Genetic partitioning of interleukin-6 signalling in mice dissociates Stat3 from Smad3-mediated lung fibrosis

Robert J. J. O'Donoghue1,2,3,4, Darryl A. Knight5, Carl D. Richards5, Cecilia M. Prêle2,3, Hui Ling Lau2,3, Andrew G. Jarnicki4, Jessica Jones4, Steven Bozinovski4, Ross Vlahos4, Stefan Thiem1, Brent S. McKenzie7, Bo Wang8, Philip Stumbles9, Geoffrey J. Laurent10, Robin J. McAnulty10, Stefan Rose-John11, Hong Jian Zhu8, Gary P. Anderson4, Matthias R. Ernst1*, Steven E. Mutsaers2,3,12**, y

Keywords: interleukin 6; pulmonary fibrosis; Smad3; Stat3; transforming growth factor beta

DOI 10.1002/emmm.201100604
Received January 18, 2011
Revised April 16, 2012
Accepted May 09, 2012

Idiopathic pulmonary fibrosis (IPF) is a fatal disease that is unresponsive to current therapies and characterized by excessive collagen deposition and subsequent fibrosis. While inflammatory cytokines, including interleukin (IL)-6, are elevated in IPF, the molecular mechanisms that underlie this disease are incompletely understood, although the development of fibrosis is believed to depend on canonical transforming growth factor (TGF)-β signalling. We examined bleomycin-induced inflammation and fibrosis in mice carrying a mutation in the shared IL-6 family receptor gp130. Using genetic complementation, we directly correlate the extent of IL-6-mediated, excessive Stat3 activity with inflammatory infiltrates in the lung and the severity of fibrosis in corresponding gp130757F mice. The extent of fibrosis was attenuated in B lymphocyte-deficient gp130757F, μMT−/− compound mutant mice, but fibrosis still occurred in their Smad3−/− counterparts consistent with the capacity of excessive Stat3 activity to induce collagen 1α1 gene transcription independently of canonical TGF-β/Smad3 signalling. These findings are of therapeutic relevance, since we confirmed abundant STAT3 activation in fibrotic lungs from IPF patients and showed that genetic reduction of Stat3 protected mice from bleomycin-induced lung fibrosis.
INTRODUCTION

Tissue fibrosis results from excessive and progressive scarring associated with destruction of normal tissue architecture and structure, and ultimately compromises organ function (Wilson & Wynn, 2009; Wynn, 2007). The clinical challenge of treating fibrotic diseases is exemplified by idiopathic pulmonary fibrosis (IPF), a heterogeneous disease unresponsive to therapy and fatal in outcome (Knight et al, 2003; Wilson & Wynn, 2009). Although the molecular mechanisms underlying initiation of IPF remain largely unknown, fibrosis is thought to arise from excessive tissue response to injury. Accordingly, effort has concentrated on the genetic dissection of steps that collectively govern normal wound healing processes and that enable re-epithelialization and extracellular matrix production to subside upon re-establishment of tissue homeostasis. Besides epithelial proliferation, differentiation and regeneration, these processes also involve stromal components, which are activated as part of the ensuing inflammatory response (Wilson & Wynn, 2009).

In IPF, epithelial injury is followed by pathologic fibrotic repair in distinct cellular foci within the lung parenchyma comprising proliferating fibroblasts and subepithelial myofibroblasts that are associated with excessive deposition of extracellular matrix proteins, including type I collagen (Cool et al, 2006; Maher et al, 2010; Moodley et al, 2003). Activation of these myofibroblasts correlates with increased levels of interleukin (IL)-1β, IL-4, IL-13, IL-17, and other inflammatory cytokines and is believed to be mediated by transforming growth factor (TGF)-β (Wilson & Wynn, 2009; Wilson et al, 2009). TGF-β and its canonical downstream signalling molecule Smad3 are central to the development and progression of fibrosis as elevated levels of TGF-β are sufficient to reproduce organ fibrosis in animal models, stimulate fibroblast differentiation and epithelial-to-mesenchymal transformation, and the observation that Smad3-deficiency confers resistance in mouse models of IPF (Bonniaud et al, 2004; Zhao et al, 2002). During inflammation, stromal cells and those of the macrophage/monocyte lineage release inflammatory cytokines of the IL-6 family, which are thought to promote fibrosis through Erk1/2 signalling-associated fibroblast proliferation (Moodley et al, 2003) and the induction of a fibrotic response that is mediated by various TGF-β family of ligands (Ogata et al, 2006). Several members of the IL-6 cytokine family, which is characterized by the shared use of the common gp130 receptor subunit, have been implicated in pulmonary fibrosis. Transgenic overexpression of IL-11 or Oncostatin M (Osm) in mice, for instance, promotes lung scarring with striking histo-pathological similarities to that observed in human disease (Bamber et al, 1998; Kuhn et al, 2000). Meanwhile, overexpression of IL-6 is a common finding in bronchoalveolar lavage (BAL) fluid of IPF patients (Mozaffarian et al, 2008), where IL6 gene polymorphisms segregate with disease severity (Pantelidis et al, 2001). Meanwhile, activation of the latent transcription factor Stat3, one of the signalling molecules engaged by gp130, has been proposed to affect fibrosis in skin and liver (Ghazizadeh et al, 2007; Ogata et al, 2006), albeit with contradicting outcomes (Mair et al, 2010). These observations therefore leave the mechanisms unresolved by which the wide-spread expression of gp130 on epithelial, stromal and hematopoietic cells, and the individual intracellular molecular components engaged by gp130 contribute to fibrosis and whether this response requires canonical TGF-β/Smad3 signalling (Knight et al, 2003).

