The anti-fibrotic effect of inhibition of TGFβ-ALK5 signalling in experimental pulmonary fibrosis in mice is attenuated in the presence of concurrent γ-herpesvirus infection

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most progressive and fatal of all fibrotic conditions, with a median survival of 3 years. The pathomechanisms involved remain poorly understood, but current hypotheses propose that this condition arises as a result of repetitive epithelial injury followed by a highly aberrant wound healing response in genetically susceptible and aged individuals (reviewed in Datta et al., 2011). The classical histopathological pattern of IPF presents as usual interstitial pneumonia (UIP), with evidence of patchy epithelial damage and hyperplasia combined with abnormal proliferation of mesenchymal cells, concomitant with overproduction and disorganized deposition of extracellular matrix (ECM). Fibrotic foci, the histopathological hallmark of UIP/IPF, comprise accumulations of fibroblasts and myofibroblasts within an extensive ECM underlying injured and reparative epithelium, and are widely considered to represent the leading edge of the fibrotic response.

Although the aetiology of IPF remains unknown, studies examining the role of infection in IPF implicate viral infections, especially human herpesviruses (HHVs), as important contributors to the initiation and progression of this condition (reviewed in Molyneaux and Maher, 2013). Current evidence, albeit from small IPF cohort studies, suggests a role for Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), human herpesvirus-8 (HHV-8) and human herpesvirus-7 (HHV-7) in the progression of fibrosis (Calabrese et al., 2013; Egan et al., 1995; Tang et al., 2003; Vergnon et al., 1984). Moreover, there is evidence linking viral infection with the incidence of acute exacerbation of IPF (AE-IPF) (Wootton et al., 2011), a life-threatening complication that presents as worsening of dyspnoea and an accelerated decline in lung function (Collard et al., 2007).

Animal models of fibrosis have established a causal role for viral infection in the progression of experimental pulmonary fibrosis (Ashley et al., 2014; McMillan et al., 2008; Mora et al., 2005; Vannella et al., 2010). Murine γ-herpesvirus 68 (MHV-68) is closely related to EBV and, like its human counterpart, infects the respiratory epithelium and establishes life-long latency in the host (Nash et al., 2001). This viral tropism for alveolar epithelial cells II (AEC II) contributes to dysregulated epithelial repair and surfactant abnormalities associated with increased apoptosis and alveolar collapse (Lawson et al., 2008). Moreover, latent viral infection alters the phenotype of infected alveolar epithelial cells and fibroblasts, leading to increased TGFβ production and activation (Stoolman et al., 2010; Vannella et al., 2010), as well as increased fibroblast responsiveness to this cytokine, especially in aged mice (Naik et al., 2011).

Current evidence supports a central role for TGFβ in the pathogenesis of fibrosis, including human and murine pulmonary fibrosis (Santana et al., 1995). TGFβ is a potent promoter of extracellular matrix production and promotes fibroblast-to-myofibroblast differentiation, as well as epithelial cell apoptosis (reviewed in Fernandez and Eickelberg, 2012). Multiple approaches that disrupt either TGFβ activation or signalling through direct
cytokine inhibition (Giri et al., 1993), Smad3 knockout (Bonniaud et al., 2004), TGF-βRII receptor knockout (Li et al., 2011), integrin αβ6 knockout (Koth et al., 2007; Morris et al., 2003) or antibody neutralization (Horan et al., 2008) have offered protection in experimental models of pulmonary fibrosis.

The aim of this study was to further our understanding of the mechanistic links between MHV-68 infection and TGFβ signalling and lung fibrosis. We first established and characterized a model of MHV-68 infection on the background of bleomycin-induced pulmonary fibrosis using standard endpoints (analysis of total lung hydroxyproline) and further investigated disease pathophysiology using ex vivo micro-computed tomography (μCT) scanning of whole lungs (Scotton et al., 2013). To investigate the potential role of TGFβ signalling in this model, we employed a highly selective, ATP-competitive activin receptor-like kinase 5 (ALK5; also known as TGF-βRI) inhibitor, SB525334, which has a proven therapeutic effect in single-hit models of experimental pulmonary fibrosis (Bonniaud et al., 2005; Scotton et al., 2013). Taken together, our data reveal previously unknown intricacies for the TGFβ signalling axis in experimental lung fibrosis, with different outcomes observed in response to ALK5 inhibition depending on the presence or absence of viral infection. These findings raise potential clinical considerations for the future targeting of the TGFβ pathway in the context of pulmonary fibrosis, including IPF.

RESULTS

Two-hit model of MHV-68 infection on the background of bleomycin-induced fibrosis

In order to establish a two-hit model of MHV-68 infection on the background of fibrosis, mice were challenged with an oropharyngeal instillation of bleomycin (25 IU/mouse) on day 0, followed by an intranasal infection with MHV-68 (1×10^5 PFU) on day 14. To further our understanding of the mechanistic links between MHV-68 infection and TGFβ signalling in this model, the ALK5 inhibitor (SB525334) was administered therapeutically from day 15 after bleomycin injury through to the end of the experiment at day 28.

