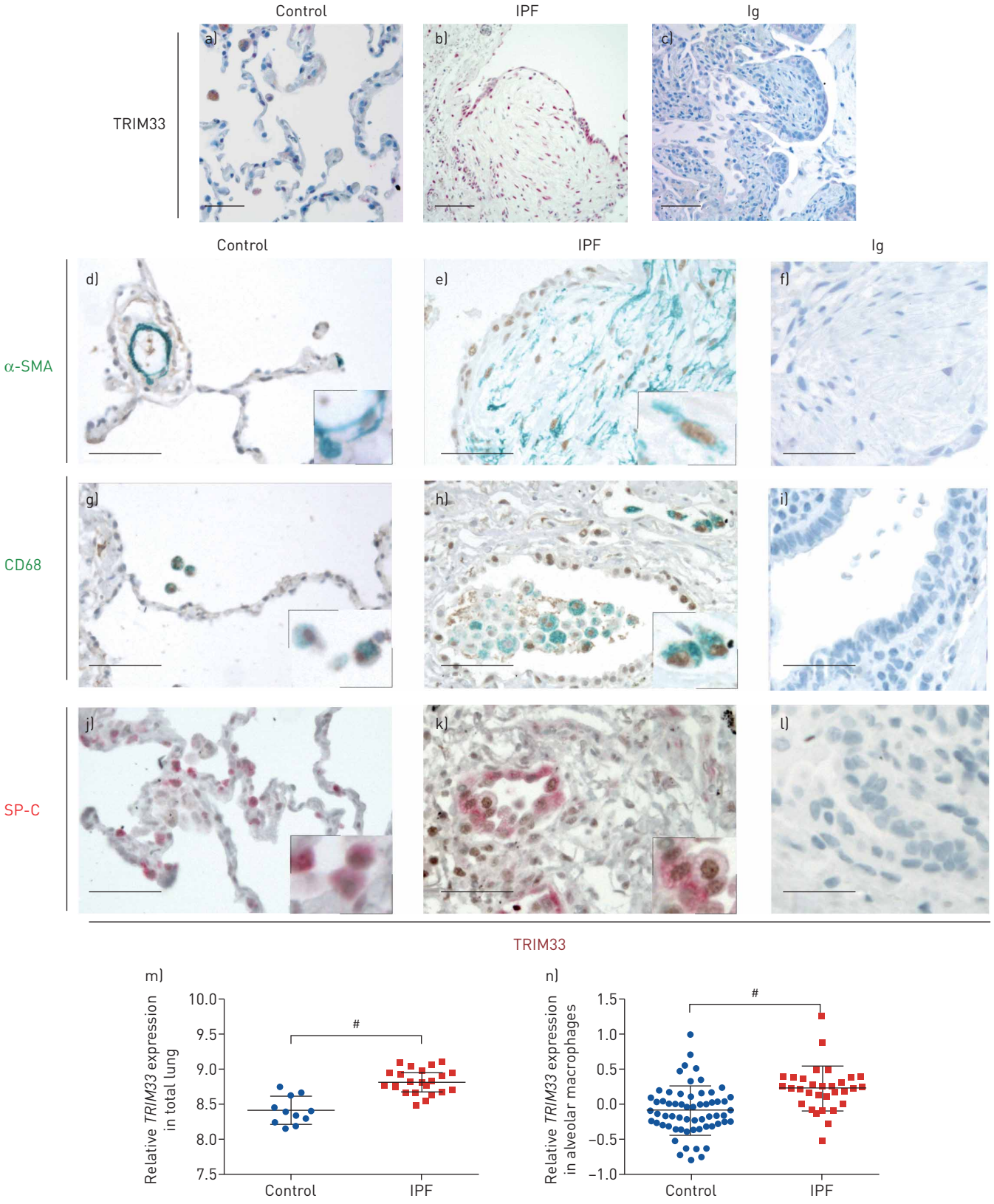

FIGURE 1 TRIM33 is upregulated in lungs of idiopathic pulmonary fibrosis (IPF) patients. a–c) TRIM33 immunostaining in lungs of a) control patients (n=4) or b) IPF patients (n=4), with fibroblastic focus; c) secondary antibody control. Scale bars: 100 µm. d–l) TRIM33 (brown), d–f) smooth muscle actin (α-SMA; green), g–i) macrosialin (CD68; green) and j–l) surfactant-associated protein C (SP-C; red) immunostaining in d, g, j) control lungs (n=3) or e, h, k) IPF lungs (n=3); f, i, l) irrelevant Ig controls. Scale bars: 100 µm. m and n) Analysis of publicly available microarray datasets, showing relative gene expression of *TRIM33*. Results expressed as median with interquartile range. #: p=0.0001, unpaired t-test with

Welch's correction. m) Accession number GSE110147, from lung samples from 22 IPF patients undergoing lung transplantation and 11 normal lung tissues flanking lung cancer resections. n) Accession number GSE49072, from alveolar macrophages isolated from 31 IPF patients and 61 normal volunteers or unaffected relatives.

Altogether, these results suggest that, although *Trim33* deficiency has no impact on TGF-β1 secretion at the basal level, yet it can promote the production of active profibrotic factors upon BLM stimulation, which in turn activates lung fibroblasts.

Haematopoietic-specific Trim33$^{-/-}$ *mice are more sensitive to BLM-induced pulmonary fibrosis*

To confirm the *in vivo* significance of TRIM33 in immune cells during pulmonary fibrosis development, we used mice with cFes-driven Cre to generate conditional *Trim33* knockout mice resulting in *Trim33* depletion specifically in the haematopoietic system: *Trim33*$^{f/f}$-Cre^{cFes} mice (hs*Trim33*$^{-/-}$ mice; figure 4a) [15]. Following BLM challenge, hs*Trim33*$^{-/-}$ mice developed more severe fibrosis compared to control mice (*Trim33*$^{f/f}$ mice), characterised by worsened pulmonary alveolar structure remodelling and increased collagen accumulation throughout the lungs (figure 4b). Picro Sirius Red staining of lung sections showed a 1.7-fold increase in lung collagen content in hs*Trim33*$^{-/-}$ mice compared to a 1.4-fold increase in control mice (supplementary figure S4a). This result was confirmed by a Sircol assay, in which we found that BLM induced a significant increase in collagen content in hs*Trim33*$^{-/-}$ mouse lungs compared to control lungs (figure 4c). In lung tissues, the increase in *Acta2* mRNA level induced by BLM was significantly higher in hs*Trim33*$^{-/-}$ mice compared to control mice (supplementary figure S4b). Similar findings were observed after repetitive systemic intravenous BLM exposure (supplementary figure S5a–c).