In this study, we use gp130 mutant mice with either deregulated Stat1/3 or Erk1/2-signalling to assess susceptibility to bleomycin administration as a widely used model that recapitulates epithelial injury-induced lung fibrosis (Moeller et al, 2008). We found that ligand-dependent excessive Stat1/3 activation, either in the hematopoietic or stromal compartments of gp130F/F mice (Jenkins et al, 2005a), increased bleomycin-induced fibrosis in an IL-6-dependent manner, and that systemic ablation of Il6 or impairment of gp130-mediated Stat3 activation, attenuated the fibrotic response. Importantly, gp130-mediated lung fibrosis occurred independently of canonical TGF-β signalling in Smad3-deficient mice, but required mature B lymphocytes and correlated with parenchymal accumulation of B-cell containing foci. With the prevalence of excessive Stat3 activation in lungs of IPF patients and the capability of therapeutically targeting components of the gp130 signalling cascade, our findings are likely to be of clinical relevance.

RESULTS

Excessive fibrotic response in mice with exaggerated IL-6-dependent Stat3 hyperactivation

To mimic the development of human inflammation-associated lung fibrosis in mice, we trans-nasally administered bleomycin to gender-matched mice (8–12 weeks of age) harbouring gp130 mutations that bias intracellular signalling either towards the Stat1/3 or Erk1/2 signalling pathways in gp130−/− mice or gp130A55R mice, respectively (Tebbutt et al, 2002) (Supporting Information Fig S1). Compared to wild-type (gp130wt) mice, bleomycin-challenged gp130F/F mice showed extensive changes to their lung architecture, including consolidation of airspaces, thickened alveolar septae, inflammation and epithelial dysplasia (Fig 1). In contrast, gp130A55R mice were completely protected from pulmonary fibrosis. We corroborated these observations by measuring hydroxyproline content of lungs as an established marker of collagen deposition 14 and 30 days following bleomycin challenge, as well as by assessing Col1a1 transcription in lungs of these mice (Fig 2A and B). We have previously shown that gp130A55R, gp130D57F and gp130D57F mice simultaneously comprise an allelic series for increasing gp130-mediated Stat1/3 signalling (attenuated in gp130A55R and excessive in gp130D57F mice) and for Erk1/2 signalling (excessive in gp130A55R and attenuated in gp130D57F mice) (Jenkins et al, 2005a; Tebbutt et al, 2002). This was confirmed by examining the abundance of transcriptionally active, tyrosine phosphorylated form of Stat3 (pStat3) in lung fibroblasts obtained from gp130D57F, gp130D57F and gp130A55R mice treated with IL-6 for 0–180 min (Supporting Information Fig S2A). The molecular rationale underpinning the reciprocal relationship between activation of the Stat1/3 and Erk1/2 signalling arises from the observation that the negative regulatory Socs3 protein
is transcriptionally induced by Stat3 and requires binding to the phosphorylated tyrosine residue in position 757 in mouse (759 in human) gp130 (Ernst & Jenkins, 2004).

In order to dissect the contribution of the individual pathways engaged by gp130 to the fibrotic response, we systemically restricted the pool of Stat3 or Stat1 available for activation using compound gp130757F mice (Ernst et al, 2008). Upon reduction of excessive Stat3 activation observed in compound gp130757F mice to levels more comparable to those observed in gp130wt mice, we detected a similar fibrotic response between gp130757F;Stat3−/− and gp130wt mice 30 days after challenge (Figs 1 and 2A,B). However, complete Stat1 ablation in gp130757F;Stat1−/− mice only provided partial protection from fibrosis (Supporting Information Fig S2B and C). These genetic observations imply that the enhanced fibrosis observed in gp130757F mice mediated by increased Stat3 activation exceeds that mediated by genetic ablation of Stat1 expression (Walters et al, 2005) and suggest a direct relationship between the severity of the fibrotic response and the extent of gp130-mediated Stat3 signalling. Consistent with this, we detected excessive Col1a1 luciferase (Col1a1-luc) reporter activity in gp130757F;Stat1−/− embryonic fibroblasts in response to stimulation with the pan-gp130 designer cytokine HYPER-IL-6 (Fig 2C). Since HYPER-IL-6 activates gp130 independently of the ligand binding
IL-6 receptor α-subunit (Fischer et al, 1997), this excludes the possibility that our observed results reflect potential differences in endogenous IL-6 receptor expression between the different genotypes. We measured mRNA levels of the IL-6 family members Il6, Il11 and Osm in the lungs of wt and gp130<sup>757F</sup> mice 3 days after bleomycin challenge and found that in bleomycin-challenged lungs of gp130<sup>757F</sup> mice, Il6 mRNA remained selectively elevated 14 days later (Fig 2D and Supporting Information Fig 2D). Trans-nasal administration of HYPER-IL-6 promoted Colla1 gene transcription profoundly in lungs of gp130<sup>757F</sup>;Stat3<sup>−/−</sup> mice (Fig 2E). The potential role of IL-6 family molecules directly promoting lung fibrosis was further supported by our observation that genetic ablation of Il6 in gp130<sup>757F</sup>;Il6<sup>−/−</sup> mice...
ameliorated the excessive fibrotic responses induced in bleomycin challenged \textit{gp130}^{757F} mice (Supporting Information Fig S3A and B).

