



**HAL**  
open science

## **Inactivation of IL-6 and soluble IL-6 receptor by neutrophil derived serine proteases in cystic fibrosis**

Eamon P. McGreal, Philip L. Davies, Wendy Powell, Stefan Rose-John, O. Bradley Spiller, Iolo Doull, Simon A. Jones, Sailesh Kotecha

► **To cite this version:**

Eamon P. McGreal, Philip L. Davies, Wendy Powell, Stefan Rose-John, O. Bradley Spiller, et al.. Inactivation of IL-6 and soluble IL-6 receptor by neutrophil derived serine proteases in cystic fibrosis. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2010, 1802 (7-8), pp.649. 10.1016/j.bbadis.2010.04.005 . hal-00600113

**HAL Id: hal-00600113**

**<https://hal.science/hal-00600113>**

Submitted on 14 Jun 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

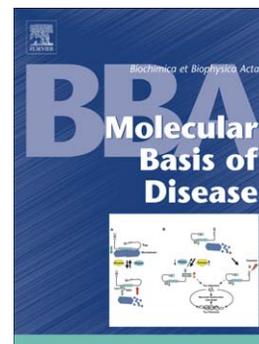
Inactivation of IL-6 and soluble IL-6 receptor by neutrophil derived serine proteases in cystic fibrosis

Eamon P. McGreal, Philip L. Davies, Wendy Powell, Stefan Rose-John, O. Bradley Spiller, Iolo Doull, Simon A. Jones, Sailesh Kotecha

PII: S0925-4439(10)00084-0  
DOI: doi: [10.1016/j.bbadis.2010.04.005](https://doi.org/10.1016/j.bbadis.2010.04.005)  
Reference: BBADIS 63092

To appear in: *BBA - Molecular Basis of Disease*

Received date: 10 February 2010  
Revised date: 1 April 2010  
Accepted date: 19 April 2010



Please cite this article as: Eamon P. McGreal, Philip L. Davies, Wendy Powell, Stefan Rose-John, O. Bradley Spiller, Iolo Doull, Simon A. Jones, Sailesh Kotecha, Inactivation of IL-6 and soluble IL-6 receptor by neutrophil derived serine proteases in cystic fibrosis, *BBA - Molecular Basis of Disease* (2010), doi: [10.1016/j.bbadis.2010.04.005](https://doi.org/10.1016/j.bbadis.2010.04.005)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

## INACTIVATION OF IL-6 AND SOLUBLE IL-6 RECEPTOR BY NEUTROPHIL DERIVED SERINE PROTEASES IN CYSTIC FIBROSIS.

Eamon P. McGreal<sup>a,\*</sup>, Philip L. Davies<sup>a</sup>, Wendy Powell<sup>a</sup>, Stefan Rose-John<sup>d</sup>, O. Bradley Spiller<sup>a</sup>, Iolo Doull<sup>c</sup>, Simon A. Jones<sup>b</sup> and Sailesh Kotecha<sup>a</sup>.

<sup>a</sup>Department of Child Health, <sup>b</sup>Department of Medical Biochemistry and Immunology, Cardiff University School of Medicine and <sup>c</sup>Cystic Fibrosis Unit, Children's Hospital for Wales, Heath Park, Cardiff CF14 4XN, UK. <sup>d</sup>Department of Biochemistry, Christian-Albrechts-Universität zu Kiel, Medical Faculty, Olshausenstraße 40, D-24098 Kiel, Germany.

**Running Title:** Soluble IL-6R degradation in CF

\*Corresponding Author:

Eamon P. McGreal,

Department of Child Health, Cardiff University School of Medicine

Heath Park, Cardiff CF14 4XN, UK

Tel: +44 (0)2920 748488

Fax: +44 (0)2920 744283

Email: mcgrealep@cf.ac.uk

**Abbreviations:** Cystic fibrosis (CF), soluble IL-6 receptor (sIL-6R), IL-6 receptor (IL-6R), soluble gp130 (sgp130), bronchoalveolar lavage fluid (BALF), neutrophil elastase (NE), cathepsin G (CG), proteinase 3 (PR3), dithiothreitol (DTT), alpha-1 anti-trypsin (AAT), matrix metalloproteinase (MMP), neutrophil derived serine protease (NSP).

**ABSTRACT**

The ability of IL-6 to signal via both membrane bound and soluble receptors is thought to explain the capacity of this cytokine to act in both the initiation and resolution of acute inflammatory responses. In cystic fibrosis (CF), poorly resolved neutrophilic inflammation of the lungs is a primary cause of morbidity and mortality. Expression of IL-6 has been reported to be low in CF lung secretions, despite ongoing inflammation, but the status of soluble IL-6 receptor (sIL-6R) in these patients is unknown. We hypothesised that sIL-6R may be an important potentiator of IL-6 activity in CF associated lung disease. IL-6, sIL-6R and sgp130 (a natural antagonist of responses mediated by the sIL-6R) were analysed by ELISA and Western blot in bronchoalveolar lavage fluid (BALF) from 28 paediatric CF patients and nine non-CF controls. Total cell counts in CF were four fold higher compared to controls (median:  $1.4 \times 10^6$  cells/ml v.  $0.35 \times 10^6$  cells/ml in controls), ( $p < 0.001$ ) and the infiltrate was dominated by neutrophils which were elevated by 89 fold ( $0.62 \times 10^6$  cells/ml v.  $0.007 \times 10^6$  cells/ml in controls), ( $p < 0.001$ ). Other markers of inflammation such as IL-8 and MCP-1 were elevated 17.5 and 3.8 fold respectively (IL-8; median: 1122 pg/ml v. 64pg/ml in controls,  $p < 0.01$  and MCP-1; median: 692 pg/ml v. 182 pg/ml in controls,  $p < 0.05$ ). IL-6, although present in 23/32 CF BALF specimens compared to 1/9 controls ( $p < 0.01$ ), was weakly expressed (median: 50pg/ml). Expression of sIL-6R and sgp130 in CF was no different to control patients. We tested whether weak expression of all three molecules was due to degradation by CF BALF. Degradative activity was observed in association with BALF elastase activity and could be specifically blocked by serine protease inhibitors. Degradation of sIL-6R by purified serine proteases (elastase, cathepsin G and proteinase 3) was also observed leading to a loss of trans-signalling activity. Interestingly, sIL-6R was protected from proteolysis by interaction with IL-6. Our data identify and define a novel protease mediated deficiency of IL-6 signalling in the CF lung.

**KEYWORDS:** Cystic fibrosis, IL-6, cytokine, inflammation, protease, neutrophil.

ACCEPTED MANUSCRIPT

## 1. INTRODUCTION

The importance of IL-6 as a central orchestrator of inflammatory responses is highlighted by its ability to influence both the initiation and resolution of inflammation at a number of sites, including the lung [1, 2]. This functional pleiotropy is thought to be due to the inducible expression of a soluble IL-6 receptor (sIL-6R) which acts as an agonist for IL-6 activity [3, 4]. IL-6 responsiveness normally requires a membrane bound receptor complex, composed of a ligand binding  $\alpha$ -chain IL-6 receptor (IL-6R) and a  $\beta$ -chain gp130 signalling receptor [4]. However, sIL-6R arising either through regulated cleavage of membrane bound receptor [5], or through *de novo* synthesis of a differentially spliced isoform (DSsIL-6R) [6, 7], interacts with IL-6 to form a highly active agonistic complex which can signal via gp130, independently of membrane anchored IL-6R. This process has been named trans-signalling [8].

In contrast to gp130, which is found on all cell types, membrane bound IL-6R is expressed by a limited spectrum of cells, primarily restricted to leukocytes and hepatocytes [9]. Induced sIL-6R expression therefore confers IL-6 responsiveness on the great majority of cell types in the body which lack a cognate membrane bound receptor [4]. Furthermore, sIL-6R enhances IL-6 activity *in vivo* by 10-100 fold [10], permitting potent responses to even very low doses of IL-6, such as those found in the CF lung. This quantitative and qualitative modulation of IL-6 activity enables sIL-6R to extensively shape inflammatory events. Specific blockade of IL-6 trans-signalling *in vivo* with a naturally occurring antagonist, soluble gp130 (sgp130), has highlighted several important activities of this pathway during acute inflammatory responses. In models of peritoneal inflammation, IL-6 trans-signalling shifts the balance of leukocyte recruitment away from neutrophil dominated recruitment in favour of mononuclear cells [1, 11] by modifying chemokine expression [12] and neutrophil apoptosis [13], a pivotal

step in the resolution of acute innate inflammatory responses. Furthermore, intratracheal administration of IL-6 attenuates LPS induced pulmonary neutrophil infiltration [2]. This is particularly relevant to CF as chronic neutrophil dominated inflammation of the lungs due to recurrent and poorly resolved infectious episodes [14, 15], an apparent propensity for excessive neutrophil recruitment by the CF lung [16] and dysregulated neutrophil apoptosis, leads to progressive lung disease and is the primary cause of morbidity and mortality in these patients [17]. Furthermore, although IL-6 is found at high concentrations in a range of inflammatory diseases [18-20] expression in lung secretions from CF patients is notably low despite evidence of significant inflammatory activity [21-23].