Total lung collagen was measured by quantifying hydroxyproline levels by reverse-phase high performance liquid chromatography (HPLC) (Fig. 1). MHV-68 infection alone [saline (Sal)+MHV68] had no significant effect on total lung collagen levels when compared to uninfected control lungs (Sal). Administration of bleomycin (Bleo) resulted in a doubling of lung collagen deposition, which was significantly attenuated by SB525334 treatment in the Bleo+SB525334 group (mean±s.e.m. of Bleo vs Bleo+SB525334, 3.8±0.4 mg vs 2.97±0.14 mg, P=0.04). MHV-68 infection on the background of existing lung fibrosis (Bleo+MHV-68) did not increase total lung collagen levels compared to the Bleo group. Interestingly, in the two-hit model, there was no difference in

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total lung collagen between the Bleo+MHV-68+SB525334 group compared with the Bleo+MHV-68 group (mean±s.e.m. of Bleo+MHV-68 vs Bleo+MHV-68+SB525334, 4±1 mg vs 3.5±0.4 mg, \( P=0.6 \)). These observations were further confirmed by the Sircol assay (supplementary material Fig. S1). Taken together, these data led us to conclude that SB525334 attenuates fibrosis in the single-hit model but that the therapeutic effect of this inhibitor is largely lost in the two-hit model.

**µCT characterization of the two-hit model**

*Ex vivo* µCT was subsequently used to further investigate the effect of SB525334 treatment in this two-hit model. Fig. 2 shows representative 3D volume reconstructions (left panels) with corresponding mid-lung coronal µCT sections (middle panels) and magnification of key pathological changes (right panels) for lungs at day 28. Sal+MHV-68 lungs were indistinguishable from Sal control lungs, with both groups displaying an equally homogenous

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**Fig. 2. µCT characterization and quantification of the pathological changes in the single- and two-hit models.** 3D volume reconstruction (left panels, dorsal view) and representative coronal µCT sections (middle panels) with higher (4×) magnification of the highlighted insert (right panel). Mice treated with saline (Sal; A) or Sal+MHV-68 (B) show normal lung morphology; Bleomycin (Bleo)-treated mice (C) show dense subpleural fibrotic lesions, which are attenuated in the Bleo+SB525334 group (D); Bleo+MHV-68 mice (E) show evidence of extensive ground-glass opacities radiating from airways and overlying areas of dense consolidation; Bleo+MHV-68+SB525334 mice (F) reveal dense consolidation with reduced areas of ground-glass opacities. InForm analysis demonstrates an increase in the percentage of abnormal lung area (G) and density (H) in Bleo lungs above the Sal control (dotted line). No significant difference was observed between the Bleo and Bleo+MHV-68 groups. Administration of SB525334 from day 15 post-bleomycin-instillation (and 1 day p.i.) significantly attenuated lung pathology in the Bleo group but not in the two-hit Bleo+MHV-68 model. The data are representative of mean±s.e.m., one-way ANOVA, *\( P<0.05 \), **\( P<0.01 \), comparison of all Bleo-challenged groups (\( n=5 \)).
appearance with a network of airways in a virtually transparent parenchyma (Fig. 2A,B). In the Bleo group, peripheral dense fibrotic lesions were clearly visible, particularly on the dorsal side of the lungs (Fig. 2C), as previously reported for oropharyngeal bleomycin instillation (Lakatos et al., 2006; Scotton et al., 2013). Coronal sections revealed prominent sub-pleural scarring, interlobular septal thickening and traction bronchiectasis (Fig. 2C). In Bleo+SB525334 lungs, scarring and fibrosis were noticeably reduced (Fig. 2D). In contrast, in the two-hit model, lungs displayed extensive areas of dense consolidation with overlapping diffuse ground-glass opacities indicative of inflammatory changes concentrated around the airways (Fig. 2E). SB525334 treatment in this two-hit model only showed a modest therapeutic effect. Analysis of lung density revealed that total lung density was increased by fourfold for the Bleo group compared with the Sal group (Fig. 2H). This increase was reduced by ~50% in the Bleo+SB525334 group, confirming the beneficial therapeutic effect of ALK5 inhibition in the single-hit model (Fig. 2H). The voxel density score was highest for the Bleo+MHV-68 two-hit lungs and this was not significantly reduced in the Bleo+MHV-68+SB525334 group.

