SUPPLEMENTAL MATERIALS AND METHODS

Materials

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich, UK. The small molecule ALK5 inhibitor, SB525334A, was a kind gift from GlaxoSmithKline, Stevenage, UK.

Bleomycin animal model of lung injury

All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals. Male C57Bl/6J mice (Charles River Laboratories, UK) were housed in individually-ventilated cages in a specific pathogen-free facility, and used when between 9 and 12 weeks of age (and approximately 25 g in weight). Bleomycin (50 IU/mouse in 50 µl sterile 0.9 % saline; Bleo-kyowa, Kyowa Hakko (UK) Ltd, UK) or saline was administered by oropharyngeal installation as described previously by Lakatos *et al* [1] under light isofluorane-induced anaesthesia. Briefly, mice were suspended vertically by placing an elasticated thread under their front teeth; the nose was lightly clamped shut using forceps, and the tongue gently pulled forward using forceps, to prevent the swallow reflex. After allowing the mouse to switch to mouth-breathing for 10-15 seconds, bleomycin/saline was slowly instilled to the back of the throat, using a pipette with a standard 200 µl yellow tip.

For real-time RT-PCR and total lung collagen measurements (4 – 8 animals per group), lungs were removed, blotted dry and the trachea and major airways were excised before the separated lobes were snap-frozen in liquid nitrogen. For micro-CT, histological and immunohistochemical analysis (3 – 4 animals per group), the

trachea was cannulated and lungs were insufflated with 4 % paraformaldehyde in PBS at a pressure of 20 cm H_2O, followed by removal of the heart and inflated lungs *en bloc* and immersion for 4 hours in fresh fixative. Subsequently, lungs were transferred to 15 % sucrose in PBS and left overnight at 4 °C before transfer to 70 % ethanol for storage at 4 °C.

ALK5 inhibition studies with SB525334A

The pro-fibrotic contribution of ALK5 during the response to bleomycin was investigated using the highly selective small molecule ALK5 inhibitor SB525334A [2], which can inhibit Smad3 phosphorylation with an IC50 of 24 nM. Fourteen days post-bleomycin, SB525334A (30mg/kg in 100 µl acidified saline/0.2% Tween 80 pH 4.1) or vehicle (acidified saline/0.2% Tween 80 pH 4.1) was administered twice daily by oral gavage, out to 28 days post-bleomycin. Mice were subsequently sacrificed and lungs were harvested as described above for lung collagen determination and micro-CT analysis.

Determination of total lung collagen

Total lung collagen was determined by measuring hydroxyproline content in aliquots of pulverized lung as described previously {Scotton, 2009 1080 /id}. Hydroxyproline was quantified by reverse-phase high performance liquid chromatography (HPLC) of 7-chloro-4-nitrobenzo-oxao-1,3,-diazole-derived acid hydrolysates. Total lung collagen Total lung collagen was calculated assuming that collagen contains 12.2% (w/w) hydroxyproline and expressed as mg per lung.

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

Histological analysis and immunostaining

Mouse lungs (*en bloc* or separated into individual lobes post fixation) were placed in processing cassettes, dehydrated through a serial alcohol gradient, and embedded in paraffin wax blocks. For standard histological processing (H&E staining or modified Martius Scarlet Blue stain for collagen) or immunohistochemical staining, paraffin sections (2 µm or 3 µm, respectively) were cut and mounted on polylysine-coated glass slides before dewaxing in xylene and rehydration in ethanol. Serial sections were cut with 2 - 3 µm separation. For immunostaining, sections were incubated with normal goat serum (DAKO, UK), then α-SMA was localized by overnight incubation with an anti-α-SMA (1:400) primary antibody. Sections were washed in TBS, then incubated with a biotinylated goat anti-rabbit secondary antibody (1:200 dilution) for 1 hour and then washed again in TBS. Sections were incubated with a streptavidin/peroxidase complex (1:200 dilution; DAKO, UK) for a further 30 minutes, followed by a solution of 600µg/ml of 3,3'-diaminobenzidine tetrahydrochloride (Vector Laboratories, UK) for color development. Sections were washed, counterstained with Gill-2 hematoxylin (Thermo-Shandon, PA, USA), dehydrated, and mounted with DPX mountant (Merck, UK). Control sections were incubated with an isotype-specific, nonimmune rabbit IgG primary antibody (DAKO, UK). All sections were subsequently scanned on a Nanozoomer and images were captured using NDP.view v1.2.36 (both from Hamamatsu Corporation, Japan). Direct

comparisons between µCT and histology were performed on the same set of lungs; post-CT, lungs were rehydrated through an ethanol gradient (100%, 90%, 80% and 70%; 2 hours in each) prior to normal histological processing. Note that processing for µCT does lead to some diminution in signal when performing immunohistochemical analysis on lung post-CT, suggesting a degree of antigen degradation.

