Deficiency of tumour necrosis factor-related apoptosis-inducing ligand exacerbates lung injury and fibrosis

Emmet E McGrath,1 Allan Lawrie,2 Helen M Marriott,1 Paul Mercer,3 Simon S Cross,4 Nadine Arnold,2 Vanessa Singleton,1 Alfred AR Thompson,1 Sarah R Walmsley,1 Stephen A Renshaw,1 Ian Sabroe,1 Rachel C Chambers,3 David H Dockrell,2 Moira K B Whyte1

ABSTRACT

Background The death receptor ligand tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) shows considerable clinical promise as a therapeutic agent. TRAIL induces leukocyte apoptosis, reducing acute inflammatory responses in the lung. It is not known whether TRAIL modifies chronic lung injury or whether TRAIL has a role in human idiopathic pulmonary fibrosis (IPF). We therefore explored the capacity of TRAIL to modify chronic inflammatory lung injury and studied TRAIL expression in patients with IPF.

Methods TRAIL−/− and wild-type mice were instilled with bleomycin and inflammation assessed at various time points by bronchoalveolar lavage and histology. Collagen deposition was measured by tissue hydroxyproline content. TRAIL expression in human IPF lung samples was assessed by immunohistochemistry and peripheral blood TRAIL measured by ELISA.

Results TRAIL−/− mice had an exaggerated delayed inflammatory response to bleomycin, with increased neutrophil numbers (mean 3.19±0.8 wild type vs 11.5±5.4×103 TRAIL−/−, p<0.0001), reduced neutrophil apoptosis (5.42±1.6% wild type vs 2.47±0.5% TRAIL−/−, p=0.0003) and increased collagen (3.45±0.2 wild type vs 5.8±1.3 mg TRAIL−/−, p=0.005). Immunohistochemical analysis showed induction of TRAIL in bleomycin-treated wild-type mice. Patients with IPF demonstrated lower levels of TRAIL expression than in control lung biopsies and their serum levels of TRAIL were significantly lower compared with matched controls (38.1±9.6 controls vs 32.3±7.2 pg/ml patients with IPF, p=0.002).

Conclusion These data suggest TRAIL may exert beneficial, anti-inflammatory actions in chronic pulmonary inflammation in murine models and that these mechanisms may be compromised in human IPF.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic scarring disease of the lung of uncertain pathogenesis and to date no therapy has convincingly improved survival or modified clinical course.1 IPF is a heterogeneous condition both in terms of clinical course and pathological appearance. This suggests it may be initiated by different forms of lung injury and alveolar epithelial injury followed by aberrant repair is currently regarded as a central pathogenic mechanism.2 The role of inflammation, and particularly of neutrophils, in IPF is controversial but neutrophilic alveolitis is a frequent feature of the disease.3,4 Moreover, higher neutrophil counts in bronchoalveolar lavage (BAL) are associated with more rapid disease progression and with worse lung function.5,6 Neutrophilic inflammation is also a component of experimental models of IPF, including bleomycin-induced lung injury in mice.7,8

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) has important roles in regulating cell survival, particularly of immune cell populations. TRAIL is a type II membrane protein that is principally expressed by leukocytes, including monocytes, macrophages, lymphoid cells and neutrophils.9 In humans, TRAIL interacts with four membrane receptors belonging to the TNF receptor family. TRAIL receptor 1 (TRAIL-R1) and TRAIL-R2 have cytoplasmic death domains and can activate caspases and nuclear factor κB (NFκB). The receptors TRAIL-R3 and TRAIL-R4 have truncated death domains and do not activate caspases; they are usually described as decoy receptors but may activate NFκB. A soluble decoy receptor for TRAIL, osteoprotegerin (OPG), is also described. Mice have

Key messages

What is the key question?

1. Does the cell death-inducing molecule tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) regulate the severity of chronic lung inflammation and does it have a role in human idiopathic pulmonary fibrosis (IPF)?