In order to further investigate differences in lung morphology, we next performed voxel density distribution analysis for each lung based on the unsegmented µCT data. The mean number of voxels per lung at each greyscale density value (0=air/black, 255=dense tissue/white) was calculated and plotted as a histogram (Fig. 3A). Statistically significant differences in the density voxel distribution between individual experimental groups were analyzed by Student’s t-test at each bin. The data are shown as graphs of probability (y-axis) versus greyscale density value (x-axis), with the significance cut-off set at 0.05 (indicated by the dotted line). (C-E) Distribution of significantly different voxel densities was visualized on representative µCT scans (red pixels): (C) Bleo and Bleo+SB525334 show voxel localization to fibrotic lesions; (D) Bleo+MHV-68 and Bleo lungs show voxel distribution in fibrotic lesions and dispersed throughout the parenchyma; (E) Bleo+MHV-68 and Bleo+MHV-68+SB525334 lungs show voxel distribution dispersed throughout the parenchyma.

**Quantification of changes observed in µCT scans**

The abnormal lung area and lung density were subsequently quantified using tissue segmentation analysis of the whole lungs. Bleomycin injury alone resulted in ~40% of the total lung volume being characterized as abnormal; this was reduced to ~15% following SB525334 treatment (Fig. 2G). In the Bleo+MHV-68 group, ~50% of the lung volume was categorized as abnormal. SB525334 treatment in this two-hit model only showed a modest therapeutic effect. Analysis of lung density revealed that total lung density was increased by fourfold for the Bleo group compared with the Sal group (Fig. 2H). This increase was reduced by ~50% in the Bleo+SB525334 group, confirming the beneficial therapeutic effect of ALK5 inhibition in the single-hit model (Fig. 2H). The voxel density score was highest for the Bleo+MHV-68 two-hit lungs and this was not significantly reduced in the Bleo+MHV-68+SB525334 group.

In order to further investigate differences in lung morphology, we next performed voxel density distribution analysis for each lung based on the unsegmented µCT data. The mean number of voxels per lung at each greyscale density value (0-255) was calculated and plotted as a histogram (Fig. 3A). Statistically significant differences in the density voxel distribution between individual experimental groups were analyzed by Student’s t-test at each bin. The data are shown as graphs of probability (y-axis) versus greyscale density value (x-axis), with the significance cut-off set at 0.05 (indicated by the dotted line). (C-E) Distribution of significantly different voxel densities was visualized on representative µCT scans (red pixels): (C) Bleo and Bleo+SB525334 show voxel localization to fibrotic lesions; (D) Bleo+MHV-68 and Bleo lungs show voxel distribution in fibrotic lesions and dispersed throughout the parenchyma; (E) Bleo+MHV-68 and Bleo+MHV-68+SB525334 lungs show voxel distribution dispersed throughout the parenchyma.
groups were evaluated by a probability t-test (Fig. 3B). A clear separation in the density distribution was observed between Sal, Bleo, and Bleo+MHV-68 lungs, with a marked shift towards higher density voxels in the Bleo group, which was further increased in the Bleo+MHV-68 group.

Significantly different voxel distributions between the Bleo and Bleo+SB525334 groups fell in the greyscale density range of 100-155; these voxels localized to fibrotic lesions in the Bleo group (Fig. 3C). Significant increases over a wide voxel range (50-115) were observed in the Bleo+MHV-68 group compared with Bleo (Fig. 3D). In the Bleo+MHV-68 lungs, these voxels corresponded to extensive areas of parenchyma with diffuse ground-glass opacities, in addition to fibrotic lesions. Treatment with SB525334 had a significant effect on a very narrow range of voxels (50-65) in the two-hit group, again indicating the lack of therapeutic effect of ALK5 inhibition on fibrosis in the two-hit model (Fig. 3E).

Quantification of inflammation in the two-hit model
The lung abnormalities mapped by µCT analysis were subsequently matched to fibrotic and inflammatory changes identified on hematoxylin and eosin (H&E) and Martius Scarlet Blue (MSB)-stained tissue sections (Fig. 4). Histological analysis confirmed dense patchy fibrosis and collagen deposition in Bleo-injured lungs, which was reduced in mice treated with SB525334. Bleo+MHV-68 lungs displayed evidence of extensive fibrotic lesions and, notably, infiltrations of mononuclear inflammatory cells that formed dense aggregates. We subsequently quantified these inflammatory cell aggregates (IAs) and found that, consistent with our radiological findings, there was little evidence of IAs in Sal+MHV68 lungs. In stark contrast, there were numerous IAs present in the Bleo+MHV-68 two-hit group and these IAs were significantly increased compared with all other experimental groups (Fig. 5A). The number of these IAs was significantly reduced in the two-hit group treated with SB525334 (mean±s.e.m. of Bleo+MHV-68 vs Bleo+MHV-68+SB525334, 1±0.25 vs 0.4±0.13 ROI/mm², P<0.05).