Confirming our *in vitro* results, TGF-β1 levels were significantly increased in the BALF from hs*Trim33*$^{-/-}$ mice compared to control wildtype mice exposed to BLM (two-fold increase) (figure 4d). In parallel, there was no difference in TNF-α or IL-6 protein level in BALF from hs*Trim33*$^{-/-}$ *versus* control mice after BLM exposure (figure 4e and f).

Altogether, these results suggest that TRIM33 significantly represses the establishment of a profibrotic microenvironment, specifically by inhibiting the production of active TGF-β1 by immune cells, including macrophages.

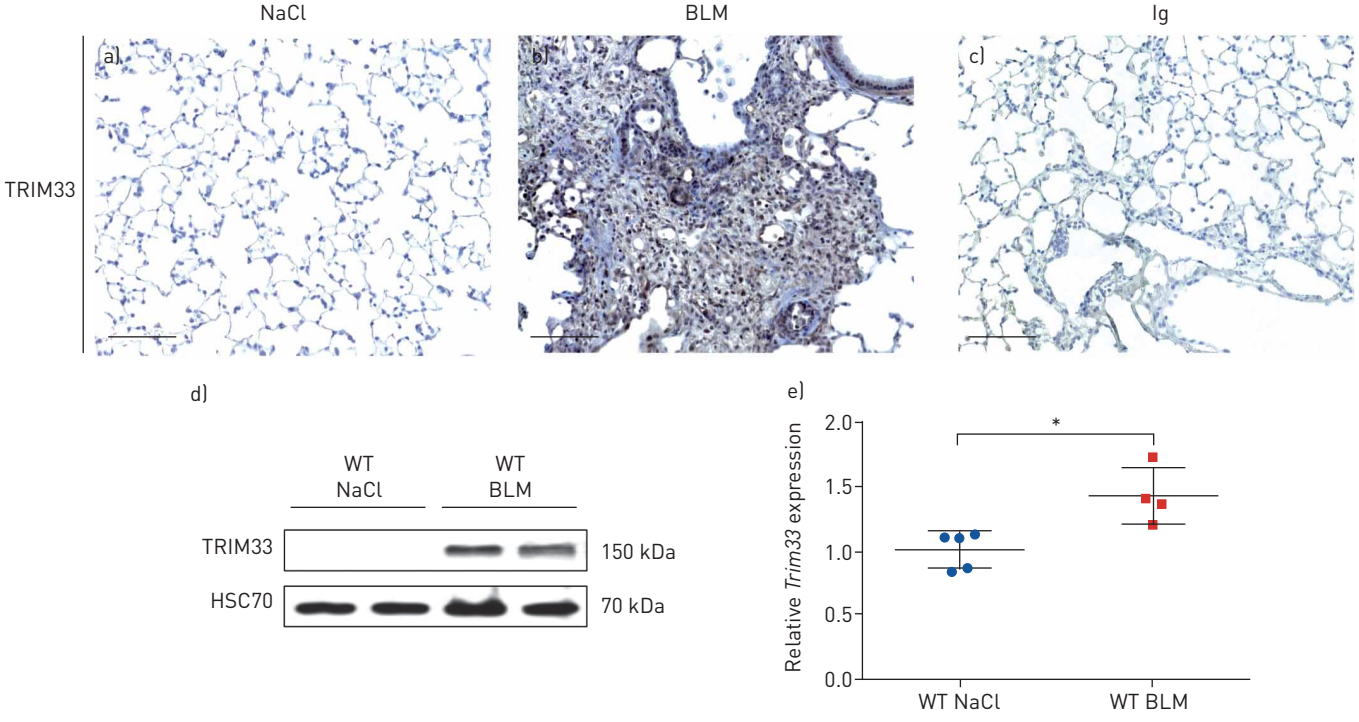

FIGURE 2 TRIM33 is upregulated during bleomycin (BLM)-induced pulmonary fibrosis. a–c) Representative images of TRIM33 immunostaining on lung sections from mice given either a) NaCl or b) BLM by intravenous injections (n=5); c) irrelevant Ig control. Scale bars: 250 µm. d) Western blot and e) mRNA levels of *Trim33* in wildtype (WT) mice given either NaCl or BLM by intravenous injection (n=4 or n=5 per group). d) HSC70 shown as loading control. e) Relative expression compared with *Rpl32* (control=1). Results expressed as median with interquartile range. *: p=0.05, nonparametric Mann–Whitney test.