Micro-CT scanning

Fixed, insufflated lungs (see earlier) were incubated for 2 hrs each in 70 %, 80 % and 90 % ethanol prior to an overnight incubation in 100 % ethanol. Lungs were subsequently transferred to 100 % hexamethyldisilazane for a further 2 hrs, prior to air-drying. Lungs were then enclosed in an expanded polystyrene tube to prevent movement, and scanned at 40 kV/100 µA, without a filter, in a Skyscan 1072 micro-CT scanner (SkyScan, Belgium), at 13.7 µm voxel size, using two frame averaging and 0.9° angular rotation step size. Reconstruction of approximately 900 sections per lung was carried out with the SkyScan NRecon software (Skyscan, Belgium). Where possible, for presentation purposes, extraneous signals from packaging material surrounding the lungs have been cropped from CT sections presented in the Figures; representative uncropped CT stacks are included in the Supplementary Results.

Micro-CT image analysis

Tissue segmentation analysis (i.e. proportion of fibrotic lung on micro-CT) was performed using InForm™ software (Caliper Life Sciences, UK). Briefly, the software was “trained” to recognise fibrotic lung, normal lung, large airways, vessels and

packaging material, using two representative micro-CT sections (8-bit greyscale) from each animal in the study. Segmentation was carried out on a medium sample area at fine resolution, to measure the tissue volume and greyscale density of fibrotic and normal lung tissue for each micro-CT section. Data for each lung (~900 sections) were then compiled into a composite measurement of fibrosis (absolute volume fibrosis x greyscale density) per whole lung.

Voxel Density Analysis

An additional approach to image analysis entailed generating composite 256-colour greyscale histograms (from 0 = black to 255 = white) for each lung (excluding the large airways), representing the frequency distribution of pixel densities (comparable to Hounsfield units). The mean number of pixels in each bin (1 greyscale unit wide) were then calculated for each group, along with a t-test distribution showing the p-value for the comparison between each animal group at each greyscale bin.

Voxel densities which showed significant differences between treated and untreated animal groups were then used to threshold the original micro-CT data, to highlight the lung where changes in tissue density had occurred.

Real-time RT-PCR

Total RNA was isolated from pulverized lung using TRIzol reagent according to the manufacturer's protocol (Invitrogen, UK). Following DNase treatment with DNA-free (Ambion, TX, USA), 1µg of RNA was used to synthesize single-stranded cDNA using qScript cDNA Supermix kit (Quanta Biosciences, USA. Real-time quantitative RT-PCR was then performed using the MESA FAST qPCR no ROX kit (Eurogentech, UK) using 1 ng of cDNA and forward and reverse primers at a final concentration of

800nM, on a Mastercycler EP Realplex 4S (Eppendorf, Germany). Cycling conditions were: 5 min at 95°C (one cycle); then 5 secs at 95°C, 45 secs at 62°C (40 cycles). Fluorescence was measured at the end of each 62°C step. Specificity of the amplification was confirmed by examining a melt curve of the reaction.

For each gene, crossing point (Cp) values were determined from the linear region of the logarithmic amplification plot. Quantitative differences in mRNA expression were determined using the Cp value for each target, normalized by subtraction of the geometric mean of the Cp values for two housekeeping genes (generating a ΔCp value): ATP Synthase 5B (ATP5B) and β2-microglobulin (B2M), identified by GeNorm analysis as the most stable housekeeping genes for this study. Relative change was determined by subtraction of the ΔCp value for the control sample from the ΔCp value for the fibrotic sample (ΔΔCp value). Fold change was subsequently calculated using the formula $2^{-\Delta\Delta Cp}$. Statistics were calculated on the ΔCp values. All primers and GeNorm kits were purchased from Primer Design, UK.