We next measured levels of immunomodulatory mediators in lung homogenates and report that viral infection in fibrotic lungs (Bleo+MHV-68) led to a significant increase in lung levels of CCL2 (Fig. 5B), IL-1β (Fig. 5D), TNFα (Fig. 5E) and IL-10 (Fig. 5F) above the levels detected for Bleo lungs. Treatment with SB525334 did not affect the levels of any of these mediators. In contrast, IFNγ was only detectable in virally-infected lungs and IFNγ levels were significantly increased in the Bleo+MHV68+SB525334 group compared to the Bleo+MHV-68 group (mean±s.e.m. of Bleo+MHV-68 vs Bleo+MHV-68+SB525334, 55.14±2.7 vs 96.7±18.6 μg/lung, P<0.05) (Fig. 5C).

Determination of viral gene expression in the two-hit model
It has been previously reported that active viral replication is required for exacerbation of experimental pulmonary fibrosis (Ashley et al., 2014; McMillan et al., 2008). We therefore evaluated the expression of three viral genes encoding the MHV-68 DNA polymerase and the viral envelope proteins glycoprotein B and M3. In accordance with previous studies (Vannella et al., 2010; McMillan et al., 2008), we show that the viral genes are readily detected in whole lung tissue at the peak of lytic infection 7 days post-infection (p.i.) (supplementary material Fig. 2A-C). By 14 days p.i. the MHV-68 infection had entered a latent phase as demonstrated by decreased levels of viral gene expression in the lung. Blocking TGFβ signalling with the ALK5...
inhibitor SB525334 had no impact on viral load in the fibrotic lungs (Fig. 6A-C).

Splenomegaly, a reliable surrogate indicator of herpes virus latent infection (Nash et al., 2001), was evident 14 days p.i. (supplementary material Fig. S2D). In all virally-infected mouse groups, spleen weight was significantly increased when compared to the saline- or bleomycin-only control groups (Fig. 6D). Moreover, splenomegaly was further increased in the Bleo+ MHV68 double-hit group compared to single-hit groups, whereas SB525334 treatment significantly reduced spleen weights.

**DISCUSSION**

This study aimed to investigate the effect of blocking TGFβ-ALK5 signalling on the progression of lung fibrosis in the presence of...
concurrent viral infection. We report that MHV-68 viral infection of the fibrotic lung did not increase lung collagen deposition above the levels observed in the bleomycin-alone injured mice, but instead led to the marked accumulation of inflammatory cells, which persisted for at least 14 days p.i. In contrast, saline control lungs inoculated with MHV-68 showed normal architecture. The potent and highly selective TGFβ-ALK5 inhibitor SB525334 (Grygielko et al., 2005) attenuated fibrosis in the single-hit bleomycin model. In contrast, TGFβ-ALK5 inhibition did not significantly block collagen accumulation in the two-hit model but led to a marked reduction in inflammatory cell infiltrates and an enhanced anti-viral cytokine response. Taken together, these data show for the first time that the therapeutic effect of TGFβ-ALK5 inhibition in lung fibrosis is curtailed in the presence of concurrent viral infection.

μCT analysis reveals key differences in morphological features between the single- and two-hit models, and the therapeutic effect of ALK5 inhibition

The traditional collagen endpoint measurement used to evaluate fibrosis, based on total lung hydroxyproline levels, has a relatively limited signal window and does not provide information regarding the specific spatial distribution of fibrotic lesions or other potential pathophysiological changes in the injured lung. To complement our biochemical analysis of lung collagen accumulation, we employed ex vivo μCT to further characterize the pathological changes in the fibrotic lung with and without concomitant viral infection. As well as providing information regarding the spatial distribution of fibrotic lesions, whole-lung μCT scanning avoids the potential sampling error associated with standard histological analysis of tissue sections. This technology has successfully been applied to the ex vivo investigation of lung architecture (Thiesse et al., 2010; Vasiliscu et al., 2012) and, more recently, as an endpoint for evaluating fibrosis in single-hit models of fibrosis, based on either bleomycin or adenoviral overexpression of TGFβ (Rodt et al., 2010; Scotton et al., 2013). In agreement with previous data from our laboratory (Scotton et al., 2013), μCT analysis accurately differentiated dense fibrotic lesions associated with bleomycin injury from normal lung morphology. μCT analysis of virally-infected fibrotic lungs revealed diffuse ground-glass opacities and dense consolidation radiating from the bronchovascular bundles, in addition to bleomycin-induced fibrotic lesions. These radiological features are highly reminiscent of those reported in patients with AE-IPF (Collard et al., 2007).

Matching μCT analysis with the histological analysis of the same lungs confirmed that high-density areas corresponded to fibrotic tissue and collagen deposition. The dispersed inflammatory changes evident on μCT scans of virally-infected fibrotic lungs were associated with mononuclear cell aggregates localized around the...
airways and vasculature. Subsequently, the use of InForm pattern-recognition software was validated in this two-hit model to quantify the changes observed throughout the µCT scans of the whole lungs. It was noted that InForm software accurately highlighted fibrotic lesions in Bleo lungs, whereas, in the two-hit model, inflammation was found to extensively overlap with fibrosis and hence the ‘abnormal lung’ fraction encompassed both types of changes in this model. The µCT analysis confirmed the collagen biochemical data and led us to conclude that, although ALK5 inhibition was effective in preventing fibrotic progression in the single-hit model, this therapeutic effect was attenuated in the two-hit model. Density distribution analysis led us to further postulate that SB525334 primarily targeted inflammatory cell infiltration (lower density voxels) rather than the fibrotic (high density voxels) response in the two-hit model.