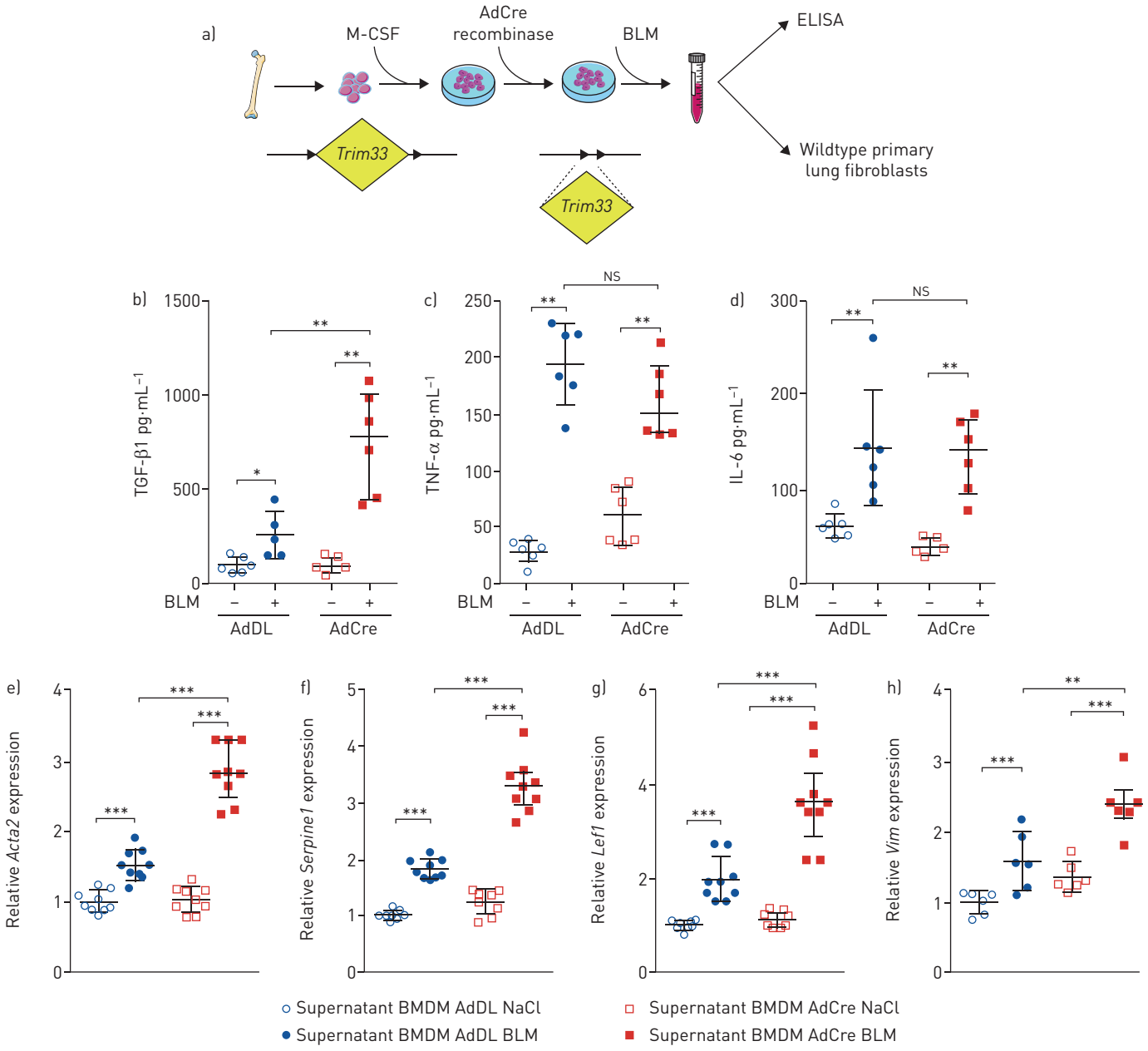

FIGURE 3 *Trim33* inhibition impairs transforming growth factor (TGF)-β1 secretion in macrophages. a) Cartoon showing the Cre-LoxP system used to deplete *Trim33* in bone marrow-derived macrophages (BMDM). M-CSF: macrophage colony-stimulating factor; AdCre: adenovirus Cre recombinase. b–d) Mouse levels of b) active TGF-β1, c) tumour necrosis factor (TNF)-α and d) interleukin (IL)-6 measured by ELISA in supernatant of BMDM from *Trim33*$^{f/f}$ mice exposed to control adenovirus vector (AdDL) or AdCre and either NaCl (–) or bleomycin (BLM; +) for 48 h at 5 mg·mL^{-1}. Results shown for n=3 each with two biological replicates. e–h) Relative mRNA levels compared with *Rpl32* (control=1) of e) actin alpha 2, smooth muscle (*Acta2*), f) serpin family E member 1 (*Serpine1*), g) lymphoid enhancer-binding factor 1 (*Lef1*) and h) vimentin (*Vim*) were analysed by quantitative PCR in murine primary pulmonary fibroblasts treated with supernatant from BMDM exposed to AdDL or AdCre and either NaCl or BLM for 48 h at 5 mg·mL^{-1}. Results shown for n=3 each with three biological replicates. b–h) Results expressed as median with interquartile range. *: p<0.05; **: p<0.01; ***: p<0.001; NS: nonsignificant; all nonparametric Mann–Whitney test.

Trim33 *depletion* ex vivo *promotes signalling pathways downstream of TGF-β1*

As TRIM33 was also overexpressed in fibroblasts from IPF patients (figure 1), we investigated the effects of TRIM33 expression in primary pulmonary fibroblasts. We isolated primary pulmonary fibroblasts from *Trim33*$^{f/f}$ mice and depleted the *Trim33* gene by infection with AdCre (or AdDL as control; figure 5a). The loss of TRIM33 expression was confirmed by Western blot (figure 5b). Treatment of control (AdDL) fibroblasts with recombinant TGF-β1 induced an upregulation of the SMAD pathway downstream gene mRNAs (*Acta2*, *Serpine1*, *Lef1* and twist family BHLH transcription factor 1 *(Twist1)*; figure 5c–f).