What is the bottom line?

1. TRAIL-deficient mice have worse lung inflammation and more lung collagen than wild-type mice and patients with IPF show lower levels of TRAIL than healthy controls.

Why read on?

TRAIL is a potential biomarker of IPF progression and correction of TRAIL deficiency might have clinical utility in treatment of IPF.
a single TRAIL death receptor, which shares 79% sequence homology with human TRAIL-R2, together with two decoy receptors. The clinical promise of TRAIL as an agent with which to manipulate apoptosis is emphasised by its use in phase I and II studies in cancer therapy. We previously showed exogenous TRAIL can accelerate apoptosis of neutrophils, a key cellular process for resolving inflammation in vivo. TRAIL is implicated in the elimination of senescent neutrophils from the human circulation. Our recent data in a murine model of acute lung injury show TRAIL regulates neutrophil lifespan at sites of inflammation. There is little data on roles of TRAIL in chronic inflammation. TRAIL can, however, modify fibrosis, since TRAIL treatment of mice has been shown to promote resolution of hepatic fibrosis and, importantly, there is evidence TRAIL may directly induce apoptosis of primary lung fibroblasts.

Because of these dual effects upon neutrophils and fibroblasts we hypothesised TRAIL may regulate chronic inflammation in the lung, modifying both the inflammatory and fibroproliferative components of this condition, and play a role in human IPF.

**MATERIALS AND METHODS**

**Animals**

TRAIL−/− mice on a C57/BL6 background were a kind gift from Amgen (Seattle, Washington, USA). All procedures were approved by the University of Sheffield Ethics Committee and were performed in accordance with the Home Office Animal (Scientific Procedures) Act 1986.

**Human subjects**

All lung tissue was supplied by the Cambridge Tissue Bank with approval from the Papworth Hospital Ethics Committee, with further details in the online supplement. Serum samples were collected from 51 patients with IPF. Controls were age and sex matched to patients with IPF and were recruited via the patient’s family physician who confirmed they did not have a diagnosis of interstitial lung disease. Clinical data, including pulmonary function tests, are provided in the online supplement. These studies were performed with the approval of the South Sheffield Research Ethics Committee.

**Bleomycin model of lung injury**

Female mice were used between 8 and 12 weeks of age, in accordance with the UK Home Office Animals (Scientific Procedures) Act, 1986. Clinical-grade bleomycin (Nippon Kayaku, Slough, UK) 1 mg/kg body weight in 30 μl of saline, or an equal volume of saline as a control, was administered by single intratracheal injection into a surgically exposed trachea under anaesthesia.

**Bronchoalveolar lavage**

Following BAL a haemocytometer total cell count was performed and differential cell counts calculated from cytospin preparations (Cytospin 3; Thermo Shandon, Runcorn, UK) stained with Diff-Quik and assessed by blinded reviewers.

**Assessment of neutrophil apoptosis**

The proportion of apoptotic neutrophils was determined by blinded reviewers, counting duplicate cytopsins stained by Diff-Quik (>300 cells per slide).

**Measurement of total lung collagen**

Total lung collagen was calculated by measuring hydroxyproline content in aliquots of pulverised lung. Details are given in the online supplement.

**Preparation of lung tissue for histological studies**

Unlavaged lungs were insufflated with 4% paraformaldehyde in phosphate-buffered saline (PBS) at a pressure of 20 cm H₂O, followed by removal of the heart and inflated lungs en bloc and immersion for 4 h in fresh fixative. Subsequently lungs were transferred into 15% sucrose in PBS and left overnight at 4°C, before transfer to 70% ethanol.

**Masson’s trichrome staining**

Unlavaged lungs were formalin fixed, processed and embedded in paraffin wax blocks. Sections of lung tissue were stained as described in the online data supplement.

**Immunohistochemistry**

Staining with specific antibodies and TUNEL staining was performed as detailed in the online data supplement.