In our study we did not demonstrate an increase in lung collagen accumulation following MHV-68 infection on the background of pre-existing fibrosis. This is not a universal finding, and others have reported exacerbation of fibrosis by MHV-68 in the context of FITC and bleomycin models of lung fibrosis (Ashley et al., 2014; McMillan et al., 2008). There could be several potential explanations, including intrinsic differences between the initiating fibrogenic insults as well as their route of administration. FITC is a fine particle that is deposited in the lung and leads to focal chronic inflammation and fibrosis (Moore and Hogaboam, 2008). In contrast, bleomycin causes initial epithelial injury by direct DNA damage and oxidative stress, which in turn triggers a robust inflammatory response leading to inflammatory cell recruitment and vascular leak (Degryse and Lawson, 2011). Increased TGFβ activity by day-14 post-injury is associated with the development of extensive patchy fibrosis (Degryse and Lawson, 2011). The route of administration of bleomycin is known to influence the spatial distribution and evolution of fibrotic lesions, with intratracheal administration resulting in localized bronchiocentric lesions and oropharyngeal administration, as used in our study, causing diffuse peripheral, subpleural lesions (Scotton and Chambers, 2010). Furthermore, whereas the intratracheal model of bleomycin-induced fibrosis resolves over time (Degryse et al., 2010), recent evidence from our laboratory demonstrated that the oropharyngeal mode of bleomycin instillation leads to persistent fibrosis and collagen deposition with little evidence of restoration of lung architecture up to at least 6 months post-injury (Scotton et al., 2013). These key differences between models might be crucial in terms of determining the subsequent effect of MHV-68 infection on the progression of the fibrotic response. In addition, we addressed the possibility that the differences between the reported studies could have arisen from using different methods of collagen quantification. In agreement with our HPLC data, standard Sircol colorimetric assay confirmed the lack of exacerbated lung collagen accumulation in our model. In contrast, all studies agree that MHV-68 infection triggered a robust and persistent inflammatory response on a background of pre-existing fibrosis.

ALK5 inhibition targets inflammatory cell infiltration in the two-hit model

MHV-68 infection alone leads to the long-term release of immunomodulatory mediators by resident and recruited cells in the lung, including: TNFα by mesenchymal cells, B cells and alveolar macrophages; CCL2 and IFNγ by alveolar macrophages; and IFNγ and IL-10 by T cells (Sarawar et al., 1996; Stoolman et al., 2010). Our two-hit model clearly demonstrates that, in the event of infection concomitant with pre-existing fibrosis, the inflammatory response is exacerbated, persistent and associated with a further increase in the accumulation of mediators that might also perpetuate the profibrotic milieu. CCL2 is readily detected in the sera and bronchoalveolar lavage fluid of IPF patients (Baran et al., 2007; Suga et al., 1999) and promotes fibrocyte and inflammatory cell recruitment (Moore et al., 2005) as well as collagen production by fibroblasts (Kim et al., 2014). In models of MHV-68-mediated exacerbation of FITC-induced fibrosis (McMillan et al., 2008) and fibrosis in latently infected lungs (Vannela et al., 2010), high levels of CCL2 and CCL12 are detected in virally-infected fibrotic lungs. Overexpression of IL-1β in vivo leads to acute alveolar and parenchymal inflammation that progresses into interstitial fibrosis with accumulation of fibroblasts and myofibroblasts in the lung (Kolb et al., 2001). Similarly, overexpression of TNFα in rat lungs leads to acute inflammation and fibrosis (Sime et al., 1998). Overexpression of IL-10 has also been linked to the development of pulmonary fibrosis in vivo (Sun et al., 2011). Although current evidence suggests that TGFβ is an important driver of lung collagen accumulation during days 14 to 28 post-bleomycin in the single-hit model (Scotton et al., 2013), it is plausible that, in the two-hit model, the TGFβ-independent, additive profibrotic actions of these mediators perpetuate ECM deposition and hence override the antifibrotic effect of SB525334.