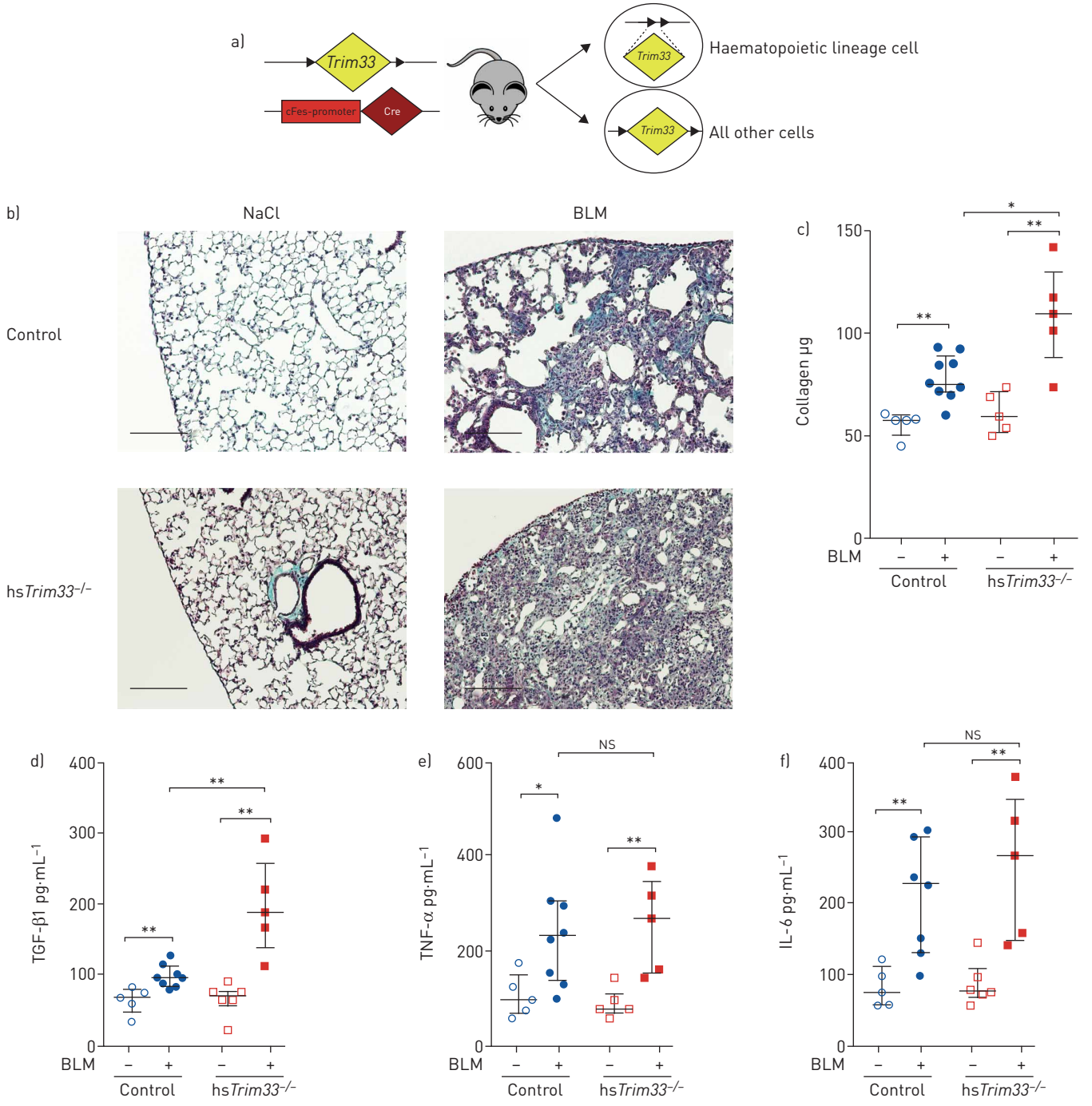

FIGURE 4 Haematopoietic-specific *Trim33*$^{-/-}$ (hs*Trim33*$^{-/-}$) mice are more sensitive to bleomycin (BLM)-induced pulmonary fibrosis. a) Cartoon showing the Cre-LoxP system used to deplete *Trim33* in haematopoietic lineage cells. b) Representative histology of control mice (*Trim33*$^{f/f}$) and hs*Trim33*$^{-/-}$ mice lungs, 21 days after intratracheal injection of NaCl or BLM (1.5 mg·kg^{-1}). Masson's trichrome staining (n=6). Scale bars: 250 µm. c) Collagen quantification using a Sircol assay on the left lung extract from control and hs*Trim33*$^{-/-}$ mice given either NaCl (–) or BLM (+), at day 21. n=4 to n=9 per group. d–f) Mouse levels of d) active transforming growth factor (TGF)-β1, e) tumour necrosis factor (TNF)-α and f) interleukin (IL)-6 were measured by ELISA in bronchoalveolar lavage fluid from control and hs*Trim33*$^{-/-}$ mice, 21 days after intratracheal instillation of BLM or NaCl. n=4 to n=8 per group. c–f) Results expressed as median with interquartile range. *: p<0.05; **: p<0.01; NS: nonsignificant; all nonparametric Mann–Whitney test.

Interestingly, the upregulation of those genes was further increased in *Trim33*-depleted fibroblasts (AdCre) compared to AdDL fibroblasts (2.98-fold increase for *Acta2*, 2.05 for *Serpine1*, 3.2 for *Lef1* and 1.8 for *Twist1*; figure 5c–f). These results were confirmed using 3D lung tissue slices generated from *Trim33*$^{f/f}$ mice and treated with AdCre (figure 5g–j). *Trim33* depletion was confirmed by quantitative PCR (figure 5g) and we showed that this depletion induced, upon TGF-β1 treatment, a significant increase in *Acta2*,