**ELISAs**

ELISAs for TRAIL and OPG were from R&D Systems (Abingdon, UK) and performed according to the manufacturer’s instructions.

**Statistical analysis**

Unless otherwise stated, results were analysed using a one-way or two-way analysis of variance as appropriate, followed by a Bonferroni’s post-test for multiple comparisons. Patient and control data for serum TRAIL and OPG levels were compared using two-tail paired t-tests. Correlations between serum TRAIL levels and total diffusing capacity of the lung for carbon monoxide (TLco) or survival were assessed using Pearson’s correlation coefficient. Results were considered significant if p<0.05.

**RESULTS**

**Bleomycin-mediated pulmonary inflammation is enhanced in TRAIL-deficient mice**

The composition of BAL was assessed at time points up to 23 days following bleomycin administration. Bleomycin instillation resulted in inflammatory cell recruitment into the lungs of challenged mice. BAL fluid from control mice (PBS instilled) consisted overwhelmingly of alveolar macrophages and contained <0.1×10⁶ neutrophils at all time points in wild-type and TRAIL−/− strains (data not shown). There was an enhanced inflammatory response in TRAIL−/− compared with wild-type mice, with significantly increased total and percentage neutrophil counts at day 7 following bleomycin instillation (mean 3.19±0.8×10⁶ in wild-type vs 11.5±5.4×10⁶ in TRAIL−/− mice, p<0.0001; mean 2.37±1.0% in wild-type vs 8.25±1.3% neutrophils in TRAIL−/− mice, p<0.0001) (figure 1A,B). Macrophages (as total or percentage count) were not significantly different between the groups of mice (figure 1C,D) but TRAIL−/− mice also had a significant increase in total lymphocytes at day 7 when compared with wild-type mice (mean 7.32±2.8×10⁴ in wild-type vs 14.6±4.8×10⁴ in TRAIL−/− mice, p=0.035) (figure 1E). To characterise the neutrophilic inflammation in more detail, we performed additional time points at day 5 and 10 and confirmed peak neutrophil counts were detected at day 7 (data not shown). Lymphocyte numbers also peaked at day 7 and were predominantly CD3+ as opposed to CD19+ cells (data not shown).

**TRAIL-deficient mice have reduced apoptosis following bleomycin-induced lung injury**

TUNEL staining of day 23 lung sections from both wild-type and TRAIL−/− mice revealed significantly fewer apoptotic cells...
in TRAIL$^{-/-}$ mice (mean 46.4±25.1 cells/HPF) in wild-type vs 8.90±6.50 cells/HPF in TRAIL$^{-/-}$ mice, p<0.0001) (figure 2). The majority of apoptotic events seen in wild-type mice were in areas with numerous influxing inflammatory cells (figure 2A). Very few such events were seen in TRAIL$^{-/-}$ mice, despite the presence of inflammatory infiltrates (figure 2B). That differences in rates of apoptosis in the inflammatory cell infiltrate explained the differences in numbers of TUNEL-positive events was apparent by analysing the numbers of apoptotic cells in BAL (figure 3). The proportion of the neutrophil BAL population after bleomycin instillation that had the microscopic appearances of apoptosis was <2% in BAL from both wild-type and TRAIL$^{-/-}$ mice at day 3. This increased as neutrophil numbers peaked at day 7 and then decreased (figure 3A). At later time points there was a significantly lower proportion of apoptotic neutrophils in the TRAIL$^{-/-}$ mice, for example, mean 5.42±1.6% in wild-type vs 2.47±0.5% in TRAIL$^{-/-}$ mice at day 7, p=0.0003. This suggested reduced apoptosis was contributing to the greater neutrophil numbers in BAL of TRAIL$^{-/-}$ mice. Despite the greater neutrophil numbers in TRAIL$^{-/-}$ mice, these mice also showed fewer absolute numbers of apoptotic neutrophils than their wild-type counterparts (figure 3B).