In contrast to the lack of therapeutic effect of ALK5 inhibition on lung collagen accumulation in the two-hit model, the Bleo+MHV-68 infected lungs harboured the highest number of inflammatory aggregates (IAs) compared with all other experimental groups, and this parameter was reduced in response to ALK5 inhibitor treatment. TGFβ plays a key immunomodulatory role, including inhibition of CD4 T-cell differentiation, IFNγ production, induction of regulatory T cells and inhibition of antigen-presenting-cell function (Odeberg and Söderberg-Nauclér, 2001). In models of Herpes simplex virus-1 (HSV-1) infection, inhibition of TGFβ signalling in immune cells leads to the expansion of natural killer (NK) cells, increased IFNγ production and hence better control of viral infection, which in turn is associated with reduced immune cell infiltration at the site of infection (Allen et al., 2011). Moreover, inhibition of TGFβ signalling decreases the viral capacity to establish latency and reduces the number of immune cells infiltrations into the primary site of infection as well as to the site of latency (Allen et al., 2011). In bone-marrow-transplantation models, latent MHV-68 infection leads to chronic and persistent pneumonitis and fibrosis, which is associated with the accumulation of macrophages and the influx of neutrophils and lymphocytes into the lung, with the latter being dominated by CD8 and CD4 T cells (Coomes et al., 2011). Importantly, blocking TGFβ signalling in T cells leads to an enhanced antiviral responses and attenuation of inflammation and fibrosis (Coomes et al., 2010, 2011). Consequently in our two-hit model, ALK5 inhibition reduced the number of IAs in the lung and attenuated splenomegaly. Interestingly, these responses were not associated with any reduction in viral gene expression in the lung; rather, they were associated with increased levels of IFNγ in the virally-infected fibrotic lungs treated with the TGFβ-ALK5 inhibitor. IFNγ does not play a direct role in viral clearance from the lung (Dutta et al., 1997) but it is a key cytokine involved in antiviral immunity, essential for CD8 T-cell-mediated responses during acute lytic infection and for CD4 T-cell-dependent control of persistent infection (Christensen et al., 1999). IFNγ-receptor-deficient mice show increased perivascular accumulations of immune cells, primarily B cells, in response to MHV-68 infection (Lee et al., 2009). It is plausible that the increase in IFNγ levels is associated with the decrease in IAs observed in our study. This might actually be beneficial to the host, because viral load and disease severity are often neither linear nor indeed associated. The immunopathology associated with the viral infection is often more damaging than the virus itself, and, in the case of γ-herpesviruses, this...
immunopathology is associated with Th1-type cytokine expression, inflammation and bystander tissue damage (Nash et al., 2001). Antiviral therapies have been shown to be beneficial in a subset of IPF patients with evidence of EBV infection (Egan et al., 2011), and attenuate fibrosis resulting from chronic MHV-68 infection in animal models (Mora et al., 2007). Our results point to an interesting prospect of combining anti-TGFβ and antiviral therapies as a potential treatment for pulmonary fibrosis.

Furthermore, the balance between the lytic and latent phases of infection is also likely to influence the progression of fibrosis. In our studies we confirmed the switch from lytic phase (7 days p.i.) to the latent phase (14 days p.i.) by measuring the spleen weights and viral gene expression in the lungs. To the best of our knowledge, this is the first report evaluating the progression of fibrosis in the two-hit model at 14 days p.i., which could be another explanation for the lack of exacerbation of collagen deposition. Previous studies (Ashley et al., 2014; McMillan et al., 2008) measured collagen deposition at 7 days p.i., at the height of the extremely cytotoxic and inflammatory lytic phase. Another study reported that latent MHV-68 infection induced a pro-fibrotic phenotype in lung tissue (Stoolman et al., 2010) and showed exacerbated fibrotic responses in the latently infected lungs (Vannella et al., 2010). This highlights the need for further longitudinal studies to assess the relative contributions of herpesvirus viral replication, latency and specific host responses on disease severity and concomitant pulmonary fibrosis.

Conclusions and implications
In conclusion, we report that MHV-68 infection on the background of pre-existing fibrosis leads to a robust and persistent inflammatory response that is reminiscent of ground glass opacities and consolidation reported in patients with AE-IPF. Targeting TGFβ-ALK5 signalling in the fibrotic lung prevents further progression of fibrosis in the single-hit model but this effect is attenuated in the presence of concurrent viral infection. In contrast, inhibiting TGFβ and antiviral therapies as a potential treatment for pulmonary fibrosis.

MATERIALS AND METHODS
MHV-68 infection on the background of pulmonary fibrosis
All studies were ethically reviewed and performed in accordance with the UK Home Office Animals for Scientific Procedures Act 1986. C57BL/6 male mice between 10 and 12 weeks of age (Charles River Laboratories, UK) were administered bleomycin (25 IU/mouse in 50 µl of sterile 0.9% saline) or saline by oropharyngeal instillation as previously described (Lakatos et al., 2006).

Two weeks after bleomycin instillation, mice were anaesthetized by intraperitoneal injection of ketamine (80 mg/kg body weight) and xylazine (8 mg/kg body weight). 1×10^6 plaque forming units (PFUs) of MHV-68 (ATCC, Manassas, VA, USA) suspended in 20 µl sterile saline were inoculated intranasally. Mice were sacrificed 7 or 14 days p.i. by intraperitoneal injection of pentobarbital and severing of the abdominal inferior vena cava.