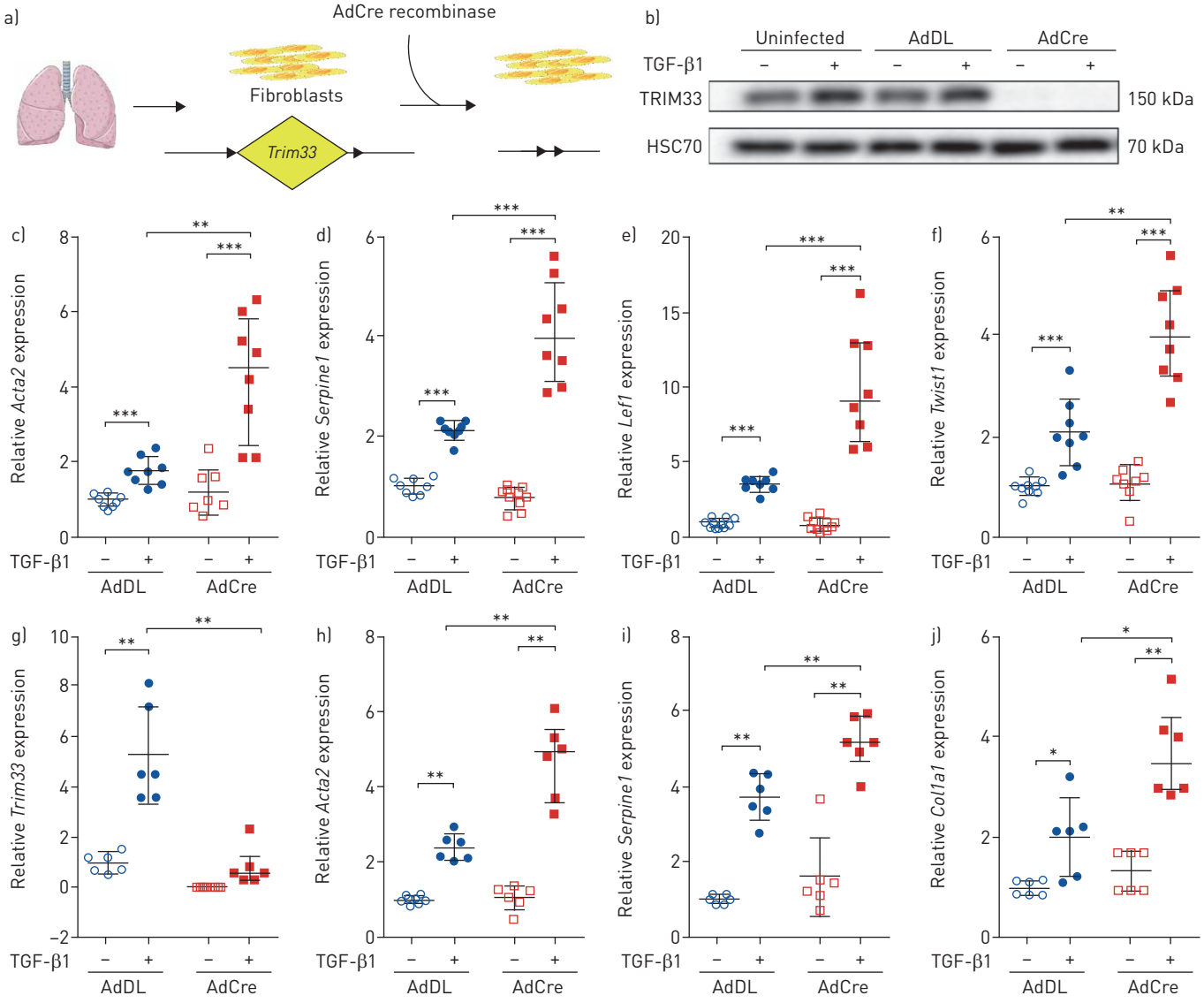

FIGURE 5 Downregulation of *Trim33* promotes pathways downstream of transforming growth factor (TGF)-β1. a) Cartoon showing the Cre-LoxP system used to deplete *Trim33* in primary lung fibroblasts. AdCre: adenovirus Cre recombinase. b) TRIM33 expression was analysed by Western blot in pulmonary primary fibroblasts generated from *Trim33*$^{f/f}$ mice, after the fibroblasts were infected with control adenovirus vector (AdDL) or AdCre and then cultured with recombinant TGF-β1 (48 h at 10 ng·mL^{-1}; n=3). Uninfected fibroblasts shown as control. HSC70 shown as loading control. c–f) mRNA levels of c) actin alpha 2, smooth muscle (*Acta2*), d) serpin family E member 1 (*Serpine1*), e) lymphoid enhancer-binding factor 1 (*Lef1*) and f) twist family BHLH transcription factor 1 *(Twist1)* were analysed by quantitative PCR of pulmonary primary fibroblasts, isolated from *Trim33*$^{f/f}$ mice, infected with AdDL or AdCre and then cultured with recombinant TGF-β1 (48 h at 10 ng·mL^{-1}; n=4 each with two biological replicates). g–j) mRNA levels of g) *Trim33,* h) *Acta2,* i) *Serpine1* and j) collagen type I alpha 1 chain (*Col1a1)* were analysed by quantitative PCR on 3D lung tissue slices generated from *Trim33*$^{f/f}$ mice, infected with AdDL or AdCre and then cultured with recombinant TGF-β1 (48 h at 10 ng·mL^{-1}; n=3 each with two biological replicates). c–j) Relative expression compared with *Rpl32* (control=1). Results expressed as median with interquartile range. *: p<0.05; **: p<0.01; ***: p<0.001; all nonparametric Mann–Whitney test.

Serpine1 and collagen type I alpha 1 chain (*Col1a1)* mRNA (1.9-fold increase expression for *Acta2*, 1.3 for *Serpine1* and 1.54 for *Col1a1*; figure 5h–j).

Therefore, TRIM33 appears to negatively control downstream signalling of TGF-β1 by blocking overactivation of the TGF-β1 pathway in fibrotic conditions.

Trim33 *inhibition in the lungs worsens BLM-induced fibrosis in mice*

To confirm the *in vivo* significance of *Trim33* expression in the lungs, we depleted *Trim33* in *Trim33*$^{f/f}$ mouse lungs by intratracheal administration of AdCre (figure 6a). We observed in our model that 29.7% of the lung cells underwent recombination 48 h after AdCre intratracheal instillation, as measured using flow cytometry (supplementary figure S6a). AdCre instillation was able to induce recombination mostly in