**TRAIL-deficient mice have increased total lung collagen**

The effect of TRAIL deficiency on total lung collagen accumulation, 16 days after intratracheal administration of bleomycin or saline, is shown in figure 4. This time point has previously identified significant differences in fibro-proliferative responses to bleomycin. TRAIL$^{-/-}$ and wild-type mice demonstrated no significant difference in lung collagen levels after saline challenge. Lung collagen accumulation was significantly increased in bleomycin-instilled wild-type mice compared with saline control (mean 2.08±0.60 mg with saline treatment vs 3.46±0.30 mg with bleomycin, p=0.0033) and was of at least equivalent magnitude to previous studies using the same experimental protocol. In comparison to wild-type mice there was a significantly greater increase in collagen accumulation following bleomycin treatment in TRAIL$^{-/-}$ mice (mean 3.46±0.3 mg in wild-type vs 5.8±1.5 mg in TRAIL$^{-/-}$ mice, p=0.005).

**TRAIL immunolocalisation following bleomycin-induced lung injury**

Immunohistochemical examination of TRAIL expression in the lung was performed at key time points in wild-type mice, with TRAIL$^{-/-}$ mice acting as controls. In wild-type animals receiving saline intratracheally, TRAIL staining of the bronchial epithelium was observed, together with some staining in alveolar type 2 pneumocytes, in keeping with previous studies. Following bleomycin administration, no specific TRAIL immunostaining was detected in TRAIL$^{-/-}$ mice (figure 5A), while in wild-type mice staining was seen in the bronchial and alveolar epithelium and also in well defined groups of cells adjacent to the bronchial epithelium and in cells in the alveolar air spaces.
These positively staining cells were identified as bronchus-associated lymphoid tissue and alveolar macrophages, as illustrated by staining with anti-CD3 and anti-galectin-3 antibodies respectively (online supplement).

Immunohistochemical expression of TRAIL in human IPF
To examine the potential role of TRAIL in human fibrotic lung disease, we examined TRAIL expression in human lung tissue specimens by immunohistochemistry. In lung sections from control patients there was positive staining for TRAIL associated with resident alveolar macrophages and in the epithelium (figure 6A,B), in keeping with findings in unchallenged wild-type mice (figure 5 and Weckmann et al19). In lung tissue obtained from patients with established IPF, TRAIL staining was detected in areas of relatively normal lung, both in alveolar macrophages and epithelial cells (figure 6C,D). In areas of dense fibrosis, however, there was little staining either of alveolar macrophages or of lymphoid tissue with variable but generally low-level staining of the respiratory epithelium (figure 6E,F).

Serum TRAIL is reduced in patients with IPF compared with controls
The soluble form of TRAIL is normally present in serum and can be detected by ELISA, as can its soluble receptor, OPG.9 20 Absolute serum levels of TRAIL were reduced in patients with IPF relative to their matched controls (p = 0.002, figure 7A) although OPG levels were not significantly altered (p = 0.230, figure 7B). The ratio of OPG:TRAIL was significantly increased (p = 0.053, figure 7C). Within the cohort of patients with IPF (online supplement), serum TRAIL levels were significantly correlated with predicted TLco (p = 0.046, figure 8), although the magnitude of the correlation was not large. There was no relationship identified between forced vital capacity (%) predicted and serum TRAIL levels and no correlation of serum OPG levels with any of these parameters (data not shown).

DISCUSSION
Neutrophils are thought to play an important role in the pathogenesis of pulmonary fibrosis. Accumulation of neutrophils and increased release of neutrophil chemotactic factors are observed...
in patients with a variety of causes of pulmonary fibrosis, including IPF. Neutrophil-dependent, T-cell independent mechanisms make an important contribution to collagen deposition in several murine models of pulmonary fibrosis while factors which reduce neutrophil accumulation and production of neutrophil chemotactic factors reduce collagen deposition. Thus accumulation of neutrophils, which can perpetuate pulmonary inflammation through the release of matrix metalloproteinases and serine proteases, can enhance pulmonary fibrosis. Conversely, compounds such as cyclin-dependant kinase inhibitors that accelerate neutrophil apoptosis reduce lung fibrosis.