In this study we examine the expression of IL-6 trans-signalling molecules in bronchoalveolar lavage fluid (BALF) from CF and controls patients. We describe the relationship between expression of these molecules and other inflammatory markers in these patient groups and define a novel deficiency of the IL-6 trans-signalling pathway in CF patients.

## 2. MATERIALS AND METHODS

### 2.1 Reagents

All reagents were from Fisher Scientific, UK unless otherwise indicated. Plasma derived AAT (Prolastin) was from Talecris Biotherapeutics, NC. GM6001 (Galardin) was from Calbiochem, UK. PMSF and 1,10-phenanthroline were from Sigma, UK. Purified neutrophil elastase (NE), cathepsin G (CG) and proteinase 3 (PR3) were from Athens Research and Technology, GA. Recombinant human IL-6, sIL-6R and sgp130 were from R&D systems, UK.

### 2.2 Patients and clinical samples

Patient material was obtained following approval from the South East Wales Research Ethics Committee. 32 samples were obtained from 28 children with CF, (median age 6.2 years, IQR: 1.8-10.4). For 13/32 samples from CF patients, bronchoalveolar lavage (BAL) was performed by flexible bronchoscopy for clinical investigation of new respiratory symptoms or persistent respiratory symptoms which were refractory to current treatment. For 18/32 samples, non-bronchoscopic lavage was performed while the patient was under general anaesthesia for a surgical procedure not related to respiratory symptoms, most commonly insertion or removal of a portacath but also for gastrostomy insertion, bowel re-anastomosis and meconium ileus surgery. BAL methodology information was unavailable for 1 CF patient in the study. BAL was performed by non-bronchoscopic lavage on nine control patients (median age 8.3 years, IQR 4.2-9.1) who were recruited from children under general anaesthetic for the surgical removal of tonsils or adenoids. Although upper airway symptoms had been present leading up to the surgery, all control children were reported to be well at the time of surgery, had no underlying chest problems and were not on medication. Lavage was performed using 1ml normal saline/kg body weight (up to a maximum of 20ml). Following

collection all BALF samples were kept on ice. Cellular material and BAL supernatants were separated by centrifugation at 500g for 5 minutes. Supernatants were stored at  $-70^{\circ}\text{C}$  in 25 $\mu\text{l}$  aliquots to avoid multiple freeze thaw cycles during subsequent analyses; storage occurred within 45 minutes of collection. Cellular material was resuspended in PBS and treated with 50 $\mu\text{g}/\text{ml}$  dithiothreitol (DTT) for 15 min at room temperature to dissociate any excess mucous prior to taking a total cell count by haemocytometer. Differential cell counts were performed by light microscopy on Wright-Giemsa stained cytopins by counting a minimum of 300 total events in four separate fields. Total cell counts were available for 26 samples and reliable differential cell counts could be enumerated for 21 CF samples and 7 controls. Where differential counts could not be obtained, this was due to the absence of a total cell count, lack of sufficient specimen for cytopins or the presence of excessive mucus and cell clumping in the sample (despite DTT treatment).

### 2.3 *Cell isolation, culture and stimulation.*

A549 alveolar epithelial cells from the European Collection of Cell Cultures (ECACC, UK) were cultured in Kaighns-F12 medium (Hyclone, UK) supplemented with 5% heat inactivated foetal calf serum (Invitrogen-Gibco, UK), 100U/ml Penicillin/Streptomycin, 1mM Sodium Pyruvate (Hyclone, UK). Cells at 70% confluency were cultured in DMEM without serum for 18 hours prior to the addition of stimulants. Cells were then cultured in serum free DMEM with 30ng/ml each of native recombinant IL-6, sIL-6R or IL-6 in combination with sIL-6R. IL-6 in combination with sIL-6R which had been treated with 500mM CG for 2h at  $37^{\circ}\text{C}$  was also used as a stimulus. CG activity was neutralised with 50 $\mu\text{g}/\text{ml}$  AAT prior to adding IL-6. Supernatants were collected at 18 hours, cleared by centrifugation and cell free aliquots were prepared for analysis.

Neutrophils were isolated from citrated human blood obtained from healthy adult donors. Red blood cells were sedimented using 6% Dextran BSS (Baxter Healthcare, IL). The leukocyte fraction was layered onto Histopaque-1077 (Sigma, UK) and centrifuged at 1,000rpm/20min. The neutrophil pellet was isolated and washed with serum free RPMI 1640. Cells were cultured at a density of  $2 \times 10^6$  cells/ml in serum free RPMI.

#### 2.4 ELISA

IL-6, sIL-6R and sgp130 were measured using DuoSet ELISA kits (R&D Systems, UK) according to manufacturer's instructions. IL-8 was measured using BD OptiEIA IL-8 ELISA kit (BD, UK) and MCP-1 was measured using the CCL2 ELISA kit (eBioscience, San Diego, CA). BALF was diluted in the range 1:3-1:12.5 v/v for CF and control samples. Post development absorbance was read at 450nm on a Dynex Revelation MRX TC spectrophotometer (Dynex Technologies Ltd., UK).

#### 2.5 Elastase activity assay

Elastase activity was determined as previously described [24]. Briefly, BALF samples were diluted 1:10 or 1:100 in assay activity buffer (0.5M NaCl, 0.1M Tris pH 7.5 with 0.05% Triton X100). A seven point standard curve was created by two fold dilution of NE stock solution from 40nM to 0.625nM. Chromogenic substrate Suc-Ala-Ala-Pro-Val-pNA (Bachem, UK) in DMSO was diluted to 2mM in activity buffer. 100 $\mu$ l of substrate was added to 100 $\mu$ l of standards or samples in a flat bottomed 96-well plate (Elkay, UK). 100 $\mu$ l of activity buffer alone was included as a negative control.

Elastase mediated conversion of the chromogenic substrate was measured using a Dynex Revelation MRX TC spectrophotometer warmed to 30°C and reading every minute at 405nm.

The change in optical density/min in BALF samples were converted to nM Elastase using the standard curve.

## 2.6 *Cleavage assays*

To measure the ability of CF BALF to cleave IL-6, sIL-6R and sgp130, 300ng/ml of recombinant IL-6, sIL-6R or sgp130 was incubated with 20% CF BALF (which had been cleared by centrifugation at 13,000 rpm/1 min) in Tris buffered saline (TBS; 154mM NaCl, 50mM Tris) for the indicated times at 37°C. For analysis of the proteolytic activity of purified proteases towards sIL-6R, 300ng/ml recombinant sIL-6R was incubated with the indicated concentrations each of purified NE, CG or PR3 in TBS for the indicated times at 37°C. Where specific protease inhibitors were used, CF BALF was pre-treated with the indicated concentrations of 1, 10-phenanthroline, GM6001, PMSF or AAT for 30 mins on ice prior to the addition of recombinant proteins. All samples were diluted with non-reducing Laemmli buffer and boiled for 3 min. Antigens were detected by SDS-PAGE followed by Western blotting.

## 2.7 *SDS-PAGE and Western blotting*

Samples containing IL-6, sIL-6R and sgp130 were resolved on 20% and 15% and 10% polyacrylamide gels respectively. Proteins were transferred to nitrocellulose membranes by electroblotting, blocked in PBS/5% milk for 30 min and incubated with goat anti-IL-6 (Peprotech), goat anti-IL-6R (R&D Systems) or rabbit anti-gp130 (Santa Cruz Biotechnology Inc., CA) for 1 hour at room temperature or overnight at 4°C. Blots were washed sequentially with PBS/0.05% Tween and PBS followed by incubation with HRPO-conjugated secondary antibodies against goat or rabbit IgG (Jackson ImmunoResearch, UK) for 1 hour at room temperature. Blots were again washed and antigens were detected using Kodak X-OMAT

film with ECL substrate (Pierce Biotechnology, IL). Blots were scanned and brightness and contrast adjusted using Adobe Photoshop. For clarity of presentation the order of individual lanes in figure 3C and 4B have been rearranged. Densitometry was performed on scanned blots using Image J (v1.37) software.