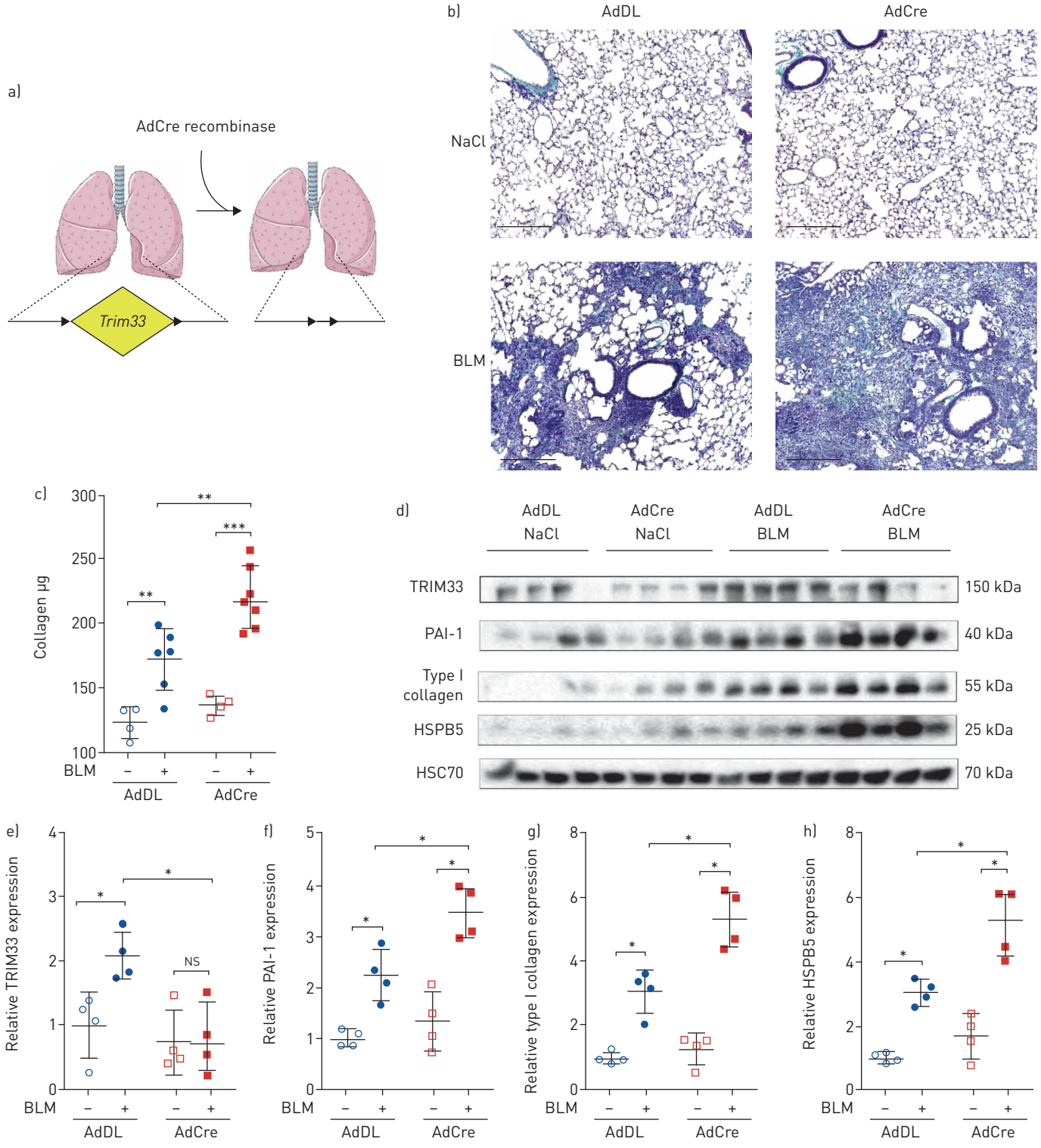

FIGURE 6 *Trim33* inhibition in the lungs worsens bleomycin (BLM)-induced fibrosis in mice. a) Cartoon showing the Cre-LoxP system used to deplete *Trim33* in mouse lung. AdCre: adenovirus Cre recombinase. b) Representative histology of *Trim33*$^{f/f}$ mouse lungs infected with control adenovirus vector (AdDL) or AdCre, then challenged 3 days later with intratracheal injection of NaCl or BLM (1.5 mg·kg^{-1}). Representative images of Masson's trichrome collagen staining at day 21 after instillation (n=6). Scale bars: 250 µm. c) Collagen quantification (21 days after BLM treatment) using a Sircol assay on the left lung from mice infected by AdDL or AdCre and given either NaCl (–) or BLM (+). n=5 to n=8 per group. d) Protein levels of TRIM33, plasminogen activator inhibitor 1 (PAI-1), type I collagen and heat shock protein B5 (HSPB5) were analysed by Western blot in lungs from mice infected by AdDL or AdCre, 21 days after NaCl or BLM treatment. HSC70 shown as loading control. e–h) Densitometry from Western blots for relative protein expression compared with HSC70 (control=1) of e) TRIM33, f) PAI-1, g) type I collagen and h) HSPB5. Results expressed as median with interquartile range. *: p<0.05; **: p<0.01; all nonparametric Mann–Whitney test.

CD326 (epithelial cell adhesion molecule)-positive cells (epithelial cells) but also in ER-TR7-positive cells (fibroblasts) and in some CD68-positive cells (macrophages) (supplementary figure S6b and c). 3 days after AdCre exposure, mice were challenged with BLM and fibrosis was assessed at 21 days post BLM. AdCre-mediated *Trim33* depletion in mice resulted in more severe fibrosis after BLM intratracheal challenge, compared to the AdDL mice expressing normal levels of TRIM33 (figure 6b and c; supplementary figure S7a). The upregulation of fibrosis-related genes induced by BLM at the mRNA level was significantly higher in AdCre mice compared to AdDL mice (1.64-fold increase for *Col1a1*, 1.41 for *Serpine1* and 1.3-fold for *Twist1*; supplementary figure S7a). Upregulation of fibrosis-related markers was also found at the protein level for plasminogen activator inhibitor 1 (PAI-1), type I collagen and HSPB5 (figure 6d and f–h). As expected, mice treated with AdDL showed an increase in TRIM33 protein level upon BLM challenge, whereas mice treated with AdCre did not (figure 6d and e). Interestingly, whereas depletion of *Trim33* potentiated fibrogenesis marker induction, in nonfibrotic conditions it did not appear to have any effect on collagen accumulation, mesenchymal differentiation or on the immune cell profile of the BALF (supplementary figure S7a and b).

TRIM33 activity and protein level are impaired by HSPB5 overexpression

TRIM33 and HSPB5 are described as key regulators of the TGF-β1/SMAD pathway. HSPB5 was upregulated following AdCre-mediated *Trim33* depletion, suggesting a mechanistic regulation between both proteins. In the pulmonary tissue of *Hspb5*-deficient mice (*Hspb5* knockout; supplementary figure S8a), TRIM33 protein level was increased compared with wildtype mice (figure 7a). This difference was further amplified following BLM challenge. At the same time, the depletion of *Hspb5* induced no change in *Trim33* mRNA level in total lung tissue (figure 7b). Similar results were found using primary pulmonary fibroblasts from *Hspb5* knockout mice treated *ex vivo* with TGF-β1 (supplementary figure S8b). However, *HSPB5* overexpression in human alveolar epithelial A549 cells induced a decrease in TRIM33 protein level (figure 7c), again without interfering with its mRNA level (figure 7d). Our data suggest that, in parallel to the induction of its expression after *Trim33* depletion, HSPB5 negatively regulates TRIM33 protein level in a post-translational manner.

