

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, ¹Ian 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 cytopins 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 H₂O, 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 H₂O, 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 peroxidase 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

1. Cretney E, Takeda K, Yagita H, et al. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. *J Immunol* 2002;**168**:1356-1361.
2. American Thoracic Society, European Respiratory Society. 2002. American Thoracic Society/European Respiratory Society international multidisciplinary consensus classification of the idiopathic interstitial pneumonias. This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS executive committee. *Am J Respir Crit Care Med* 2001;**165**:277-304
3. Dockrell DH, Marriott HM, Prince LR, et al. Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection. *J Immunol* 2003;**171**:5380-5388.
4. Rowe SJ, Allen L, Ridger VC, et al. Caspase-1-deficient mice have delayed neutrophil apoptosis and a prolonged inflammatory response to lipopolysaccharide-induced acute lung injury. *J Immunol* 2002;**169**:6401-6407.

5. Mutsaers SE, Foster ML, Chambers RC, et al. Increased endothelin-1 and its localization during the development of bleomycin-induced pulmonary fibrosis in rats. *Am J Respir Cell Mol Biol* 1998;**18**:611-619.
6. Lendrum AC, Fraser DS, Slidders W, et al. Studies on the character and staining of fibrin. *J Clin Pathol* 1962;**15**:401-413.
7. Yang X, Long L, Southwood M, et al. Dysfunctional Smad signaling contributes to abnormal smooth muscle cell proliferation in familial pulmonary arterial hypertension. *Circ Res* 2005;**86**:1053-1063.
8. Lawrie A, Waterman E, Southwood M, et al. Evidence of a role for osteoprotegerin in the pathogenesis of pulmonary arterial hypertension. *Am J Pathol* 2008;**172**:256-264.

Table 1. Clinical characteristics of IPF patients and controls

Characteristic	Controls	Patients	P value
Age (y)	69.5 ± 9.2	67.6 ± 9.0	0.082
% Male	55	55	
% Predicted TLCO	N/A	43.9 ± 18.4	
% Predicted FVC	N/A	87.8 ± 22.5	
Survival (months)	N/A	49.6 ± 43.5	
Serum TRAIL (□g/ml)	38.1 ± 9.6	32.3 ± 7.2	0.002
Serum OPG (□g/ml)	2740 ± 1260	3038 ± 1219	0.230
Smoking Pack Years	7 ± 31	15 ± 32	0.459

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.