**Excessive fibrotic response in \textit{gp130}^{757F} mice depends on mature B lymphocytes**

Injury-dependent induction of cytokines primes the subsequent inflammatory response that precedes the development of pulmonary fibrosis (Wilson & Wynn, 2009). We therefore analysed BAL fluid from bleomycin-challenged mice and observed augmented cytokine accumulation, in particular of IL-1, IL-6, IL-13, and GCSF, in \textit{gp130}^{757F} mice when compared to \textit{gp130}^{wt}, \textit{gp130}^{757F};\textit{Stat3}^{+/-}, or \textit{gp130}^{DStat} mice (Supporting Information Fig S4A and B). However, at the height of the inflammatory response 3 days after bleomycin administration (Moeller et al, 2008; Wilson & Wynn, 2009), there was no correlation between the number of inflammatory cells contained within BAL fluid of bleomycin-challenged mice (Fig 3A and Supporting Information Fig 4C) and extent of fibrosis 30 days later (Figs 1 and 2A and Supporting Information Fig 3A and B). Foci of inflammatory cells within the lung parenchyma were most prominent in \textit{gp130}^{757F} mice compared to all other genotypes of mice consistent with the severe fibrosis observed in these mice (Fig 3B and Supporting Information Fig S5A). Collectively, these data suggest that attenuated Stat3 activation may enable egression of inflammatory cells into BAL fluid, while excessive Stat3 activity may promote their retention in the lung parenchyma.

![Figure 3](https://www.embomolmed.org/EMBO_Mol_Med_2012_4_939-951/article-figures/)

**Figure 3. Increased lymphocytes are associated with inflammation and lung fibrosis.**

A. Changes to cell numbers in BAL fluid of wild-type (\textit{wt}), \textit{gp130}^{757F} (757F), \textit{gp130}^{DStat} (\Delta\textit{Stat}) and \textit{gp130}^{757F};\textit{Stat3}^{+/-} (757F;\textit{Stat3}^{+/-}) mice 3 days after bleomycin administration relative to saline-treated mice of the same genotype. \(n = 4\) mice. MØ, macrophages; PMN, polymorphonuclear cells and LYM, lymphocytes.

B. Distribution of inflammatory cells in foci within the lung parenchyma of mice 3 days after bleomycin treatment. Cell types were identified by histological appearance (PMN) or immunohistochemical staining for F4/80 (MØ), or CD3 and B220 (LYM). \(n = 4\) mice.

C. Female \textit{wt} or 757F mice (\(n > 3\) per group) were reconstituted with bone marrow from 757F, \textit{wt} or \DeltaStat mice and lungs were collected challenged 21 days after bleomycin or saline administration. Bars indicate percent change in collagen content in lung homogenates between saline and bleomycin treated mice. The range of collagen between saline and bleomycin treated mice was 7.089–31.658 mg. Empty bars show lung collagen levels in bleomycin challenged mice that have not undergone bone marrow transplant.

D. qPCR analysis of \textit{Col1a1} mRNA expression in lung homogenates 30 days after bleomycin challenge of \textit{wt}, 757F, \textit{gp130}^{757F};\textit{Rag1}^{+/-} (757F;\textit{Rag1}^{+/-}) and \textit{gp130}^{757F};\textit{mMT}^{+/-} (757F;\textit{mMT}^{+/-}) mice. \textit{Col1a1} signals were normalized to 18S and expressed relative to saline-treated mice of the same genotype. \(n = 4\) mice. All data are expressed as mean \pm SEM with \(p < 0.05\), \(p < 0.01\) and \(p < 0.001\) using Bonferroni multiple comparisons test.
Stat3 mediates lung fibrosis independent of Smad3

Since bleomycin-induced lung fibrosis depends on the preceding inflammatory response (Moeller et al, 2008), we performed adoptive bone marrow transfer experiments to compare the contributions to disease between the hematopoietic and the parenchymal compartment. We excluded a major effect from the irradiation process, since the fibrotic response remained indistinguishable between non-irradiated and non-reconstituted bleomycin challenged gp130 WT or gp130F mice and their syngeneically reconstituted counterparts (Fig 3C). However, in reconstituted gp130 WT hosts, but not in gp130F hosts, we observed a gradual attenuation of bleomycin-induced hydroxyproline accumulation that correlated with the extent by which gp130-dependent Stat3 activation could occur in donor cells (i.e. excessive in gp130 WT and attenuated in gp130F bone marrow cells). Furthermore, when reconstituted with bone marrow proficient for gp130-dependent Stat3 signalling (i.e. gp130 WT or gp130F cells), gp130 WT mice were more susceptible to fibrosis than gp130F hosts. Collectively, these observations suggest that excessive Stat3-mediated Stat3 signalling in the lung parenchyma of gp130 WT hosts promotes fibrosis, which is further amplified by excessive Stat3 signalling in bone marrow-derived cells.