### 2.8 *Measurement of apoptosis*

Neutrophil apoptosis was assessed by flow cytometry on a BD FACSCalibur (Becton Dickinson, MA, USA) using Annexin V-PE (MBL, MA) and ToPro-3 (Invitrogen, UK) at the indicated timepoints. Supernatants were harvested at the indicated time points, centrifuged and cell free aliquots stored at -20°C for future analysis.

### 2.9 *Statistics*

Statistics were calculated using GraphPad Prism (v5). Unless otherwise indicated, differences of the medians of non-parametric data were analysed using either Mann-Whitney (for two groups) or the Kruskal-Wallis test with Dunn's multiple comparison test (for more than two groups). For the presentation of data with zero values on graphs with log scales in figures 1C and 2A and B, data were transposed by the addition of 0.1 (ELISA data) or 0.0001 (PMN data); statistics were calculated with non-transposed data.

### 3. RESULTS

#### 3.1 *Inflammatory markers are elevated in CF BALF compared to controls.*

Total and differential cell counts were enumerated in BALF specimens from CF and control patients. Total cell counts were elevated 4 fold in BALF from CF patients compared to controls (CF:  $1.4 \times 10^6$  cells/ml; IQR: 0.54-3.0 v. Controls:  $0.35 \times 10^6$  cells/ml; IQR: 0.12-0.49), ( $p < 0.001$ ), (Figure 1A). Elevated cell counts in CF were dominated by neutrophils which were elevated 89 fold compared to controls (CF:  $0.62 \times 10^6$  cells/ml; IQR: 0.16-2.2 v. Controls  $0.007 \times 10^6$  cells/ml; IQR: 0.0008-0.01), ( $p < 0.001$ ), (Figure 1B). MNCs were more modestly elevated by 3.5 fold in CF (CF:  $0.34 \times 10^6$  cells/ml; IQR: 0.1-0.65 v. Controls  $0.096 \times 10^6$  cells/ml; IQR: 0.02-0.25), ( $p < 0.05$ ), (Figure 1C). The expression of key chemokines was measured in 32 CF BALF specimens and 9 controls. Elevated PMN counts in CF BALF were mirrored in expression of the neutrophil chemoattractant IL-8 which was elevated 17.5 fold compared to controls (CF: 1122 pg/ml; IQR: 323-2133 v. Controls 64 pg/ml; IQR: 46-691), ( $p < 0.01$ ), (Figure 1D). The mononuclear cell chemoattractant, MCP-1 was also elevated in CF BALF, albeit more modestly at 3.8 fold (CF: 692 pg/ml; IQR: 278-1128 v. Controls 182 pg/ml; IQR: 110-345), ( $p < 0.05$ ), (Figure 1E).

#### 3.2 *Expression of IL-6 and its soluble receptors sIL-6R and sgp130 in BALF from CF patients*

Expression of IL-6, sIL-6R and sgp130 is upregulated in many inflammatory diseases but has not been examined in CF to date. In this study, modest quantities of IL-6 were detected in 22/32 CF samples (median concentration 50pg/ml; IQR: 0-74) but were nevertheless elevated compared to controls ( $p < 0.01$ ) where IL-6 was detected in only one sample (Figure 2A). Levels of sIL-6R in BALF samples from CF patients were not significantly different from those observed in BALF from control patients (median concentration for CF 456pg/ml; IQR:

228-704, median concentration for controls 349pg/ml; IQR: 162-522), (Figure 2B). sIL-6R was detected in all samples tested, except one CF sample. Similar to sIL-6R, sgp130 levels in CF patients were not significantly different to control patients (median concentration for CF 6210pg/ml; IQR: 4196-8473, median concentration for controls 5056pg/ml; IQR: 4153-6317), (Figure 2C). sgp130 was detected in all samples tested.

### 3.3 BALF from CF patients degrades IL-6, sIL-6R and sgp130

Unregulated protease activity is well described in the lungs of CF patients and we hypothesised that proteolytic destruction of IL-6 trans-signalling molecules may explain the low levels observed in CF BALF. Initially the concentration of NE in BALF was assessed as a marker of unregulated proteolytic activity. Free NE activity was found in 18/32 CF BALF samples analysed (median activity in positive samples: 81nM, IQR: 37-643nM). In the 21 CF BALF samples where accurate differential counts were available, NE activity was associated with greater numbers of PMN in BALF ( $p < 0.01$ ) (Figure 3A). NE activity was not detected in any of the control BALF samples. To test the ability of CF BALF to degrade IL-6, sIL-6R and sgp130, 300ng/ml of each molecule was added to a 20% BALF solution in TBS and samples were incubated at 37°C for 60 min. Samples were analysed by Western blot to assess antigen loss after 60 min. The capacity to degrade IL-6, sIL-6R and sgp130 was associated with NE activity in BALF samples (Figures 3B, C and D). Densitometry was performed on blots to calculate antigen loss for IL-6 and sIL-6R over this period. For both antigens, BALF samples with detectable NE contained significantly greater degradative activity when compared to CF BALF samples without NE activity (IL-6;  $p < 0.001$ , sIL-6R;  $p < 0.01$ ) or control BALF samples (IL-6;  $p < 0.05$ , sIL-6R;  $p < 0.001$ ), (Figures 3E and F).

### 3.4 Degradation of IL-6 and sIL-6R is due to serine proteases in CF BALF

Matrix metalloproteases (MMPs) and neutrophil derived serine proteases (NSPs) are two major families of proteolytic enzymes participating in CF lung pathology. To investigate whether either of these enzyme groups were responsible for the observed degradation of IL-6 and sIL-6R we tested the ability of a number of protease inhibitors to protect exogenously added proteins from degradation by CF BALF. Serine protease inhibitors AAT (50µg/ml) and PMSF (2mM) both protected IL-6 and sIL-6R from degradation by CF BALF (Figure 4A and B). By contrast the broad spectrum matrix metalloproteinase (MMP) inhibitors GM6001 (25µM) and 1,10-phenanthroline (5mM) did not offer any protection in parallel experiments when used at doses previously shown to effectively inhibit MMPs *in vitro* [25, 26].

### 3.5 Supernatants from *in vitro* aged neutrophils contain free elastase activity and degrade sIL-6R *in vitro*.

The CF lung contains large numbers of neutrophils. Previous studies have shown that these cells are a major source of sIL-6R during inflammatory responses. However, in diseases such as CF, large numbers of necrotic neutrophils persist in the lung, releasing serine proteases into the local airway. We addressed the capacity of protease release under these circumstances to degrade sIL-6R. Neutrophils isolated from the blood of normal healthy volunteers were cultured for up to 24 hours. Cell viability was assessed by flow cytometry using Annexin-V/ToPro3 at 0, 6, 12, and 24 hours and supernatants were collected to determine NE activity and capacity to degrade sIL-6R. As expected, cultured neutrophils moved from a predominantly viable phenotype (annexinV<sup>-</sup>/ToPro3<sup>-</sup>) to an apoptotic phenotype (annexinV<sup>+</sup>/ToPro3<sup>-</sup>) over the first 12h progressing to a late apoptotic/necrotic phenotype (annexinV<sup>+</sup>/ToPro3<sup>+</sup>) by 24h (Figure 5A). Supernatants from these cells were analysed for NE activity. NE activity in these samples increased over the 24 hour period indicating the

release of NE by neutrophils as they progress through apoptosis to necrosis (Figure 5B). The ability of these supernatants to degrade sIL-6R was tested. As predicted from the NE activity in these samples, degradation of exogenously added sIL-6R was observed in a time dependent manner (Figure 5C). Interestingly, not all supernatants from these experiments were capable of degrading sIL-6R which may reflect the wide range of NE activity detected in these supernatants and the relatively low level of NE activity released from these neutrophils relative to that seen in CF BALF.