We examined the role of TRAIL in chronic inflammation in the lung using the well established bleomycin model. This model reproduces many of the histological features of IPF and is associated with recruitment of inflammatory cells, including neutrophils, into the injured lung during the first 7 days, with fibrotic change and deposition of matrix occurring up to day 82, 6 Conversely, compounds such as cyclin-dependant kinase inhibitors that accelerate neutrophil apoptosis reduce lung fibrosis. 13

TRAIL-deicient mice showed biochemical evidence of increased collagen deposition. In both the bleomycin model and human pulmonary fibrosis tissue, there is increased deposition of collagen and other molecules of the extracellular matrix by lung fibroblasts. Previous work by Yurovsky showed TRAIL induces apoptosis of primary lung fibroblasts derived from patients with IPF. The increased collagen lung we observed in the absence of TRAIL could, therefore, result from the lack of its pro-apoptotic effects on pulmonary fibroblasts, and/or the increased inflammatory drive to fibroproliferation consequent upon increased numbers of neutrophils.

TRAIL is widely expressed on leucocyte populations and, in the normal human lung, TRAIL expression has been detected on alveolar septa, bronchial epithelium and vascular endothelium. In bleomycin-treated wild-type mice, TRAIL was also detected in a population of CD3-positive, peribronchial inflammatory cells with the appearances of bronchus associated lymphoid tissue and in galectin-3-positive air-space cells with the

Figure 4 Lung collagen accumulation in response to bleomycin is increased in tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-deficient mice. (A–C) Representative lung tissue sections stained with Masson’s Trichrome from wild-type mice at day 7 and 23 after saline and bleomycin instillations. (A) Lung architecture was normal in TRAIL−/− mice given saline. (B) and (C) Extensive patchy fibrotic foci with increased deposition of collagen were seen in wild-type mice given bleomycin at day 7 and 23, respectively. (D) Total lung collagen as measured by reverse phase high-performance liquid chromatography quantimation of lung hydroxyproline in acid hydrolysates of pulverised lung. Data represent the mean ± SEM of values obtained in groups of five mice. Wild-type and TRAIL−/− mice instilled with saline had similar collagen levels in the lungs. Wild-type mice instilled with bleomycin had a significant increase in collagen compared with the saline control (*p = 0.0033). TRAIL−/− mice had a significant increase in collagen compared with wild-type mice instilled with bleomycin (**p = 0.005) and with saline controls (**p = 0.0004).
The characteristic appearance of alveolar macrophages. These findings were in keeping with BAL analysis in which macrophages and lymphocytes were found to express TRAIL by flow cytometry (data not shown). Macrophage expression of death receptor ligands has been shown to regulate apoptosis induction in a variety of inflammatory cells. A role for macrophage Fas ligand (FasL) induction of target cell killing has been described and for TNFα acting in concert with integrins in the macrophage induction of apoptosis in neutrophils. We did not detect neutrophil expression of TRAIL in our experiments, in keeping with other recent data. The distribution of TRAIL expression in wild-type mice following bleomycin lung injury, with ongoing expression on epithelial cells but upregulated expression on inflammatory cells, is very different from that in control samples and in LPS-mediated lung injury or allergen challenge models in which TRAIL expression is predominantly on the airway epithelium. In a recently described model of influenza-mediated inflammatory lung disease, TRAIL was expressed on alveolar macrophages and also on a small proportion of natural killer cells in BAL. Our data suggest that macrophage-expressed and possibly lymphocyte-expressed TRAIL could play an important role in regulating the inflammatory response in chronic pulmonary inflammation.