The striking correlation between focal lymphocytic accumulation in the lung parenchyma of gp130 WT mice and the severity of their fibrotic response (Figs 2A and 3B) is consistent with a role for lymphocytes during development of bleomycin-induced lung fibrosis and their prominent abundance in fibrotic tissues of IPF patients with non-specific interstitial pneumonia (Keogh & Limper, 2005; Wilson & Wynn, 2009). Indeed, we observed a profound increase in B220-positive cells in bleomycin-challenged gp130 WT mice, which persisted for 30 days (Supporting Information Fig S5A and B). Furthermore, mature lymphocytes exacerbate fibrosis in gp130 WT mice, since fibrosis in T and B lymphocyte compound-deficient gp130 WT;Rag1−/− mice was reduced and comparable to disease in bleomycin challenged wild-type mice (Supporting Information Fig S5D). This observation was corroborated at the level of Col1α1 gene transcription which was similar in wild-type and gp130 WT;Rag1−/− mice (Fig 3D). Moreover, we also observed reduced fibrosis and collagen transcription in gp130 WT;μMT−/− compound mutant mice deficient in mature B lymphocytes. Collectively, our findings suggest that bone marrow derived B lymphocytes promote bleomycin-induced fibrosis in susceptible gp130 WT hosts (Fig 3D and Supporting Information Fig S5D).

TGF-β response is blunted in gp130 WT mice

The development of experimental fibrosis correlates with elevated TGF-β levels, which is thought to molecules link the activity of inflammatory cytokines to the resulting fibrotic response (Bonniaud et al, 2005; Gauldie et al, 2007). This concept is supported by the observation that two distinct Smad3 null mutations protect mice from bleomycin-induced lung fibrosis (Bonniaud et al, 2004; Zhao et al, 2002) as well as TGF-β-induced lung fibrosis (Bonniaud et al, 2004; Bonniaud et al, 2005). Using recombinant TGF-β1 as a reference, we detected elevated levels of TGF-β activity in serum from gp130 WT mice when assayed on NIH3T3 cells expressing the pCAGA12-luc reporter plasmid that records Smad3-dependent gene transcription (Supporting Information Fig S6A). However, when assaying for TGF-β-induced phosphorylation of Smad3 (pSmad3), we observed reduced abundance of this transcriptionally active form of Smad3 in lung fibroblasts from gp130 WT mice compared to those prepared from gp130 WT, gp130 Stat3 or gp130 WT;Stat3−/− mice (Supporting Information Fig S6B). Similarly, the capacity of TGF-β to induce Col1α1 gene expression was reduced in lung fibroblasts from gp130 WT mice when compared to those obtained from either gp130 WT or gp130 WT;Stat3−/− mice (Supporting Information Fig S6C), suggesting that excessive Stat3 activation decreases TGF-β responsiveness of primary lung fibroblast from gp130 WT mice. Indeed, this observation is consistent with our previous findings that excessive Stat3 activity blunted TGF-β-induced signalling (i.e. Smad2 phosphorylation) and transcriptional response (i.e. pCAGA12-luc activity) in mouse embryo fibroblasts and gastric epithelium of gp130 WT mice due to enhanced transcriptional induction of the TGF-β signalling antagonist Smad7 (Jenkins et al, 2005a).

Fibrosis in gp130 WT mice occurs independently of Smad3

Since gp130 WT mice develop a more profound fibrotic response to bleomycin despite their attenuated TGF-β responsiveness, we next determined genetically whether their enhanced lung fibrosis could occur independently of canonical TGF-β signalling. We therefore challenged gp130 WT and gp130 WT;Smad3−/− mice with bleomycin and observed 21 days later in both genotypes of mice a profound fibrotic response that was characterized by excessive hydroxyproline accumulation and collagen deposition in the pulmonary interstitium (Fig 4A and B). In gp130 WT mice, however, ablation of Smad3 (Smad3−/−) reduced bleomycin-dependent lung fibrosis in mice as previously reported (Bonniaud et al, 2004; Zhao et al, 2002) when compared to the fibrotic lesions and excessive collagen deposition observed in Smad3 proficient wild-type mice 30 days after bleomycin challenge (Fig 4A and B).

To ascertain that excessive Stat3 activation could mediate bleomycin-induced fibrosis independently of Smad3, we extended this observation to a physiologically more relevant setting where excessive Stat3 signalling occurred in gp130 WT mice in response to prolonged exposure to gp130 cytokines rather than through engagement of mutant gp130 WT receptors. Since prolonged exposure of mice to Osm and other gp130 cytokines promotes excessive collagen production and fibroblast proliferation (Mozaffarian et al, 2008; Scaffidi et al, 2002), we transnasally administered a replication-deficient adenovirus encoding murine Osm to wild-type mice and demonstrated an increase in transcriptionally active phosphorylated Stat3 (Supporting Information Fig S6D). Furthermore, continuous exposure of Smad3−/− mice to Osm induced severe subepithelial and interstitial pulmonary fibrosis 14 days later that was comparable to the response seen in wild-type mice (Fig 4C). This was in contrast to Smad3 deficiency conferring protection from bleomycin-induced lung fibrosis in gp130 WT mice (Fig 4A and B).
Excessive STAT3 phosphorylation in human IPF tissue