*3.6 The three neutrophil derived serine proteases, NE, CG and PR3 degrade sIL-6R in a time and dose dependent manner leading to a loss of activity.*

Neutrophils express three closely related serine proteases, NE, CG and PR3, in a co-ordinated fashion. AAT and PMSF inhibit all three proteases to different extents. The susceptibility of IL-6 to cleavage by all three proteases has previously been shown [27] however we wished to determine the relative ability of each of these proteases to degrade sIL-6R. NE, CG and PR3, were used to treat 300ng/ml sIL-6R. A range of protease concentrations between 15 and 1000nM were used to reflect the original concentration of active NE measured in CF BALF (IQR: 37-643nM, reported in section 3.3). When sIL-6R was measured by Western blot, a time and dose dependent degradation of sIL-6R by all three proteases was evident (Figure 6A). On the basis of molar equivalence, CG was the most potent protease with maximal degradation occurring within 60 minutes at a concentration of 250nM. NE also demonstrated potent activity at 250nM but required up to 4 hours for almost complete degradation of sIL-6R to occur. PR3 was the least active protease with minimal degradation of sIL-6R occurring in the presence of 1000nM protease after 1 hour and complete degradation being observed after 4 hours in the presence of 500nM of protease. As CG was the most potent of the serine proteases in these experiments, its effect on sIL-6R bio-activity was tested. sIL-6R treated

with 500nM CG for 2 hours at 37° was compared to native sIL-6R for its ability to induce MCP-1 production in the A549 alveolar type-II epithelial cell line when combined with IL-6. In this system, IL-6 has a modest impact on MCP-1 production compared to untreated cells (MCP-1 production in response to IL-6 was set at 100% and used to standardise between experiments). sIL-6R when combined with IL-6 enhanced the MCP-1 production (225%, range 187-293%). Treatment of sIL-6R with CG almost completely abolished IL-6 trans-signalling activity in this assay (130%, range 119-143%) production compared to native sIL-6R ( $p=0.038$ ), (Figure 6B).

### 3.7 IL-6 protects sIL-6R from degradation by serine proteases

Ligand-receptor interactions commonly result in conformational changes. We hypothesised that such conformational changes might alter the susceptibility of the IL-6/sIL-6R complex to serine proteases. To test this, 300ng/ml of sIL-6R was incubated with 250nM NE and CG or 500nM PR3 in the presence or absence of IL-6. sIL-6R was modestly protected from degradation in the presence of 300ng/ml IL-6 and almost completely protected from NE and PR3 when 3000ng/ml was used (Figure 7A). IL-6 offered relatively poor protection against CG, although a lower molecular weight form of sIL-6R remained in the presence of higher concentrations of IL-6 (Figure 7A). The ability of IL-6 to protect sIL-6R from degradation by CF BALF, where multiple proteases may be present at the same time, was also tested. In line with our observations with purified proteases, we observed that a 10-fold excess of IL-6 significantly protected sIL-6R from degradation in two of the three samples tested (Figure 7B), indicating that in certain situations this protective effect may operate *in vivo*.

To further investigate the importance of ligand/receptor interactions on susceptibility to protease digestion, we took advantage of HYPER IL-6, a recombinant molecule consisting of a truncated sIL-6R molecule covalently linked to IL-6 by a short peptide [28]. As IL-6 and

sIL-6R are unable to dissociate in this state, we postulated that HYPER IL-6 should also be resistant to serine protease mediated degradation. When exposed to NSPs, HYPER-IL-6 was almost completely protected from degradation by both CG (250 nM) and PR3 (500nM) (Figure 8A) when compared to sIL-6R alone. Following treatment with NE, a distinctive lower molecular weight band was visible by Western blot (Figure 8A). Further analysis of this band revealed that it was immunoreactive with antibodies against both IL-6 and sIL-6R (Figure 8B), indicating that it represented a truncated form of the HYPER-IL-6 molecule retaining components of both IL-6 and sIL-6R.

#### 4. DISCUSSION

We have assessed the expression of IL-6, sIL-6R and the inhibitor of IL-6 trans-signalling, sgp130, in the lungs of patients with CF. Three important observations arise from this study. First, in light of previous reports describing unusually low IL-6 expression in lung secretions from CF patients, we show that NSPs in CF BALF are capable of proteolytically degrading IL-6. Secondly, we have for the first time described the expression of the IL-6 agonist, sIL-6R, and the potent antagonist of IL-6 trans-signalling responses, sgp130 in this patient group; the absence of an upregulated expression profile for these molecules in CF BALF compared to controls was unexpected in light of the evident inflammation, and contrasts with the situation in many other inflammatory diseases. Finally, we have identified neutrophil derived serine protease activity as the major mediator of degradation of IL-6, sIL-6R and sgp130 in the CF lung.

Although this is the first investigation of sIL-6R and sgp130 expression in CF patients, previous reports have described extremely low or undetectable IL-6 in induced sputum from CF [21-23] and others which could detect IL-6 found it to be expressed at levels not significantly different from disease free control patients [29]. Here, the concentration of IL-6 detected in BALF from CF patients, although significantly different from control patients, was much lower than IL-6 values reported in other inflammatory lung diseases such as diffuse bronchiectasis [23], pneumonia [30], asthma [31] and neonatal chronic lung disease [20]. The reported deficiency of IL-6 in CF patients is unlikely to reflect defective IL-6 production, as airway epithelial cells from these patients spontaneously express more IL-6 than healthy control cells [32] and circulating IL-6 can be detected in CF serum at levels which correlate with the acute phase C-reactive protein [33]. Furthermore, as shedding of sIL-6R from the surface of neutrophils and other cells has been identified as a major source of this molecule

during inflammation [1, 11, 34, 35], the low level of sIL-6R in neutrophil dominated CF BALF was unexpected. Likewise, elevated expression of both sIL-6R and sgp130 has been reported in a number of diseases [36] reflecting their importance in modulating the activity of IL-6.

Unregulated proteolytic activity is a known contributor to pathology in the CF lung and is implicated in the reduced expression and activity of a number of important components of the immune response [37]. Targeting of IL-6 by NSPs [27] has previously been suggested as an explanation for the surprisingly low pulmonary expression of IL-6 in CF patients [23] however, this is the first study to show specific proteolysis of this cytokine by serine proteases in CF BALF. We also show that the ability of IL-6 to mediate normal inflammatory responses is further compromised by the destruction of sIL-6R and sgp130 in the CF lung. Degradation of both IL-6 and sIL-6R is prevented by the serine protease inhibitors AAT and PMSF, but not by the pan-MMP inhibitors, GM6001 and 1,10-phenanthroline. Using purified protease preparations, we have shown that sIL-6R is also susceptible to degradation by all three proteases with a hierarchy of activity; CG>NE>PR3. At  $10^2$ - $10^3$  nM, the concentrations of purified NSPs needed to achieve degradation of sIL-6R were broadly similar to the concentrations of active NE detected in the CF BALF specimens ( $10^1$ - $10^3$  nM). This study has not investigated the possibility that proteases found in CF BALF may act upon IL-6 signalling molecules in an additive or synergistic fashion as has previously been shown for NE and CG [38]. However, all three enzymes are found in the same neutrophil azurophilic granules and their expression is similarly regulated. Consequently, the occurrence of free CG and PR3 activity in lung secretions from CF patients mirrors that of NE [39, 40] and so they are likely to be able to act co-operatively upon target substrates. Taken together, these data suggest that all three NSPs participate in IL-6 and sIL-6R degradation in vivo. Data from

A549 cells presented here shows that this degradation is sufficient to functionally inactivate the trans-signalling capacity of sIL-6R and does not merely represent a discrete cleavage event leading to loss of antigenicity with the antibody used for Western blots.

The release of large amounts of NSPs into the extracellular environment is by no means an inevitable feature of neutrophil biology. The majority of NSPs found in the CF lung are released from necrotic neutrophils which persist in the CF lung due to a failure of normal apoptotic cell clearance [41]. In our study, even the relatively modest NE activity released into supernatants from  $2 \times 10^6$  neutrophils is capable of degrading sIL-6R. Interestingly, Chalaris et al. have described shedding of intact sIL-6R as part of the normal programme of neutrophil apoptosis [11]. However in our study, degradation was associated with the emergence of necrotic neutrophils and release of NE. Notably, Chalaris et al. used much shorter culture times than those used here which may have led to the emergence of a predominantly apoptotic phenotype in that study. Such cells are likely to have limited NSP exposure compared to the conditions used here, particularly the longer culture times and serum free conditions used to more closely reflect the protease/anti-protease imbalance prevalent in the CF lung [42]. It is also important to point out that CF neutrophils, in contrast to neutrophils from healthy donors used here, exhibit accentuated NE secretion and an exaggerated activation profile [43, 44] which may result in more profound degradation by these cells.