TRAIL is not the only death receptor ligand shown to induce apoptosis of inflammatory leucocytes. FasL and TNFα have well characterised roles in acceleration of neutrophil apoptosis, but

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**Figure 5** Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) expression localises to bronchus associated lymphoid tissue (BALT) and alveolar macrophages. Lung sections 16 days after bleomycin instillation were immunohistochemically stained for TRAIL. (A) Lung section demonstrating the presence of alveolar macrophages and BALT tissue and absence of specific TRAIL immunostaining in a TRAIL−/− mouse. (B) and (C) Lung sections (representative of sections from five mice) demonstrating TRAIL expression localised to epithelium and BALT in a wild-type mouse. (D) Lung section demonstrating TRAIL expressing alveolar macrophages in a wild-type mouse.

**Figure 6** Immunohistochemical localisation of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) in human lung specimens. Immunostaining for TRAIL on representative lung tissue sections from human lung material. (A) and (B) Intense immunostaining for TRAIL in control lung was associated with macrophages and alveolar epithelium. Representative images are shown from lung sections of three individuals. (C–F) Analysis of lung tissue from patients with idiopathic pulmonary fibrosis. Representative images are shown from lung sections of five individuals. (C, D) Areas of lung with relatively normal architecture showed TRAIL staining similar to that from control lung samples whereas matched samples from fibrotic areas of the same individuals (E, F) demonstrated almost no positive immunostaining for TRAIL. Scale bars for panels (A), (C) and (E) represent 100 μm and for (B), (D) and (F) 50 μm.
primary cell types are described. We previously showed TRAIL induces apoptosis of transformed cells, although effects on some receptor. The TRAIL system has since been implicated in the had no chemotactic effects in human neutrophils in vitro but did in other cell types with TRAIL treatment in vivo. In contrast, FasL induces apoptosis of bronchial epithelial cells and cytokoticity in other cell types with TRAIL treatment in vivo. In contrast, FasL induces apoptosis of bronchial epithelial cells and pulmonary overexpression of TNFα induces chronic pulmonary inflammation and fibrosis.

Recent work in a murine model of asthma showed increased pulmonary epithelial expression of TRAIL and that inhibition of TRAIL function attenuated allergic inflammation. In contrast, we found the absence of TRAIL is associated with enhanced inflammation in the lung and that TRAIL is expressed on macrophage and lymphoid cells. Moreover, absence of TRAIL results in more severe inflammation and fibrosis, suggesting this ligand may have a role in ameliorating non-allergic inflammation. In keeping with this, recent studies of TRAIL-R-deficient mice showed development of severe pneumonitis following ionising radiation that was absent from wild-type mice.

We show there may be a relative paucity of TRAIL expression in the fibrotic regions of interstitial lung disease in man. Lung samples from patients with IPF showed low levels of TRAIL expression in densely fibrosed areas, with relative preservation of TRAIL expression in more histologically normal areas. Circulating levels of soluble TRAIL were lower in patients with IPF than in controls, whereas levels of the soluble receptor, OPG, were unchanged. The magnitude of difference seen in TRAIL levels, although relatively small, was comparable to other studies using the same ELISA and equivalent to the changes seen in patients with atherosclerosis compared with healthy controls, where lower levels of TRAIL have been shown to be of prognostic significance.

The biological roles of TRAIL in human IPF clearly require further exploration. In the bleomycin model, deficiency of TRAIL leads to increased inflammation and collagen deposition, suggesting TRAIL expression by leucocytes and/or tissue cells acts to limit these features of disease and thus might act as a disease modifier, influencing disease progression. TRAIL also has possible utility as a biomarker of disease progression in IPF, which could be addressed in longitudinal studies. Whether TRAIL has a role in susceptibility to disease is unknown, although functional genetic polymorphisms of TRAIL and its receptors exist and could be studied in patients with IPF and control populations. It is also conceivable that treatment with exogenous TRAIL might have a beneficial anti-inflammatory and antifibrotic effect in IPF. Forms of recombinant TRAIL and TRAIL-R2 agonistic antibodies are in clinical trials in a range of malignant diseases and could be considered for a non-malignant disease, such as IPF, with an equally poor prognosis.
Interstitial lung disease