Statistical analysis

Statistical analysis was performed using Sigmaplot 12.3 software. All differences in mRNA levels were calculated from ΔCp values. Differences between two groups were evaluated by a two-tailed Student's t-test. Multiple group comparisons were evaluated using one- or two-way ANOVA as appropriate, with Holm-Sidak post hoc testing. Percentages of counts (binomial) were transformed to arcsin values (Gaussian) prior to statistical analysis. Differences in body weight gain/loss between mouse groups were evaluated with a repeated measures ANOVA with Bonferroni post hoc testing. Pearson's correlation was used to determine the statistical significance of correlated data. A p-value of less than 0.05 was considered

significant.

SUPPLEMENTAL RESULTS

Classical measures of the natural history of the response to bleomycin

Our long-standing model of bleomycin-induced lung fibrosis (BILF) involved surgical tracheostomy and direct intratracheal injection of bleomycin [3;4]. With the advent of the oropharyngeal aspiration method described by Lakatos et al [1] for the delivery of silica to mouse lungs, we undertook to adapt the approach for bleomycin administration - resulting in a technically less demanding, less invasive and more reproducible model of BILF [5]. Typical endpoints for the inflammatory and fibrotic phases were 7 and 14 days, respectively, associated with a 50 to 60 % increase in the amount of total lung collagen. We chose to prolong our existing BILF model, to assess changes in lung parameters over an extended timecourse, from 3 days to 28 days post bleomycin (n = 11 - 14 mice per treatment per timepoint, of which 3 were used for histology). In a separate experiment, we also assessed mice 3 months (n = 12 mice per group, of which 4 were used for histology/micro-CT) and 6 months after injury (n = 4 mice per group, for histology/micro-CT only).

As expected, oropharyngeal bleomycin delivery of 50 IU/mouse resulted in a significant loss of starting body weight during the acute inflammatory phase (approximately 10% weight loss at 7 days post bleo), which was recovered by day 28 (Supplementary Figure 1A). At three or six months after bleomycin, there was no significant difference in body weight between the saline and bleomycin groups (3 months: 135% vs 128% of starting weight; 6 months: 152% vs 147% of starting weight).

HPLC determination of hydroxyproline content demonstrated that total lung collagen was significantly increased above baseline from day 10 onwards (Figure 1B). Of interest, when expressed as the amount of collagen per milligram of lung tissue (wet weight), early timepoints post-bleomycin reported a decreased value (presumably due to the degree of lung oedema), which reached equivalence with the saline group at day 28, then exceeded the control group at 3 months post bleo (Supplementary Figure 1B). There was a significant correlation between the lung weight and total lung collagen at day 28 ($r^2 = 0.69$; $p<0.01$).

In contrast, lung collagen determination using the commonly used Sircol assay gave a markedly different profile and magnitude of the changes in lung collagen (Supplementary Figure 1C). The Sircol readout was increased at day 7, and remained elevated out to 3 months – but only accounted for a fraction of the lung collagen as determined by HPLC. In fact, at 28 days, Sircol detected around 9% of the total lung collagen present. No correlation was found between total lung collagen (Sircol determination) and lung weight at day 28 (data not shown) or between total lung collagen (Sircol determination) and total lung collagen (HPLC determination) at day 28 (data not shown).

An increase in collagen mRNA levels preceded the observed changes in protein, with a significant upregulation evident as early as three days post bleomycin. At three months post bleomycin, the raised Col1a1 mRNA signature had returned to baseline (Supplementary Figure 1D).

Histologically, the early changes in the lung architecture reflected the acute inflammation followed by a transition to a more fibrotic microenvironment (Supplementary Figure 2). Predominantly neutrophilic infiltrates were clearly apparent at day 3, progressing to mononuclear cell infiltrates, increased vascular

leak and haemorrhage at day 7. From day 10, lymphocytic aggregates were abundant, closely associated with increasingly fibrotic lesions containing alpha-smooth muscle actin+ myofibroblasts (data not shown) and deposition of collagen (stained blue by modified Martius Scarlet Blue [MSB] stain). At days 21 and 28 post bleomycin, the cellularity of the fibrotic lesions and lymphoid aggregates appeared to decrease, suggesting a dispersal of the mononuclear cell infiltrate. Day 28 lungs were characterized by dense lesions containing frequent mature (dark blue) collagen fibrils, completely obliterating the normal lung architecture and extending from the bronchi to the pleural surface. However, in all histological sections examined, fibrotic lesions were interspersed with areas of overtly normal lung tissue.