Proteases such as the MMPs have well acknowledged roles in influencing normal and pathological inflammatory responses through the activation and inactivation of inflammatory mediators [45]. Even during well regulated inflammatory responses in otherwise healthy individuals, limited exposure to free or cell associated NSP activity is likely to occur. It is

possible that the NSP mediated destruction of IL-6 trans-signalling molecules described here is merely a pathological manifestation of an otherwise important role played by these enzymes in shaping and regulating normal inflammatory responses. In this scenario, complexes formed between IL-6 and sIL-6R may significantly prolong the activity of IL-6 during inflammatory responses. The ability of excess IL-6 to protect sIL-6R from degradation by NE and PR3 is particularly interesting. This contrasts with a previous study which described the inactivation of IL-6 by inflamed cerebrospinal fluid [27] where a complex between IL-6 and sIL-6R was shown to protect IL-6 from inactivation by CG but not NE or PR3. Here we observed that conversely, IL-6 offers relatively poor protection to sIL-6R against CG. Furthermore, the sIL-6R which remains following exposure to CG in the presence of IL-6 was of a slightly lower molecular weight (as in figure 7A), suggesting that even when IL-6 confers protection, limited truncation of sIL-6R still occurs. The potency of CG towards IL-6/sIL-6R complexes may be explained by previous data which show that CG can attack IL-6 at the Phe<sup>78</sup>-Asn<sup>79</sup> bond [27]. As this site is crucial for interactions with sIL-6R [46, 47], damage here may preclude substantial interactions with sIL-6R leading to relatively ineffective protection against this NSP. Although not addressed here, it is possible that sgp130 may also play a protective role in complex with IL-6/sIL-6R and may itself be protected from the proteolysis we have described in this study. In CF, it is likely that the overwhelming NSP activity would leave little scope for secreted IL-6 or liberated sIL-6R to form protective complexes, perhaps explaining why we see so little of these molecules in CF BALF. Interestingly however, the recombinant IL-6/sIL-6R fusion molecule, HYPER-IL-6, displayed enhanced resistance to all proteases tested including CG. As the Phe<sup>78</sup>-Asn<sup>79</sup> bond of IL-6 is likely to be in close proximity to sIL-6R in this molecule, CG may be unable to attack the complex. We also noted that HYPER-IL-6 was susceptible to NE proteolysis, leading to the formation of a truncated molecule retaining components of both IL-6 and sIL-

6R. This may reflect altered activity of NE towards the shortened sIL-6R sequence used to express HYPER-IL-6 [28]. Given the discrete cleavage pattern of HYPER-IL-6 in response to NE, it is likely that completely protease resistant versions of this molecule could be engineered. The development of protease resistant molecules which can regulate leukocyte infiltration *in vivo* may offer an attractive avenue for therapeutic intervention.

In conclusion, this study has described a functional deficiency of the IL-6 trans-signalling pathway in the lungs of patients with CF. This is the first analysis of the complete IL-6 trans-signalling pathway in CF patients and is the first description of a deficiency of this pathway in any population. These findings have a number of important implications. Firstly, experimental blockade of IL-6 trans-signalling in murine models of peritoneal inflammation results in exaggerated and protracted neutrophil infiltration as well as reduced neutrophil apoptosis [1, 12]. It is intriguing to suggest that trans-signalling may similarly regulate inflammation in the lung, particularly since intra-tracheal administration of IL-6 has been shown to attenuate LPS induced neutrophil infiltration [2]. In this scenario, it is possible that a deficiency of IL-6 trans-signalling may partially explain the protracted and persistent neutrophil recruitment in lungs of CF patients. Secondly, the identification of serine protease activity as the underlying cause of this deficiency and the identification of enhanced resistance to proteolysis in the recombinant IL-6/sIL-6R fusion protein further supports the use of protease inhibitors such as AAT in the treatment of CF and opens the possibility of future therapeutic strategies using recombinant protease resistant molecules such as HYPER IL-6 to break the cycle of inflammatory cell recruitment in these patients.

**5. ACKNOWLEDGMENTS:** This study was partly supported by a grant from the Harold Hyam Wingate Foundation (EPMG). SK and PLD have received funding from Arriva Pharmaceuticals Inc.

ACCEPTED MANUSCRIPT

## 6. REFERENCES

- [1] S.M. Hurst, T.S. Wilkinson, R.M. McLoughlin, S. Jones, S. Horiuchi, N. Yamamoto, S. Rose-John, G.M. Fuller, N. Topley, S.A. Jones, IL-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation, *Immunity*, 14 (2001) 705-714.
- [2] T.R. Ulich, S. Yin, K. Guo, E.S. Yi, D. Remick, J. del Castillo, Intratracheal injection of endotoxin and cytokines. II. Interleukin-6 and transforming growth factor beta inhibit acute inflammation, *Am J Pathol*, 138 (1991) 1097-1101.
- [3] A. Mackiewicz, H. Schooltink, P.C. Heinrich, S. Rose-John, Complex of soluble human IL-6-receptor/IL-6 up-regulates expression of acute-phase proteins, *J Immunol*, 149 (1992) 2021-2027.
- [4] T. Taga, M. Hibi, Y. Hirata, K. Yamasaki, K. Yasukawa, T. Matsuda, T. Hirano, T. Kishimoto, Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130, *Cell*, 58 (1989) 573-581.
- [5] J. Mullberg, H. Schooltink, T. Stoyan, M. Gunther, L. Graeve, G. Buse, A. Mackiewicz, P.C. Heinrich, S. Rose-John, The soluble interleukin-6 receptor is generated by shedding, *Eur J Immunol*, 23 (1993) 473-480.
- [6] S. Horiuchi, Y. Koyanagi, Y. Zhou, H. Miyamoto, Y. Tanaka, M. Waki, A. Matsumoto, M. Yamamoto, N. Yamamoto, Soluble interleukin-6 receptors released from T cell or granulocyte/macrophage cell lines and human peripheral blood mononuclear cells are generated through an alternative splicing mechanism, *Eur J Immunol*, 24 (1994) 1945-1948.
- [7] J.A. Lust, K.A. Donovan, M.P. Kline, P.R. Greipp, R.A. Kyle, N.J. Maihle, Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor, *Cytokine*, 4 (1992) 96-100.
- [8] S. Rose-John, P.C. Heinrich, Soluble receptors for cytokines and growth factors: generation and biological function, *Biochem J*, 300 ( Pt 2) (1994) 281-290.
- [9] S.A. Jones, P.J. Richards, J. Scheller, S. Rose-John, IL-6 transsignaling: the in vivo consequences, *J Interferon Cytokine Res*, 25 (2005) 241-253.
- [10] M. Peters, S. Jacobs, M. Ehlers, P. Vollmer, J. Mullberg, E. Wolf, G. Brem, K.H. Meyer zum Buschenfelde, S. Rose-John, The function of the soluble interleukin 6 (IL-6) receptor in vivo: sensitization of human soluble IL-6 receptor transgenic mice towards IL-6 and prolongation of the plasma half-life of IL-6, *J Exp Med*, 183 (1996) 1399-1406.
- [11] A. Chalaris, B. Rabe, K. Paliga, H. Lange, T. Laskay, C.A. Fielding, S.A. Jones, S. Rose-John, J. Scheller, Apoptosis is a natural stimulus of IL6R shedding and contributes to the proinflammatory trans-signaling function of neutrophils, *Blood*, 110 (2007) 1748-1755.
- [12] R.M. McLoughlin, S.M. Hurst, M.A. Nowell, D.A. Harris, S. Horiuchi, L.W. Morgan, T.S. Wilkinson, N. Yamamoto, N. Topley, S.A. Jones, Differential regulation of neutrophil-activating chemokines by IL-6 and its soluble receptor isoforms, *J Immunol*, 172 (2004) 5676-5683.
- [13] R.M. McLoughlin, J. Witowski, R.L. Robson, T.S. Wilkinson, S.M. Hurst, A.S. Williams, J.D. Williams, S. Rose-John, S.A. Jones, N. Topley, Interplay between IFN-gamma and IL-6 signaling governs neutrophil trafficking and apoptosis during acute inflammation, *J Clin Invest*, 112 (2003) 598-607.
- [14] T.Z. Khan, J.S. Wagoner, T. Bost, J. Martinez, F.J. Accurso, D.W. Riches, Early pulmonary inflammation in infants with cystic fibrosis, *Am J Respir Crit Care Med*, 151 (1995) 1075-1082.
- [15] M.W. Konstan, K.A. Hilliard, T.M. Norvell, M. Berger, Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation, *Am J Respir Crit Care Med*, 150 (1994) 448-454.