References


Figure 1
Deficiency of TNF-Related Apoptosis Inducing Ligand (TRAIL) exacerbates lung injury and fibrosis

1Emmet E. McGrath, 2Allan Lawrie, 1Helen M. Marriott, 3Paul Mercer, 4Simon S. Cross, 2Nadine Arnold, 1Vanessa Singleton, 1A. A. Roger Thompson, 1Sarah R. Walmsley, 3Ian Sabroe, 3Rachel Chambers, 2David H. Dockrell, 1Moira K.B. Whyte.

Departments of 1Infection and Immunity, 2Cardiovascular Sciences and 4Neuroscience, University of Sheffield, Sheffield, S10 2RX, and 3Centre for Respiratory Research, University College, London WC1E 6JJ, United Kingdom.

Online Data Supplement
**Methods**

**Animals**

TRAIL−/− mice on a C57/BL6 background were a kind gift from Dr Mark Smyth and Amgen (Seattle, USA).[1] All procedures were approved by the University of Sheffield Ethics Committee and were performed in accordance with the Home Office Animal (Scientific Procedures) Act 1986.

**Human Subjects**

Fibrotic lung tissue was removed from individuals undergoing lung biopsy or transplant surgery who had a pre-operative clinical diagnosis of IPF according to ATS/ER Consensus criteria and histologic confirmation of usual interstitial pneumonia (UIP).[2] Control tissue was from tissue distal to resected primary lung tumour, in patients with no clinical evidence of IPF or other fibrotic lung disease. All lung tissue was supplied by the Cambridge Tissue Bank with the approval of the Papworth Hospital Ethics Committee. Serum samples were collected from 31 IPF patients fulfilling ATS/ERS Consensus criteria. 31 controls were age and sex-matched to IPF patients and were recruited via the patient’s family physician, who confirmed they did not have a diagnosis of interstitial lung disease. Clinical data and pulmonary function tests are provided in Table 1. These studies were performed with the approval of the South Sheffield Research Ethics Committee.

**Bleomycin Model of Lung Injury**

Female mice were housed in a specific pathogen–free facility and used when between 8 and 12 weeks of age, in accordance with the UK Home Office Animals (Scientific Procedures) Act, 1986. Clinical-grade bleomycin (Nippon Kayaku Co. Ltd., Slough, UK) 1 mg/kg body weight in 30 µl of saline, or an equal volume of saline as a
control, was administered by single intratracheal injection into a surgically exposed trachea under anaesthesia as previously described.[3]

**Bronchoalveolar lavage (BAL)**

Mice were sacrificed 3-23 days after treatment with an overdose of sodium pentobarbitone and BAL was performed as previously described.[3] A total of 10 µl of BAL was diluted in 90 µl of 3% acetic acid for a hemocytometer total cell count. Differential cell counts were calculated from cytospin preparations (Cytospin 3; Thermo Shandon, Runcorn, U.K.) made from 100 µl of each BAL sample, stained with Diff-Quik and assessed by blinded reviewers.

**Assessment of neutrophil apoptosis**

The proportion of apoptotic neutrophils was determined by blinded reviewers, counting duplicate cytospins stained by Diff-Quik (>300 cells per slide). In keeping with previous work, we found the morphological features of apoptotic and non-apoptotic murine neutrophils could be clearly distinguished by light microscopy.[4]

**Measurement of Total Lung Collagen**

Total lung collagen was calculated by measuring hydroxyproline content in aliquots of pulverized lung as described previously[5]. Hydroxyproline levels were quantitated by reverse-phase high performance liquid chromatography (HPLC) of 7-chloro-4-nitrobenzo-oxao-1,3-diazole-derived acid hydrolysates. Hydroxyproline reacts with 7-chloro-4-nitrobenzo-oxao-1,3-diazole to generate a chromophore which has maximum light absorbance at 495 nm. Total lung collagen was determined under the assumption that lung collagen contains 12.2% (w/w) hydroxyproline and the results were expressed as milligrams of collagen per lung.[5]

**Preparation of Lung Tissue for Histological Studies**
Unlavaged lungs were insufflated with 4% paraformaldehyde in phosphate-buffered saline (PBS) at a pressure of 20 cm H$_2$O, followed by removal of the heart and inflated lungs en bloc and immersion for 4 hours in fresh fixative. Subsequently lungs were transferred into 15% sucrose in PBS and left overnight at 4°C, before transfer to 70% ethanol.