ALK5-inhibitor study
The highly selective ALK5 inhibitor SB525334 (Grygielko et al., 2005) was a kind gift from Novartis, Horsham, UK. The compound (30 mg/kg body weight in 100 µl acidified saline/0.2% Tween 80 pH4.1) or vehicle (acidified saline/0.2% Tween 80 pH4.1) was administered from day 15 post-bleomycin-instillation, which corresponds to 1 day p.i., twice daily by oral gavage for the remaining duration of the experiment. Treatment combinations are summarized in Table 1.

For measurements of total collagen and inflammatory mediators, the lungs were snap-frozen in liquid nitrogen, weighed and pulverized to homogeneity. For µCT, histological and immunohistochemical analysis, the lungs were insufflated with 4% paraformaldehyde at a constant pressure of 20 cm H2O, fixed for 24 h then stored in 70% ethanol. Spleens were snap-frozen in liquid nitrogen and weighed.

Determination of total lung collagen
Total lung collagen was calculated by measuring hydroxyproline content in aliquots of pulverized lung. Hydroxyproline was quantified by reverse-phase HPLC of NBD-Cl-derived acid hydrolysates of the pulverized lung and the value used to calculate total lung collagen based on the average hydroxyproline content of collagen (12.2%).

Total lung collagen was also measured using the Sircol assay (Biocolor Ltd, UK) according to the manufacturer’s instructions. Briefly, a small quantity of pulverized lung was accurately weighed and acid-pepsin extracted. The quantity of collagen was calculated in mg per lung.

Micro-computed tomography (µCT) imaging
Insufflated lungs were incubated for 2 h each in increasing concentrations of ethanol (70%, 80%, 90%), then 100% ethanol overnight before being transferred to 100% hexamethyldisilazane for another 2 h and then air-dried. Lungs were scanned in a SkyScan 1072 µCT scanner (SkyScan, Kontich, Belgium) at 40 kV/100 µA, without a filter, using two frame averaging at a 0.49° angular rotation step size, and a voxel size set to 12.8 µm with typical Modulation Transfer Function (MTF) of around 10%; spatial resolution was in the region of 20–30 µm. Scan time was around 10 min, allowing high-resolution visualization and good throughput. Reconstruction was carried out with the SkyScan NRecon software (SkyScan, Kontich, Belgium). Also see Scotton et al. (2013) for further details.

**Table 1. Summary of experimental groups**

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Treatment</th>
<th>Total mice per group</th>
<th>End-point analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sal</td>
<td>–</td>
<td>–</td>
<td>Biochemistry: 3; µCT/histology: 2</td>
</tr>
<tr>
<td>Sal+SB525334</td>
<td>–</td>
<td>–</td>
<td>Biochemistry: 3; µCT/histology: 2</td>
</tr>
<tr>
<td>Sal+MHV-68</td>
<td>–</td>
<td>–</td>
<td>Biochemistry: 4; µCT/histology: 2</td>
</tr>
<tr>
<td>Sal+MHV-68+SB525334</td>
<td>+</td>
<td>+</td>
<td>Biochemistry: 6; µCT/histology: 5</td>
</tr>
<tr>
<td>Bleo</td>
<td>+</td>
<td>+</td>
<td>Biochemistry: 13; µCT/histology: 5</td>
</tr>
<tr>
<td>Bleo+SB525334</td>
<td>+</td>
<td>+</td>
<td>Biochemistry: 13; µCT/histology: 5</td>
</tr>
<tr>
<td>Bleo+MHV-68</td>
<td>+</td>
<td>–</td>
<td>Biochemistry: 13; µCT/histology: 5</td>
</tr>
<tr>
<td>Bleo+MHV-68+SB525334</td>
<td>+</td>
<td>+</td>
<td>Biochemistry: 13; µCT/histology: 5</td>
</tr>
</tbody>
</table>

On day 0, mice received bleomycin at a dose of 25 IU/mouse or saline via the oropharyngeal route. On day 14, mice were anaesthetised and inoculated intranasally with MHV-68 (1×10^6 PFU) or saline. From day 15, mice received SB525334 at a dose of 30 mg/kg body weight or vehicle treatment (acidified saline/0.2% Tween 80 pH 4.1), twice daily through oral administration (per os). Final n numbers are given in the end-point analysis column.
µCT image analysis
Tissue segmentation analysis was performed using InForm™ software (PerkinElmer, UK) as previously described (Scotton et al., 2013). The software training algorithm was set to discriminate between normal versus abnormal lung and gate out any non-lung tissue as tested on three representative µCT sections (8-bit greyscale) from each animal in the study until over 90% accuracy was achieved. Saline control and Bleo+MHV-68 lungs were set as standard for normal and abnormal lung categories, respectively. All µCT sections (~900 sections per lung) were subsequently segmented using the same algorithm on a medium sample area at fine resolution. The output measurements were pixel area and pixel density for each category that were then compiled into a composite measurement of abnormal lung volume expressed as a percentage of total lung volume, and greyscale density expressed as total lung density.