- [16] M.S. Muhlebach, P.W. Stewart, M.W. Leigh, T.L. Noah, Quantitation of inflammatory responses to bacteria in young cystic fibrosis and control patients, *Am J Respir Crit Care Med*, 160 (1999) 186-191.
- [17] D.R. Koehler, G.P. Downey, N.B. Sweezey, A.K. Tanswell, J. Hu, Lung inflammation as a therapeutic target in cystic fibrosis, *Am J Respir Cell Mol Biol*, 31 (2004) 377-381.
- [18] E. Bucchioni, S.A. Kharitonov, L. Allegra, P.J. Barnes, High levels of interleukin-6 in the exhaled breath condensate of patients with COPD, *Respir Med*, 97 (2003) 1299-1302.
- [19] C.E. Hack, E.R. De Groot, R.J. Felt-Bersma, J.H. Nuijens, R.J. Strack Van Schijndel, A.J. Eerenberg-Belmer, L.G. Thijs, L.A. Aarden, Increased plasma levels of interleukin-6 in sepsis, *Blood*, 74 (1989) 1704-1710.
- [20] S. Kotecha, L. Wilson, A. Wangoo, M. Silverman, R.J. Shaw, Increase in interleukin (IL)-1 beta and IL-6 in bronchoalveolar lavage fluid obtained from infants with chronic lung disease of prematurity, *Pediatr Res*, 40 (1996) 250-256.
- [21] C. Colombo, D. Costantini, A. Rocchi, L. Cariani, M.L. Garlaschi, S. Tirelli, G. Calori, E. Copreni, M. Conese, Cytokine levels in sputum of cystic fibrosis patients before and after antibiotic therapy, *Pediatr Pulmonol*, 40 (2005) 15-21.
- [22] C.L. Ordonez, A.I. Kartashov, M.E. Wohl, Variability of markers of inflammation and infection in induced sputum in children with cystic fibrosis, *J Pediatr*, 145 (2004) 689-692.
- [23] E. Osika, J.M. Cavallion, K. Chadelat, M. Boule, C. Fitting, G. Tournier, A. Clement, Distinct sputum cytokine profiles in cystic fibrosis and other chronic inflammatory airway disease, *Eur Respir J*, 14 (1999) 339-346.
- [24] P.L. Davies, N.C. Maxwell, S. Kotecha, O.B. Spiller, Monoclonal anti-neutrophil elastase antibody characterisation: ability to block function, detect free versus serpin-complexed enzyme and stain intracellular granules, *J Immunol Methods*, 336 (2008) 175-182.
- [25] G. Mautino, N. Oliver, P. Chanez, J. Bousquet, F. Capony, Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics, *Am J Respir Cell Mol Biol*, 17 (1997) 583-591.
- [26] B.C. Sondergaard, K. Henriksen, H. Wulf, S. Oestergaard, U. Schurigt, R. Brauer, I. Danielsen, C. Christiansen, P. Qvist, M.A. Karsdal, Relative contribution of matrix metalloprotease and cysteine protease activities to cytokine-stimulated articular cartilage degradation, *Osteoarthritis Cartilage*, 14 (2006) 738-748.
- [27] U. Bank, B. Kupper, D. Reinhold, T. Hoffmann, S. Ansorge, Evidence for a crucial role of neutrophil-derived serine proteases in the inactivation of interleukin-6 at sites of inflammation, *FEBS Lett*, 461 (1999) 235-240.
- [28] M. Fischer, J. Goldschmitt, C. Peschel, J.P. Brakenhoff, K.J. Kallen, A. Wollmer, J. Grotzinger, S. Rose-John, I. A bioactive designer cytokine for human hematopoietic progenitor cell expansion, *Nat Biotechnol*, 15 (1997) 142-145.
- [29] T.L. Bonfield, J.R. Panuska, M.W. Konstan, K.A. Hilliard, J.B. Hilliard, H. Ghnaim, M. Berger, Inflammatory cytokines in cystic fibrosis lungs, *Am J Respir Crit Care Med*, 152 (1995) 2111-2118.
- [30] C. Monton, A. Torres, M. El-Ebiary, X. Filella, A. Xaubet, J.P. de la Bellacasa, Cytokine expression in severe pneumonia: a bronchoalveolar lavage study, *Crit Care Med*, 27 (1999) 1745-1753.
- [31] J.C. Virchow, Jr., C. Kroegel, C. Walker, H. Matthys, Inflammatory determinants of asthma severity: mediator and cellular changes in bronchoalveolar lavage fluid of patients with severe asthma, *J Allergy Clin Immunol*, 98 (1996) S27-33; discussion S33-40.
- [32] T.L. Bonfield, M.W. Konstan, M. Berger, Altered respiratory epithelial cell cytokine production in cystic fibrosis, *J Allergy Clin Immunol*, 104 (1999) 72-78.
- [33] L.S. Nixon, B. Yung, S.C. Bell, J.S. Elborn, D.J. Shale, Circulating immunoreactive interleukin-6 in cystic fibrosis, *Am J Respir Crit Care Med*, 157 (1998) 1764-1769.

- [34] M.I. Gomez, S.H. Sokol, A.B. Muir, G. Soong, J. Bastien, A.S. Prince, Bacterial induction of TNF-alpha converting enzyme expression and IL-6 receptor alpha shedding regulates airway inflammatory signaling, *J Immunol*, 175 (2005) 1930-1936.
- [35] S.A. Jones, S. Horiuchi, N. Topley, N. Yamamoto, G.M. Fuller, The soluble interleukin 6 receptor: mechanisms of production and implications in disease, *Faseb J*, 15 (2001) 43-58.
- [36] S. Rose-John, J. Scheller, G. Elson, S.A. Jones, Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer, *J Leukoc Biol*, 80 (2006) 227-236.
- [37] C.T. Pham, Neutrophil serine proteases: specific regulators of inflammation, *Nat Rev Immunol*, 6 (2006) 541-550.
- [38] C. Boudier, C. Holle, J.G. Bieth, Stimulation of the elastolytic activity of leukocyte elastase by leukocyte cathepsin G, *J Biol Chem*, 256 (1981) 10256-10258.
- [39] F. Rubio, J. Cooley, F.J. Accurso, E. Remold-O'Donnell, Linkage of neutrophil serine proteases and decreased surfactant protein-A (SP-A) levels in inflammatory lung disease, *Thorax*, 59 (2004) 318-323.
- [40] V. Witko-Sarsat, L. Halbwachs-Mecarelli, A. Schuster, P. Nusbaum, I. Ueki, S. Canteloup, G. Lenoir, B. Descamps-Latscha, J.A. Nadel, Proteinase 3, a potent secretagogue in airways, is present in cystic fibrosis sputum, *Am J Respir Cell Mol Biol*, 20 (1999) 729-736.
- [41] R.W. Vandivier, V.A. Fadok, P.R. Hoffmann, D.L. Bratton, C. Penvari, K.K. Brown, J.D. Brain, F.J. Accurso, P.M. Henson, Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis, *J Clin Invest*, 109 (2002) 661-670.
- [42] P. Birrer, N.G. McElvaney, A. Rudeberg, C.W. Sommer, S. Liechti-Gallati, R. Kraemer, R. Hubbard, R.G. Crystal, Protease-antiprotease imbalance in the lungs of children with cystic fibrosis, *Am J Respir Crit Care Med*, 150 (1994) 207-213.
- [43] S. Brockbank, D. Downey, J.S. Elborn, M. Ennis, Effect of cystic fibrosis exacerbations on neutrophil function, *Int Immunopharmacol*, 5 (2005) 601-608.
- [44] C. Taggart, R.J. Coakley, P. Grealley, G. Canny, S.J. O'Neill, N.G. McElvaney, Increased elastase release by CF neutrophils is mediated by tumor necrosis factor-alpha and interleukin-8, *Am J Physiol Lung Cell Mol Physiol*, 278 (2000) L33-41.
- [45] A.M. Manicone, J.K. McGuire, Matrix metalloproteinases as modulators of inflammation, *Semin Cell Dev Biol*, 19 (2008) 34-41.
- [46] M. Ehlers, J. Grotzinger, M. Fischer, H.K. Bos, J.P. Brakenhoff, S. Rose-John, Identification of single amino acid residues of human IL-6 involved in receptor binding and signal initiation, *J Interferon Cytokine Res*, 16 (1996) 569-576.
- [47] M. Kalai, F.A. Montero-Julian, J. Grotzinger, V. Fontaine, P. Vandebussche, R. Deschuyteneer, A. Wollmer, H. Brailly, J. Content, Analysis of the human interleukin-6/human interleukin-6 receptor binding interface at the amino acid level: proposed mechanism of interaction, *Blood*, 89 (1997) 1319-1333.