**Masson’s Trichrome Staining**

Unlavaged lungs were fixed via the trachea with 10% buffered formalin at 20 cm H$_2$O, processed and embedded in paraffin wax blocks. 5 µm thick sections of lung tissue were dewaxed in xylene, rehydrated with ethanol then washed in PBS. Sections were stained in celestine blue solution (0.5% celestine blue and 5% (w/v) ferric ammonium sulphate for 10 seconds, both in water; and 14% (v/v) glycerin) (all from Sigma-Aldrich) before being immersed in Mayer’s hematoxylin for 10 seconds, followed by immersion in 1% (w/v) Ponceau red in water (both from BDH/Merck, Poole, Dorset, UK) for 6 minutes. Sections were then differentiated in 1% (w/v) phosphomolybdic acid in water, before counterstaining with 0.5% (v/w) soluble methyl blue in 2.5% (v/v) acetic acid in water (both from Sigma-Aldrich). Following staining, sections were added to increasing concentrations of ethanol and xylene, facilitating dehydration, before they were mounted in DPX (BDH/Merck). To demonstrate fibrin fibers, a modified staining technique, involving a mixture of Ponceaux de Xylidine and Acid Fuchsin, based on that of Lendrum et al was used.[6]

**Immunohistochemistry.**

Individual lobes of mouse lungs were serially sectioned at 5 µm and processed, using 0.6 mol/L sodium citrate buffer (pH 6) antigen retrieval where required, as previously described.[7] Serial sections were stained with monoclonal antibodies to TRAIL
(Novocastra, Peterborough, UK) as previously described.[8] Immunohistochemical markers for murine lymphoid cells (anti-CD3; DakoCytomation, Ely, Cambridgeshire, UK) and macrophages (anti-galectin-3; DakoCytomation) were also used. Antibodies were incubated for 1 hour and labeled using a streptavidin ABC peroxidase technique (Novocastra) and visualized with 3,3’-diaminobenzidine (DakoCytomation). Tissue blocks from patients with IPF were serially sectioned and stained with anti-TRAIL antibodies (Novocastra). TUNEL staining was performed using an ApopTag peroxidise in situ apoptosis detection kit (Chemicon International) as per the manufacturer’s instructions). Numbers of apoptotic events were counted per high power field, for a minimum of 10 randomly chosen fields by blinded reviewers. Data were expressed as mean±SEM TUNEL positive cells per high power field.
References


Table 1. Clinical characteristics of IPF patients and controls

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<td>Survival (months)</td>
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<td>Serum TRAIL (g/ml)</td>
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All parameters are expressed as mean +/- standard deviation except for smoking pack years, which are expressed as median +/- interquartile range. Statistical comparisons were performed using paired two-tailed t tests, except for smoking pack years for which a Mann-Whitney test was performed.
Figure 1. Anti-CD3 and galectin-3 immunohistochemical analysis of lung tissue.

A and B, Lung sections from wild type and TRAIL−/− mice respectively 16 days after bleomycin instillation were immunohistochemically stained with anti-CD3. The collections of cells previously described earlier stained positive in both groups of mice confirming the identification of BALT tissue. C and D, Lung sections from wild type and TRAIL−/− mice respectively 16 days after bleomycin instillation were also immunohistochemically stained with anti-galectin 3 which confirmed the cells described in the alveolar spaces were correctly identified as alveolar macrophages.