To explore the potential relevance of our findings in the gp130757F mouse model to human IPF, we first confirmed that excessive Stat3 activation in fibrotic lungs of bleomycin challenged gp130757F mice was dependent on IL-6 by monitoring expression of the bona fide Stat3 target gene Soc3 (Kidder et al., 2008; Snyder et al., 2008). While we observed an IL-6-independent transient increase in Soc3 expression 3 days after the bleomycin challenge (Fig 5A), Soc3 expression remained elevated up to 30 days only in IL-6-proficient gp130757F mice if Stat3 expression was not genetically depleted (i.e. in gp130757F;Stat3−/− mice). These observations correlated with our findings of accumulation of phosphorylated Stat3 within cells that collectively comprise the fibrotic areas of either bleomycin-challenged gp130757F or gp130wt mice, which also extended to cells immediately adjacent to the collagenous deposits (Fig 5B). Next, we retrospectively investigated STAT3 activation in lung sections from IPF patients that were clinically diagnosed with usual interstitial pneumonia (IPF-UIP, Supporting Information Table T1). Since we detected pronounced pSTAT3 staining in the parenchymal cells adjacent to collagenous foci in these biopsies (Fig 5C), we categorized these cells by concordant immunoreactivity for pan-cytokeratin or CD45 as being of epithelial and haematopoietic origin, respectively. This analysis revealed pSTAT3 nuclear staining in cells co-expressing pan-cytokeratin in addition to extensive regions of cells with nuclear pSTAT3 staining that was discordant with cells staining for pan-cytokeratin or CD45.
Figure 5.
Adjacent lung sections stained either with Masson’s trichrome or phosphorylated Stat3 (pStat3) of lungs from B, C.

Figure 5. Lung fibrosis is associated with activation of gp130-Stat3 signalling cascade

Haematoxylin and eosin (H&E) stained and dual fluorescence immunohistochemical labelled pSTAT3 (red) and pan-cytokeratin (green) or CD45 (green), respectively, in lung sections from IPF-UIP patients. Scale bar = 100 μm (H&E), = 20 μm (immunofluorescence).

DISCUSSION

Although a timely resolution of acute inflammation in response to (bleomycin-induced) injury enables restoration of normal tissue architecture, an unrelenting inflammatory response undermines the healing process and culminates in tissue fibrosis (Wilson & Wynn, 2009; Wynn, 2007). Previous studies have attempted to experimentally replicate excessive inflammation by overexpressing inflammatory cytokines including TNF-α or IL-1β (Kolb et al., 2001; Sime et al., 1998). Here, we provide a model where the increased sensitivity of mutant gp130757F receptors mediates cytokine-dependent Stat3 activation in mice akin to a smouldering asymptomatic inflammation triggered by prolonged intranasal administration of Osm to wild-type mice or excessive abundance of IL-6 or OSM associated with the pathogenesis of fibrosis in IPF patients (Lesur et al., 1994; Mozaffarian et al., 2008; Xing et al., 1994).

While the exact mechanism of action by which excessive Stat3 activation promotes fibrosis remains to be further elucidated, our observation in bone-marrow reconstituted mice indicates a shared contribution of non-haematopoietic (most likely pulmonary epithelium and fibroblast) and haematopoietic cells types. Furthermore, our data also suggests that excessive Stat3 activation promotes tissue retention of innate immune cells and increases the numbers of immature and mature lymphocytes in gp130757F mice (Jenkins et al., 2005b). Consistent with this, others have reported that blocking the interaction between the Stat3 target gene Icam-1 and lymphocytes decreased lymphocyte retention in the normal pulmonary vasculature (Klemm et al., 2000). Although IL6 is a well characterized inflammatory target gene for TNF-α, and bleomycin-induced fibrosis is ameliorated in Tnfr−/− mice (Ortiz et al., 1998), B cell infiltrates also fuel IL-6-mediated and Stat3-dependent cancer growth following the release of lymphotoxin (LT) (Ammirante et al., 2010). Intriguingly, bleomycin-induced fibrosis is not only prevented in Tnfr−/−;Llxα−/− mice (Piguet et al., 1997), but also depends on the presence of CD19-positive B-cells in wild-type mice (Komura et al., 2008). Consistent with these observations, we provide here genetic evidence that the bleomycin-induced fibrotic response in gp130757F mice requires mature B lymphocytes. This correlates with exacerbated accumulation of B220+ cells and with enhanced abundance of the Th2 cytokine IL-13 in the lung, which in itself not only requires Stat3 for its effective production (Stritesky et al., 2011), but also promotes fibrosis (Fichtner-Feigl et al., 2006). It is therefore tempting to speculate that, for instance, the combined activity of LT and TNF-α, through induction of gp130-activating cytokines, indirectly promotes Stat3 signalling and the excessive matrix production that underpins lung fibrosis. This view is consistent with observations that the lower respiratory tract of IPF patients shows excessive TNF-α, LT-α and IL-6 expression (Lesur et al., 1994; Pantelidis et al., 2001) and that the fibrotic response correlates with B lymphocyte accrual in lungs from non-specific interstitial pneumonia patients (Keogh & Limper, 2005). Furthermore, exogenous administration of IL-6 family cytokines may short-circuit the need for lymphocytes, because in their absence in Rag1−/− mice, Osm can promote lung fibrosis (Mozaffarian et al., 2008). Indeed, IL-6-dependent Stat3 activation also promotes the production of two other potential
Stat3 mediates lung fibrosis independent of Smad3

...to link signalling from IL-6 family cytokines to stimulation of IPF. Recent findings further strengthen a rationale to exploit Stat3 as a therapeutic target for bleomycin-induced fibrosis, namely miR-21 and the generation of IL-17A-producing cells (Liu et al., 2010; Wilson et al., 2009). Together with our observations of excessive pSTAT3 accumulation in human IPF biopsies, these recent findings further strengthen a rationale to exploit Stat3 as an attractive signalling node for novel therapeutic strategies for IPF.