**FIGURE LEGENDS****Figure 1:** *Total and differential cell counts in BALF from CF and control patients:*

A) Total cell counts were available in BALF from 26 CF and 9 control patients. B) and C) Cytospins prepared from BALF from CF and control patients were stained with Wright-Giemsa and used to enumerate PMN and MNC numbers in each BALF sample following light microscopy. Relative numbers of PMNs, MNCs and other cell types were counted in all available samples. Cell counts were used to calculate total PMN and MNC numbers in the original BALF sample. Differential cell counts were available in 21 CF patients and 7 controls. D) IL-8 and E) MCP-1 concentrations were measured in 32 BALF from samples from CF patients and 9 BALF samples from control patients using ELISA. Horizontal lines indicate medians. Horizontal lines indicate medians. \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ .

**Figure 2:** *IL-6, sIL-6R and sgp130 expression in BALF from CF and control patients.* A) IL-6, B) sIL-6R and C) sgp130 concentrations were measured in 32 BALF from samples from CF patients and 9 BALF samples from control patients using ELISA. Horizontal lines indicate medians. \*\* =  $p < 0.01$ .

**Figure 3:** *Neutrophil elastase present in CF BALF is associated with degradation of IL-6, sIL-6R and sgp130.* A) In the 21 CF BALF samples where accurate differential cell counts were available, NE activity was more prevalent in samples with high PMN counts ( $p < 0.01$ ). B), C) and D) CF BALF samples with high NE activity (1,2 & 3) or no detectable activity (4, 5 & 6) were tested for their ability to degrade IL-6, sIL-6R and sgp130. BALF was spiked with 300ng/ml IL-6, sIL-6R or sgp130 and antigen recovery was measured at 0 and 60 minutes by Western blot using a specific polyclonal antibody. Control reactions in the absence of BALF are depicted in the first lane (No BALF). E) and F) All available BALF samples were tested for their ability to degrade IL-6 and sIL-6R by Western blot. % Antigen loss was calculated following densitometry of scanned blots. CF BALF with active elastase possessed significantly greater degradative activity compared to either NE negative samples (IL-6:  $p < 0.001$ , sIL-6R:  $p < 0.01$ ) or non-CF control samples (IL-6:  $p < 0.05$ , sIL-6R:  $p < 0.001$ ) Data presented in panel C are from separate experiments which were each individually controlled but are presented in this way to allow comparison with panels B and D. (+ve, positive; -ve, negative).

**Figure 4:** *The ability of CF BALF to degrade IL-6 and sIL-6R is inhibited by serine protease inhibitors.* CF BALF with confirmed degradative activity towards IL-6 and sIL-6R was treated with the MMP inhibitors 1,10-phenanthroline (5mM) or GM6001 (25µM) or the serine protease inhibitors PMSF (2mM) or AAT (50µg/ml) or left untreated. Samples were subsequently spiked with 300ng/ml IL-6 (panel A) or sIL-6R (panel B) and antigen recovery was measured by Western blot at 0 and 60 minutes. Data shown are representative of 3 independent experiments with BALF from 3 separate CF patients. The order of lanes in panel B has been rearranged to allow easier comparison with panel A.

**Figure 5:** *Ageing neutrophils release NE and are capable of degrading sIL-6R.*

A) Neutrophils isolated from the blood of healthy adult donors were cultured at a density of  $2 \times 10^6$  cells/ml in serum free RPMI-1640. Cellular viability was measured by flow cytometry at 0, 6, 12 and 24h using Annexin-V and ToPro-3. Neutrophils became progressively apoptotic (Annexin-V+/ToPro-3-) over the 24 hour time course, with late apoptotic/necrotic cells (Annexin-V+/ToPro-3+) emerging as soon as 6 hours of culture. B) NE activity was measured in the supernatants of these cells collected at each of the timepoints. NE activity was evident as soon as 6 hours of culture, reaching a peak at 24 hours and mirroring the presence of necrotic cells in the culture. C) Supernatants harvested from neutrophils at each of the timepoints were spiked with 150ng/ml sIL-6R. sIL-6R spiked supernatants were incubated at 37°C for 60 minutes and sIL-6R antigenicity was measured by Western blot. Supernatants harvested at 12 and 24 hours degraded sIL-6R in two of the four donors.

**Figure 6:** *Purified neutrophil derived serine proteases degrade sIL-6R in a dose and time dependent manner.* A) 300ng/ml sIL-6R was incubated with a range of concentrations (15nm-1000nM) of purified NE, CG or PR3. Samples were incubated at 37°C for 0, 60, 120 and 240 min. Integrity of sIL-6R was measured by Western blot using a specific polyclonal antibody. Western blots are representative of at least three independent experiments. B) 300ng/ml sIL-6R was treated with or without 500nM CG for 120 min at 37°C. 50µg/ml AAT was used to neutralise serine protease activity prior to the addition of 300ng/ml IL-6. Stimuli were diluted 1/10 into serum free DMEM which was used to stimulate A549 cells for 18 hours. Control stimulations with IL-6, CG or sIL-6R in the presence of AAT were also performed. IL-6 trans-signalling activity was determined by measuring MCP-1 production

by ELISA. Means and standard deviations for four independent experiments are presented. Data are presented as % MCP-1 expression compared to IL-6 alone (mean MCP-1 expression in response to IL-6 = 1146pg/ml). CG treatment of sIL-6R significantly inhibited its ability to induce the expression of MCP-1 by A549 cells.

**Figure 7:** *IL-6 protects sIL-6R from proteolytic degradation by NSPs and CF BALF.*

A) 300ng/ml sIL-6R was incubated with 250nM CG or NE or 500nM PR3 for 60 min at 37°C in the presence or absence of 300ng/ml or 3µg/ml IL-6 or BSA. sIL-6R antigen recovery was measured by Western blot using a specific polyclonal antibody. 3µg/ml IL-6 offered significant protection against all three NSPs, but most significantly against NE and PR3. No protection was observed when BSA was used at 300ng/ml or 3µg/ml. Western blots are representative of at least three independent experiments. B) 300ng/ml sIL-6R in the presence or absence of 3µg/ml IL-6 was incubated for 60 minutes at 37°C with 20% CF BAL fluid previously shown to contain high NE activity. sIL-6R recovery in all samples was measured by Western blot using a specific polyclonal antibody. Western blots are representative of at least three independent experiments.

**Figure 8:** *The recombinant IL-6/sIL-6R fusion molecule HYPER-IL-6 is resistant to NSP mediated degradation.*

A) 300ng/ml sIL-6R (left panel) or HYPER-IL-6 (right panel) was exposed to 250nM CG or NE or 500nM PR3 for 120 min at 37°C. Antigen integrity was measured by Western blot using antibodies against sIL-6R in samples taken at 0 and 120 minutes. One of three independent experiments is presented. B) HYPER-IL-6 was treated with or without NSPs as described in experiment A. Reactivity with antibodies against sIL-6R or IL-6 was tested in samples taken at 0 and 120 minutes using specific antibodies against sIL-6R (left panel) or IL-6 (right panel). One of three independent experiments is presented.