Voxel density distribution analysis
Frequency distribution of voxel densities in unsegmented lung was analyzed by generating composite 256-colour greyscale histograms (from 0—black to 255—white). The mean number of voxels in each bin (1 greyscale unit wide) was calculated along with statistical analysis of differences between the experimental groups.

Histology
Paraformaldehyde-fixed lungs were dehydrated and embedded in paraffin wax blocks. For standard histological processing, 3.5-μm paraffin sections were mounted on polylysine-coated glass slides and dewaxed. Hematoxylin and eosin (H&E) and modified trichrome (Martius Scarlet Blue [MSB]) staining was performed using an automated Sakura Tissue-Tek DRS 2000 Multiple Slide Stainer. All sections were subsequently scanned on a Nanozoomer and images were captured using NDP.view v.1.2.36 (both from Hamamatsu Corporation, Hamamatsu, Japan).

Direct comparisons between µCT and histology were performed on the same set of lungs: post-µCT lungs were rehydrated through an ethanol gradient (100%, 90%, 80% and 70% for 2 h in each) prior to standard processing as above.

Inflammatory cell aggregate (IA) counts
IAs were identified and quantified in H&E sections using Nuance® FX Multispectral Tissue Imaging Software (PerkinElmer, UK). The software unmixed and enhanced the areas of intense haematoyxlin staining that corresponded to IAs and quantified them as regions of interest per area of the lung section (ROU/mm²; five whole histological sections per animal).

Measurements of inflammatory markers
Lung powders were homogenized in PBS/1% Triton X (Sigma, UK)/ protease inhibitor cocktail (Roche, UK) using a freeze-thaw cycle. CCL2 DuoSet ELISA Development kits were purchased from R&D Systems, USA, and used according to the manufacturer’s instructions. The optical density was measured using a plate reader (Multiskan MCC/340, Tietertek) at dual wavelength A1: 450 nm and A2: 540 nm. Mouse pro-inflammatory Panel 1 V-Plex Plus Kit was purchased from Meso Scale Discovery (Rockville, MD, USA) for quantification of the following ten cytokines that are important in infection and inflammation: IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70 and TNF-α. The kit was used according to manufacturer’s protocol. Sector Imager 600 MSD plate reader and MSD Discovery Workbench software were used to record and analyze the results.

RT-PCR for viral mRNA
Total RNA from frozen powdered lung tissue was isolated with TRIzol reagent as per the manufacturer’s protocol (Invitrogen). RNA was DNase-treated using a DNasefree kit (Ambion). Real-time RT-PCR was conducted using the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, UK) with cycling conditions as follows: 1 cycle of 50°C for 2 min and 95°C for 2 min; 45 cycles of 95°C for 5 s, 55°C for 5 s and 72°C for 15 s. The specificity of the PCR product was confirmed by melting-curve analysis. The gB, DNApol and M3 primer sequences were previously published (McMillan et al., 2008). For each gene, crossing point (Cp) values were determined from the linear region of the amplification plot and normalized by subtraction of the geometric mean of the crossing point (Cp) values for two housekeeping genes: ATP synthase 5B (ATP5B) and calnexin (CANX), identified by GeNorm analysis as the most stable housekeeping genes for this study. Relative expression was subsequently calculated using the 2-ΔCt approach. All primers and GeNorm kits were purchased from Primer Design (Southampton, UK).

Statistical analysis
All data are presented as mean values±s.e.m., unless indicated otherwise. Statistical analysis was performed between two treatment groups by Student’s t-test, and between multiple treatment groups by one-way analysis of variance (ANOVA), using GraphPad Prism 5 software. A P-value of <0.05 was considered significant.

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Competing interests
R.C.C. has received research funding from GlaSCOSmithKline and Novartis but has no competing interests to declare in terms of the work described in this article. G.J. was a full-time employee of Novartis Institutes for Biomedical Research during the study and has no competing interests to declare with respect to this work. C.J.S. has received research funding from GlaSCOSmithKline and Boehringer Ingelheim, but has no competing interests to declare regarding the work in this manuscript.

Author contributions
N.S., C.J.S., P.F.M., A.E.W., G.J. and R.C.C. conceived and designed the studies; N.S., R.E.A., L.F. and C.J.S. performed experiments; N.S., A.E.W., B.H., C.J.S. and R.C.C. analyzed and interpreted the data; N.S., C.J.S. and R.C.C. drafted and edited the manuscript; R.C.C. provided the funding. All authors reviewed the manuscript.

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Supplementary material
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