Here, we provide genetic evidence for the capacity of Stat3 to link signalling from IL-6 family cytokines to stimulation of Col1a1 expression and parenchymal collagen deposition independently of canonical TGF-β/Smad3 signalling. Previous reports have described the capability of IL-6 to stimulate collagen deposition in the skin and have identified putative Stat3 binding sites in the Col1a1 and Col3a1 promoters (Lim et al., 2006). Consistent with this, we observed reduced bleomycin-induced fibrosis in the absence of IL-6 and that gp130-mediated increase in collagen gene reporter activity in fibroblasts was dependent on Stat3. By contrast, transgenic expression of IL-6 in the Clara cells of the uninjured mouse lung was associated with airspace enlargement in older mice (Kuhn et al., 2000), reminiscent of the distinct emphysematous and inflammatory changes in lungs of naïve 6 months old gp130<sup>757F</sup> mice (Ruwanpura et al., 2011). Although the gp130<sup>757F</sup> mice used in this study were less than 3 months old and occasionally showed mild air space enlargement (Fig 1; Ruwanpura et al., 2011), there was no evidence for increased IL-6 levels (Fig 2D and Supporting Information Fig S4A) at this stage. Since IL-6 expression rapidly increased after bleomycin challenge, we surmise that excessive IL-6 within the injured lung promotes fibrosis.

Although the development of experimental lung fibrosis in wild-type mice is inhibited in the absence of canonical TGF-β/Smad3 signalling (Bonniaud et al., 2004; Zhao et al., 2002), the protective effect arising from its ablation is overcome in situations of excessive Stat3 activation that results from mutant gp130<sup>757F</sup> receptors during bleomycin-induced lung injury or from the sustained presence of IL-6 family cytokines, including Osm. Although the inflammatory response elicited by systemic LPS administration induces the production of the transcriptionally active Smad2 splice variant Smad2<sup>ΔExon3</sup> (Dunn et al., 2005), which can replicate Smad3 transcriptional activity (Yagi et al., 1999), it remains unknown whether Smad2<sup>ΔExon3</sup> can mediate activation of the Col1a1 gene promoter. Notwithstanding that canonical TGF-β signalling integrates various inflammatory and regenerative stimuli that promote wound healing and the deposition of de novo tissue matrix, our data provide a strong rationale for targeting the gp130/Stat3 signalling axis as a complementary approach to current clinical trials focusing on components of the TGF-β signalling cascade.

**MATERIALS AND METHODS**

**Mice and treatments**

All mutant gp130<sup>ΔExon3</sup> and gp130<sup>757F</sup> mice along with their corresponding Stat3<sup>−/−</sup>, Stat1<sup>−/−</sup>, Il6<sup>−/−</sup>, Rag1<sup>−/−</sup>, mt<sup>−/−</sup> or Smad3<sup>−/−</sup> counterparts were between the ages of 8 and 12 weeks and were propagated on a mixed 129/Sv x C57BL/6 genetic background (Ehlich et al., 1993; Ernst et al., 2008; Jenkins et al., 2005a; Tebbutt et al., 2002). All animals were housed under specific pathogen-free conditions and experimentation was approved by the Institute’s Animal Ethics Committee.

We transnasally delivered a bolus of 50 µl bleomycin (0.05 U/mouse; Blenoxane, Bristol-Myers Squib, New York, USA), or 2 µg HYPER-IL-6 every second day for 14 days to anaesthetized mice. We trans-nasally delivered 5 x 10<sup>7</sup> PFU adenoviral vector in 30 µl PBS and collected lungs 14 days later (Langdon et al., 2003). Lethally irradiated mice...
The paper explained

PROBLEM:
Idiopathic pulmonary fibrosis (IPF) is a heterogeneous disease with an incidence of approximately 1 per 10,000, equivalent to many cancers, and which is unresponsive to therapy and fatal in outcome. Although fibrosis is thought to arise from excessive tissue response to injury, the molecular mechanisms underlying the initiation of IPF remain largely unknown.

RESULTS:
In the present study we utilize mutant mice carrying engineered mutations in gp130, the interleukin (IL)-6 co-receptor, to provide genetic evidence for the causal involvement of IL-6-dependent Stat3 signalling in this disease in an established preclinical setting. Using a genetic complementation approach, we define a functional requirement for IL-6, Stat3 and mature B-lymphocytes for the development of disease. Significantly, we provide genetic and biochemical evidence that Stat3-driven lung fibrosis can occur by a mechanism independent of signalling through the canonical transforming growth factor (TGF)-β/Smad3 pathway. We also document excessive Stat3 activation as a common feature in human patients with IPF and provide evidence that reduction of systemic Stat3 expression in mice decreases susceptibility to bleomycin-induced fibrosis.