Next, we further investigated the protein–protein interaction between HSPB5 and TRIM33. Biolayer interferometry experiments using recombinant purified proteins showed that TRIM33 was able to bind directly to both HSPB5 (with a dissociation constant (K_d) of 2.91×10^{-9} M) and SMAD4 (with a K_d of 1.28×10^{-9} M) (supplementary figure S8c and d). Furthermore, by fluorescence lifetime imaging microscopy - Förster resonance energy transfer (FLIM-FRET) analysis, we showed that HSPB5 overexpression decreased SMAD4–TRIM33 interaction (supplementary figure S8e). In line with these results and the previously described inhibitory role of HSPB5 on TRIM33 activity, we demonstrated that HSPB5 was able to reduce the sumoylation of TRIM33, which is essential for its E3 ubiquitin ligase activity on SMAD4 (figure 7e).

Altogether, these results demonstrate a feedback loop between HSPB5 and TRIM33, respectively positive and negative regulators of the SMAD pathway. We have shown that the TRIM33 level is impaired upon HSPB5 overexpression. Furthermore, we have uncovered that HSPB5 promotes TRIM33 sumoylation and thus inhibits its binding to SMAD4, leading to enhanced TGF-β1 signalling.

Discussion

TRIM33 is an E3 ubiquitin ligase known as a negative regulator of TGF-β1 signalling, one of the most potent profibrotic signals, through SMAD4 ubiquitination and turnover of TGF-β receptors [6, 8]. We have shown that TRIM33 is upregulated in both IPF patients and rodent models of lung fibrosis. *Trim33* depletion *in vitro* and *in vivo* caused an exacerbation of fibroblast activation, collagen deposition and pro-fibrogenic factor secretion under profibrotic conditions (*i.e.* on BLM or TGF-β1 exposure), suggesting a protective role of TRIM33 in lung fibrosis. Therefore, the upregulation of TRIM33 may be viewed as a failing attempt to prevent fibrosis progression in IPF and models of lung fibrosis. TRIM33 inhibitory properties on TGF-β1 signalling might be due to an insufficient level of TRIM33 expression or an intrinsic inhibition of its activity. The present works sheds light on this regulation.

Analysis of publicly available datasets demonstrated an upregulation of *TRIM33* in IPF lungs. However, we hypothesise that the level of upregulation compared to control lungs remains moderate and insufficient to exert its antifibrotic action. Nevertheless, datasets only recapitulate mRNA expression, which may not be representative of the real level of TRIM33, either at the protein level or in terms of activity in the IPF lungs. Our data showed that TRIM33 protein is markedly upregulated, mainly in myofibroblasts and macrophages and also in alveolar epithelial cells, suggesting that TRIM33 may encounter post-translational regulation that prevents its antifibrotic functions. Our team previously demonstrated that HSPB5 upregulation in IPF induced a decrease in the interaction between TRIM33 and SMAD4, hampering the ubiquitination and the nuclear export of the latter [5]. In this context, HSPB5 may serve as a physiological