SUPPLEMENTAL REFERENCES

1. Lakatos HF, Burgess HA, Thatcher TH, et al. Oropharyngeal aspiration of a silica suspension produces a superior model of silicosis in the mouse when compared to intratracheal instillation. *Exp Lung Res* 2006; **32**: 181-99.

2. Grygielko ET, Martin WM, Tweed C, et al. Inhibition of gene markers of fibrosis with a novel inhibitor of transforming growth factor-beta type I receptor kinase in puromycin-induced nephritis. *J Pharmacol Exp Ther* 2005; **313**: 943-51.

3. Howell DC, Johns RH, Lasky JA, et al. Absence of proteinase-activated receptor-1 signaling affords protection from bleomycin-induced lung inflammation and fibrosis. *Am J Pathol* 2005; **166**: 1353-65.

4. Keerthisingam CB, Jenkins RG, Harrison NK, et al. Cyclooxygenase-2 deficiency results in a loss of the anti-proliferative response to transforming growth factor-beta in human fibrotic lung fibroblasts and promotes bleomycin-induced pulmonary fibrosis in mice. *Am J Pathol* 2001; **158**: 1411-22.

5. Scotton CJ, Krupiczojc MA, Konigshoff M, et al. Increased local expression of coagulation factor X contributes to the fibrotic response in human and murine lung injury. *J Clin Invest* 2009; **119**: 2550-2563.

SUPPLEMENTAL FIGURE LEGENDS

Supplementary Figure 1. Body weight loss, increase in total lung collagen and Col1a1 mRNA levels following bleomycin instillation. (A) Body weight decreased following bleomycin injury, with a nadir around day 7 or 8, followed by a steady return to starting body weight by day 28. (B) Total lung collagen is shown in Figure 1B; however, the amount of lung collagen per milligram of lung tissue (wet weight) was lower at early time points, reaching equivalence with control lungs at day 28. (C) Lung collagen (determined by Sircol assay) was increased from day 7 onwards with a broadly similar pattern to that determined by HPLC, but it significantly underestimated the absolute amount of lung collagen. (D) Col1a1 mRNA levels (QPCR analysis) were significantly elevated as early as 3 days following bleomycin instillation, but returned to baseline at 3 months, suggesting a cessation of active fibrotic change at this timepoint. All data are mean ± SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (ANOVA) versus matched timepoint control. n = 8 - 11 mice per group.

Supplementary Figure 2. Histological analysis of the temporal response to bleomycin. Shown are standard H&E and MSB stained sections from day 3 to day 28 post bleomycin. A day 3 post-saline lung is shown for comparison. Histologically, the early changes in the lung architecture reflected the acute inflammation followed by a transition to a more fibrotic microenvironment. Neutrophilic infiltrates were clearly apparent at day 3, progressing to mononuclear cell infiltrates, increased vascular leak and haemorrhage at day 7. From day 10, lymphocytic aggregates were abundant, closely associated with increasingly fibrotic lesions and deposition of collagen (stained blue by modified Martius Scarlet Blue [MSB] stain). At days 21 and

28 post bleomycin, the cellularity of the fibrotic lesions and lymphoid aggregates appeared to decrease, suggesting a dispersion of the mononuclear cell infiltrate. Day 28 lungs were characterized by dense lesions containing frequent mature (dark blue) collagen fibrils, completely obliterating the normal lung architecture and extending from the bronchi to the pleural surface. However, in all histological sections examined, fibrotic lesions were interspersed with areas of overtly normal lung tissue. All sections have individual scale bars.

Supplementary Video 1. Representative micro-CT stack, 14d post-saline, with uncropped packaging material.

Supplementary Video 2. Representative micro-CT stack, 14d post-bleomycin, with uncropped packaging material.

Supplementary Video 3. Representative micro-CT stack, 21d post-bleomycin, with uncropped packaging material.

Supplementary Video 4. Representative micro-CT stack, 28d post-bleomycin, with uncropped packaging material.

Supplementary Video 5. Representative micro-CT stack, 84d post-bleomycin, with uncropped packaging material.

Supplementary Video 6. Representative micro-CT stack, 168d post-bleomycin, with uncropped packaging material.

Supplementary Video 7. Virtual bronchoscopy of a lung, 14d post-saline.

Supplementary Video 8. Virtual bronchoscopy of a lung, 168d post-bleomycin.