IMPACT:
Previous studies have demonstrated that canonical TGF-β/Smad3 signalling is pivotal to the pathogenesis of pulmonary fibrosis. Our study demonstrates that therapeutic targeting of IL-6/Stat3 signalling and/or B-lymphocytes may ultimately afford more efficacious treatments for IPF and related diseases than those directed solely against TGF-β/Smad3 signalling.

Cytokines, plasmids and antibodies
The pCol1a1-luc and p(CAGA)12-luc constructs and the production of HYPER-IL-6 has been described (Buttner et al, 2004; Fischer et al, 1997). Recombinant human IL-6 and human TGF-β1 were from Bender Medsystems (Vienna, Austria) and Sigma–Aldrich (St Louis, USA), respectively. Antibodies directed against B220 were from Becton Dickinson Biosciences (San Jose, USA), CD3 from Serotec (Kidlington, UK), F4/80 from Abcam (Cambridge, UK), α-tubulin (Sigma–Aldrich), CD45 and cytokeratin AE1/AE3 from Dako (Glostrup, DK), phosphorylated Stat3 (Cell Signaling Technology, Danvers, USA) and β-actin, Stat3 or phosphorylated Smad3 (Santa Cruz Biotechnology).

Collagen quantification, lung fibroblast preparation and cellular assays
We analysed hydroxyproline content of lung homogenates as described (Mutsaers et al, 1998). We digested lungs in 0.25% trypsin-EDTA (Invitrogen Life Technologies, Carlsbad, USA) at 37°C for 30 min and removed remaining connective tissue. Digests were finely minced and incubated in 1 mg/ml collagenase 1 (Invitrogen Life Technologies) for 1 h at 37°C. Pelleted cells were used for establishing primary lung fibroblast cultures in DMEM (Invitrogen Life Technologies) for 1 h at 37°C. Pelleted cells were stained with Quik-Dip (Scot Scientific, Taren Point, Australia) and differential cell counts were reconstituted with bone marrow (Ernst et al, 2008) at least 30 days before challenging with bleomycin. BAL fluid was collected by endotracheal instillation of three 0.4 ml aliquots of PBS to recover approximately 1 ml of fluid, pelleted cells were stained with Quik-Dip (Scot Scientific, Taren Point, Australia) and differential cell counts performed.

Cytokines, plasmids and antibodies
The pCol1a1-luc and p(CAGA)12-luc constructs and the production of HYPER-IL-6 has been described (Buttner et al, 2004; Fischer et al, 1997). Recombinant human IL-6 and human TGF-β1 were from Bender Medsystems (Vienna, Austria) and Sigma–Aldrich (St Louis, USA), respectively. Antibodies directed against B220 were from Becton Dickinson Biosciences (San Jose, USA), CD3 from Serotec (Kidlington, UK), F4/80 from Abcam (Cambridge, UK), α-tubulin (Sigma–Aldrich), CD45 and cytokeratin AE1/AE3 from Dako (Glostrup, DK), phosphorylated Stat3 (Cell Signaling Technology, Danvers, USA) and β-actin, Stat3 or phosphorylated Smad3 (Santa Cruz Biotechnology).

Collagen quantification, lung fibroblast preparation and cellular assays
We analysed hydroxyproline content of lung homogenates as described (Mutsaers et al, 1998). We digested lungs in 0.25% trypsin-EDTA (Invitrogen Life Technologies, Carlsbad, USA) at 37°C for 30 min and removed remaining connective tissue. Digests were finely minced and incubated in 1 mg/ml collagenase 1 (Invitrogen Life Technologies) for 1 h at 37°C. Pelleted cells were used for establishing primary lung fibroblast cultures in DMEM (Invitrogen Life Technologies) for 1 h at 37°C. Pelleted cells were stained with Quik-Dip (Scot Scientific, Taren Point, Australia) and differential cell counts performed.

Cytokines, plasmids and antibodies
The pCol1a1-luc and p(CAGA)12-luc constructs and the production of HYPER-IL-6 has been described (Buttner et al, 2004; Fischer et al, 1997). Recombinant human IL-6 and human TGF-β1 were from Bender Medsystems (Vienna, Austria) and Sigma–Aldrich (St Louis, USA), respectively. Antibodies directed against B220 were from Becton Dickinson Biosciences (San Jose, USA), CD3 from Serotec (Kidlington, UK), F4/80 from Abcam (Cambridge, UK), α-tubulin (Sigma–Aldrich), CD45 and cytokeratin AE1/AE3 from Dako (Glostrup, DK), phosphorylated Stat3 (Cell Signaling Technology, Danvers, USA) and β-actin, Stat3 or phosphorylated Smad3 (Santa Cruz Biotechnology).

Collagen quantification, lung fibroblast preparation and cellular assays
We analysed hydroxyproline content of lung homogenates as described (Mutsaers et al, 1998). We digested lungs in 0.25% trypsin-EDTA (Invitrogen Life Technologies, Carlsbad, USA) at 37°C for 30 min and removed remaining connective tissue. Digests were finely minced and incubated in 1 mg/ml collagenase 1 (Invitrogen Life Technologies) for 1 h at 37°C. Pelleted cells were used for establishing primary lung fibroblast cultures in DMEM (Invitrogen Life Technologies) for 1 h at 37°C. Pelleted cells were stained with Quik-Dip (Scot Scientific, Taren Point, Australia) and differential cell counts performed.