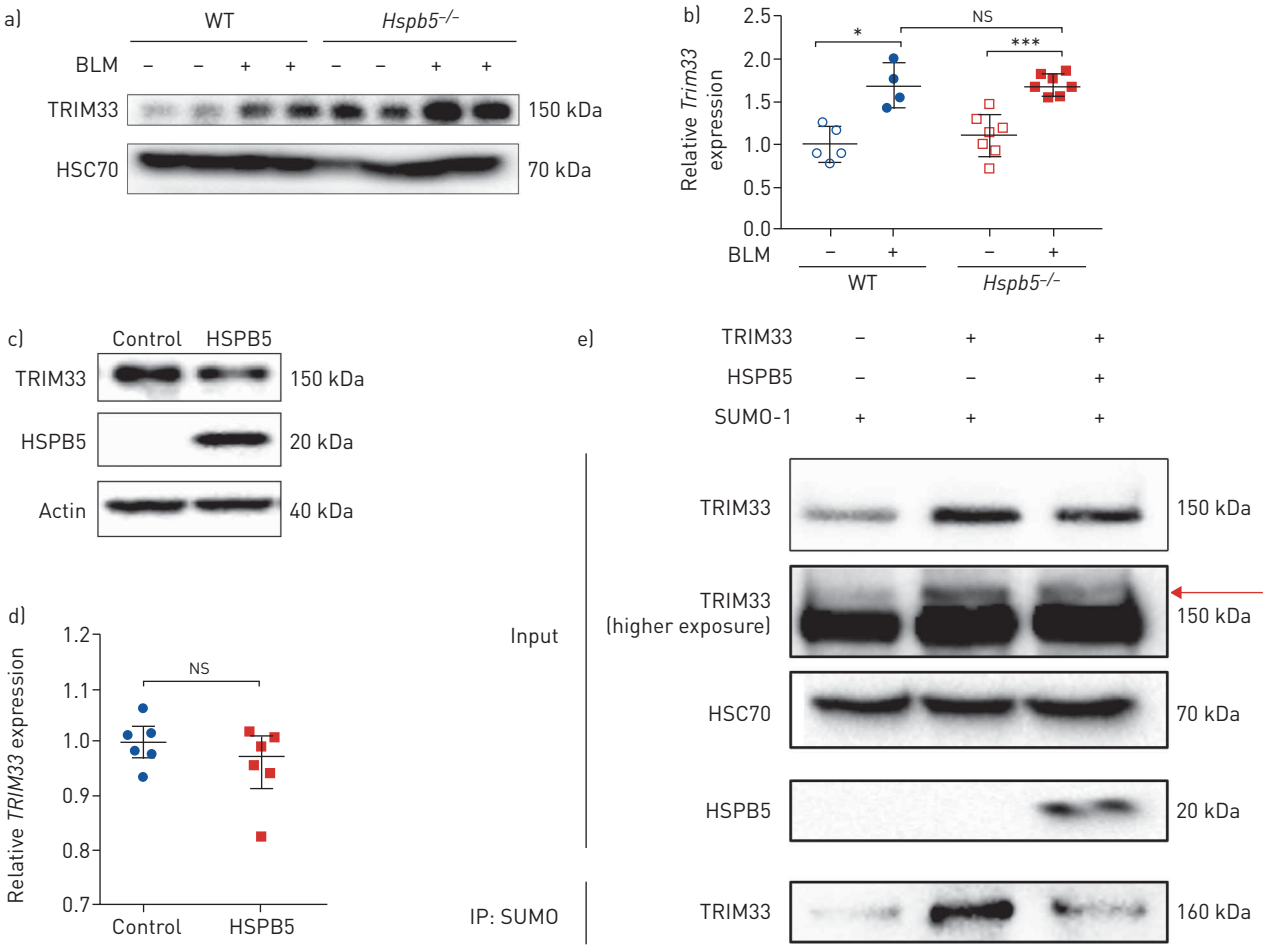

FIGURE 7 TRIM33 activity and protein level are impaired by heat shock protein B5 (HSPB5) overexpression. a) Protein levels of TRIM33 analysed by Western blot (HSC70 shown as loading control) and b) mRNA levels of *Trim33* analysed by quantitative PCR, in lungs from SV129 control wildtype (WT) mice or SV129 *Hspb5*$^{-/-}$ mice, 21 days after either NaCl (–) or bleomycin (BLM; +) treatment. b) n=4 to n=8 per group. c) Protein levels of TRIM33 (actin shown as loading control) and d) mRNA levels of *TRIM33* in A549 cells transfected with a plasmid encoding HSPB5, or the empty vector as control. n=3. b and d) Relative expression compared with *Rpl32* (control=1). Results expressed as median with interquartile range. *: p<0.05; ***: p<0.001; NS: nonsignificant; all nonparametric Mann–Whitney test. e) Pull-down of SUMO-1-his in A549 cells after transfection with either TRIM33 and/or HSPB5 and/or SUMO-1, as indicated (n=3). The arrow indicates a potential sumoylated form of TRIM33.

inhibitor of the TRIM33 negative control on TGF-β1 signalling. Furthermore, we showed that TRIM33 is able to bind to both HSPB5 and SMAD4 directly. Within this complex, HSPB5 appears to reduce the level of sumoylation of TRIM33, therefore reducing its binding and ability to ubiquitinate SMAD4 and resulting in an exacerbation of the profibrotic TGF-β1 signalling. We believe that the inhibition of the interaction between TRIM33 and HSPB5 may be able to restore TRIM33 repressor effects on the TGF-β1 pathway and may be of interest for the treatment of IPF.

Among lung cells, we demonstrated that alveolar macrophages are one of the main cell types expressing TRIM33 in human and rodent lung fibrosis. While the role of inflammation in pulmonary fibrosis remains controversial [20] and despite the lack of efficacy of anti-inflammatory therapies on IPF progression, unbiased approaches have unravelled a significant immune component in IPF and alveolar macrophages appear to be critical for the development of lung fibrosis [21, 22]. Macrophage activation is heavily involved in fibrogenesis regulation in various tissues, including the liver, kidney and lungs [23], mainly through the secretion of TNF-α, IL-6 and, most importantly, TGF-β1 [24], the latter being able to modulate fibroblast proliferation and ECM production [25]. As macrophages are able to produce a variety of profibrotic signals, we hypothesised that TRIM33 may have a key role in controlling the macrophage secretory profile in a fibrotic context. While TRIM33 has been demonstrated to play a role in the inflammatory response in BMDM [26], we have shown here, in fibrotic conditions, that *Trim33* deficiency promotes active TGF-β1 secretion by BMDM. This depletion does not modify the secretion of pro-inflammatory cytokines TNF-α and IL-6, demonstrating a rather specific mechanism towards the TGF-β1 pathway. Remarkably, we demonstrated that *Trim33* deficiency does not interfere with *Tgfb1* gene

expression. TGF-β1 is primarily produced in an inactive form that requires processing inside and outside the cell in order to be secreted and activated. As a consequence, TGF-β1 activity is mainly regulated by post-translational modifications. Therefore, our data suggest that *Trim33* depletion may interfere indirectly with the mechanisms involved in TGF-β1 maturation rather than with *Tgfb1* gene expression.

Myofibroblasts are of major importance in pulmonary fibrosis, as they are the main secretor of ECM. They can originate from various cell types, including resident fibroblasts, fibrocytes and differentiated fibroblasts *via* cellular reprogramming of epithelial cells [27]. TRIM33 has been demonstrated to be involved in mesenchymal differentiation in the oncology field. For instance, *TRIM33* depletion in epithelial cells has been linked to enhanced mesenchymal differentiation in several cancer models including nonsmall cell lung cancer [28], NMuMG mammary epithelial cells [29] and renal cell carcinoma [30]. In lung fibrosis conditions, we have demonstrated that *Trim33* depletion, in primary pulmonary fibroblasts as well as in *ex vivo* cultured lung tissue, promotes mesenchymal differentiation. In addition, depletion of *Trim33* in the lungs by intratracheal administration of AdCre increased sensitivity to BLM-induced fibrosis with an increase in ECM deposition. These results may suggest a crucial role of TRIM33 in the activation of mesenchymal differentiation, leading to an increase in the amount of myofibroblasts and subsequent exaggerated ECM deposition.

It is noteworthy that TRIM33 antibodies are found in the serum of patients in pathological conditions such as polymyositis and dermatopolymyositis. These diseases are associated with cancer but also with ILDs [11]. These ILDs present as nonspecific interstitial pneumonia or with a pattern of usual interstitial pneumonia, as observed in IPF [31]. Although the pathogenic role of TRIM33 antibodies is beyond the scope of the current work, we believe that they should be further explored to better understand their protective or aggravating role in IPF.

Thus, we hypothesise that TRIM33 is induced in profibrotic conditions as part of a negative feedback loop to slow down an exacerbated activation of TGF-β1 signalling. However, in several pathological conditions, such as IPF, the negative control exerted by TRIM33 is swallowed, due either to an inefficient upregulation or to an inhibition by other upregulated proteins in fibrotic conditions, such as HSPB5.

Conclusion

Our work demonstrates that TRIM33 is overexpressed in the lung during fibrotic conditions and we have shown that TRIM33 has a protective role against fibrogenesis by inhibiting the TGF-β1 pathway independently of inflammation. We believe that TRIM33 expression may represent an attempt to resolve the fibrotic process, which is overwhelmed during IPF. We believe that the complex interactions between TRIM33, SMAD4 and HSPB5 may represent key targets in the prevention of progression of fibrosis in cases of induced lung fibrosis, as in iatrogenic diseases or in IPF.

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