Cytokines, plasmids and antibodies
The pCol1a1-luc and p(CAGA)12-luc constructs and the production of HYPER-IL-6 has been described (Buttner et al, 2004; Fischer et al, 1997). Recombinant human IL-6 and human TGF-β1 were from Bender Medsystems (Vienna, Austria) and Sigma–Aldrich (St Louis, USA), respectively. Antibodies directed against B220 were from Becton Dickinson Biosciences (San Jose, USA), CD3 from Serotec (Kidlington, UK), F4/80 from Abcam (Cambridge, UK), α-tubulin (Sigma–Aldrich), CD45 and cytokeratin AE1/AE3 from Dako (Glostrup, DK), phosphorylated Stat3 (Cell Signaling Technology, Danvers, USA) and β-actin, Stat3 or phosphorylated Smad3 (Santa Cruz Biotechnology).

Collagen quantification, lung fibroblast preparation and cellular assays
We analysed hydroxyproline content of lung homogenates as described (Mutsaers et al, 1998). We digested lungs in 0.25% trypsin-EDTA (Invitrogen Life Technologies, Carlsbad, USA) at 37°C for 30 min and removed remaining connective tissue. Digests were finely minced and incubated in 1 mg/ml collagenase 1 (Invitrogen Life Technologies) for 1 h at 37°C. Pelleted cells were used for establishing primary lung fibroblast cultures in DMEM (Invitrogen Life Technologies) for 1 h at 37°C. Pelleted cells were stained with Quik-Dip (Scot Scientific, Taren Point, Australia) and differential cell counts performed.

Cytokines, plasmids and antibodies
The pCol1a1-luc and p(CAGA)12-luc constructs and the production of HYPER-IL-6 has been described (Buttner et al, 2004; Fischer et al, 1997). Recombinant human IL-6 and human TGF-β1 were from Bender Medsystems (Vienna, Austria) and Sigma–Aldrich (St Louis, USA), respectively. Antibodies directed against B220 were from Becton Dickinson Biosciences (San Jose, USA), CD3 from Serotec (Kidlington, UK), F4/80 from Abcam (Cambridge, UK), α-tubulin (Sigma–Aldrich), CD45 and cytokeratin AE1/AE3 from Dako (Glostrup, DK), phosphorylated Stat3 (Cell Signaling Technology, Danvers, USA) and β-actin, Stat3 or phosphorylated Smad3 (Santa Cruz Biotechnology).
pan-cytokeratin, CD45 and phosphorylated STAT3 following antigen retrieval with 10 mM citrate buffer pH 6.

**Patient samples**

Tissue biopsies from four male patients with diagnosed IPF-UIP (age range of 59–69 years, mean of 63 years) and appropriate controls were used in this study. Human ethics approval for this study was provided through Bellberry Limited for work carried out at Sir Charles Gairdner Hospital, WA, Australia. All human samples used in this study were retrospective paraffin embedded tissue samples taken for diagnostic purposes. The collection and use of these samples for this study was consistent with Section 3.2.4 of the National Health and Medical Research Council of Australia, Australian Health Ethics Committee guidelines for research involving humans and Section 25 of the World Medical Association, Declaration of Helsinki.

**Statistical analysis**

Data are expressed as mean ± SEM, with *p* < 0.05, **p** < 0.01, ***p*** < 0.001. Statistical analysis was performed using one-way analysis of variance and Bonferroni’s multiple comparisons post-test.

For more detailed Materials and Methods see the Supporting Information.

**Author contributions**

RJJO’d, DAK, CDR, HJZ, GPA, ME, SEM designed research; RJJO’d, HLL, AGJ, CDR, CMP, JJ, SB, RV, ST, BW, PS, RJMcA, SEM performed research; BSMcK, GJL, SRJ, ME contributed new reagents and analytical tools; RJJO’d, SEM, ME wrote the paper.

**Acknowledgements**

The authors would like to acknowledge Dr Toby Phesse and Dr Tracy Putoczki for critical reading of the manuscript and the technical assistance of Ms Dianne Grail, Ms Danielle Copeman, Ms Lovisa Dousha, Dr Vance Matthews, Dr Deborah Strickland and the Centre for Microscopy, Characterization and Analysis of the University of Western Australia. This work was supported by funds from the Operational Infrastructure Support Program provided by the Victorian Government, the National Health and Medical Research Council (NHMRC) of Australia, grants 3030137 and 458703, and is solely the responsibility of the institution or individual authors and does not reflect the views of NHMRC. RJJO’d was in part supported through an Australian Postgraduate Award from the University of Western Australia, Lung Institute of Western Australia PhD top-up scholarship and an unrestricted research grant from Roche awarded to GPA. DAK is a Canada Research Chair and Michael Smith Foundation Senior Scholar supported by a Canadian Institute of Health Research grant and gifts from the Raphal Dhillon fund for IPF research. ME is a Senior Research Fellow of the NHMRC.

Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

**References**


Robert J. J. O'Donoghue et al.