Figure 1

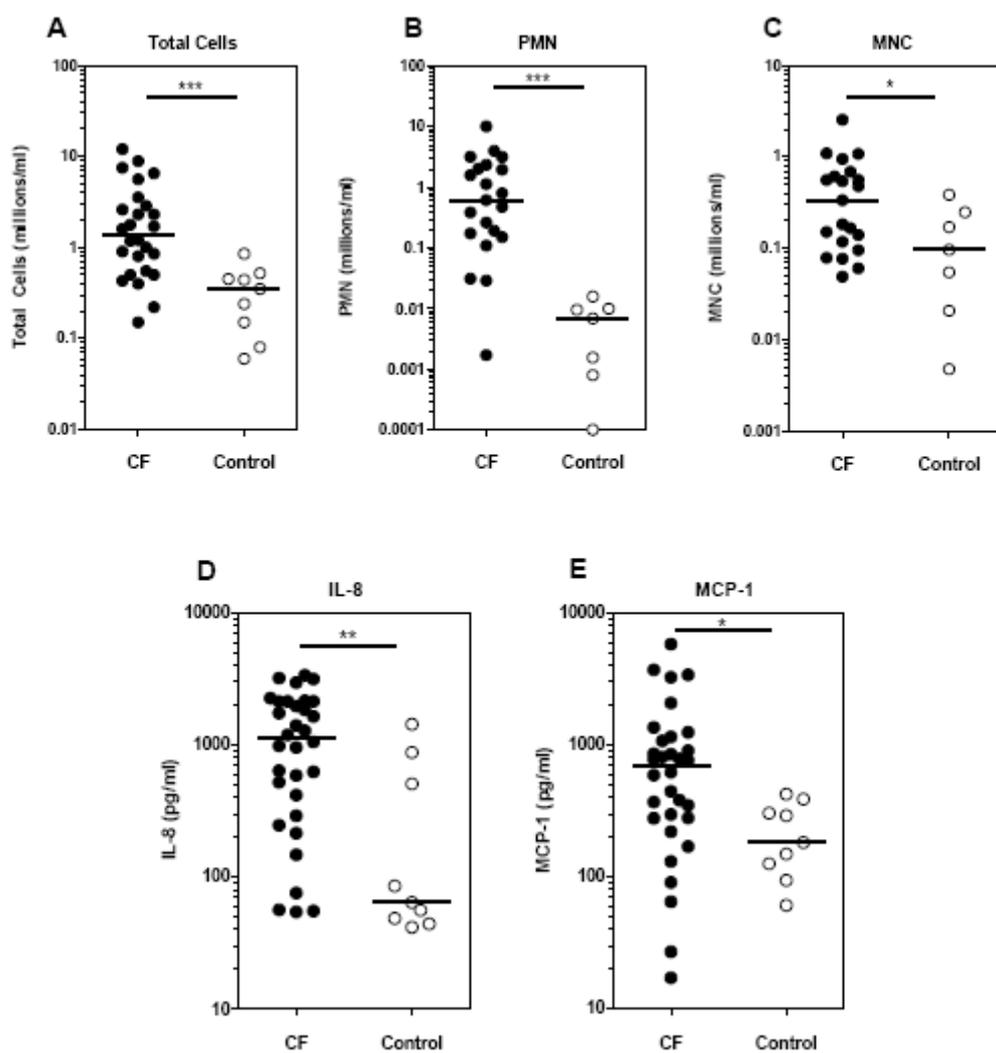


Figure 2

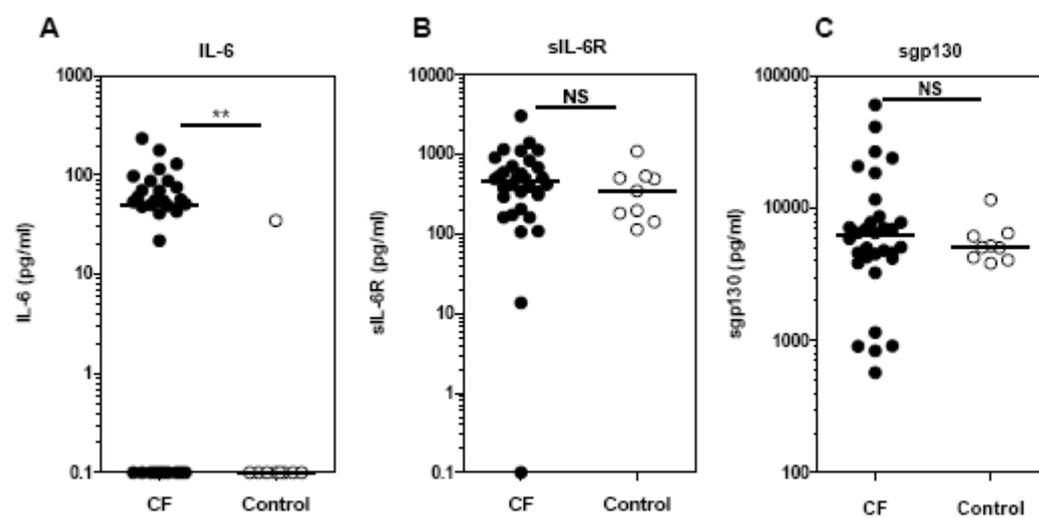


Figure 3

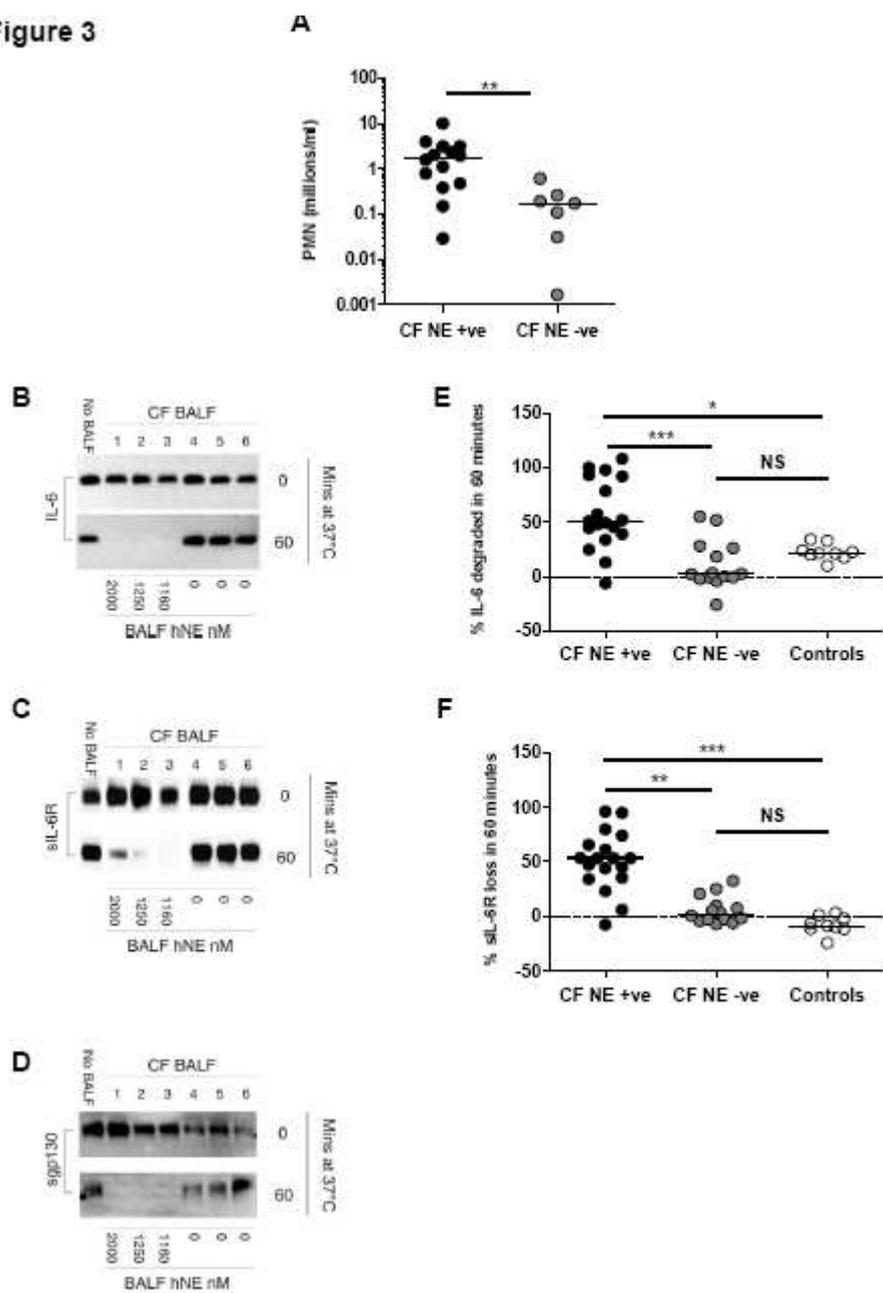


Figure 4

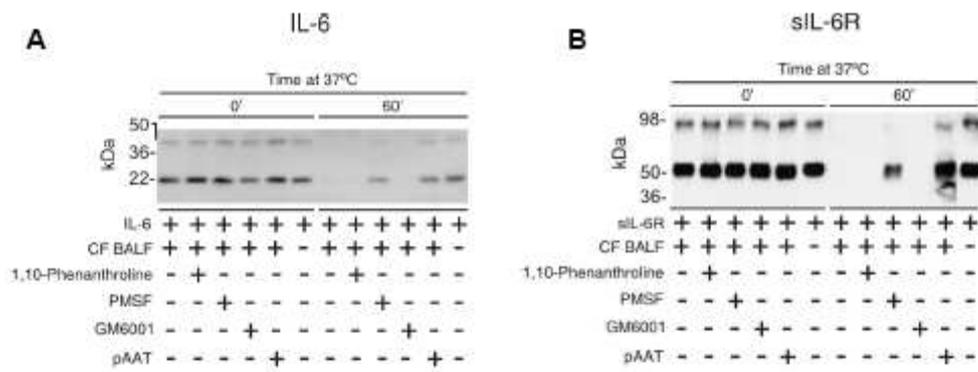


Figure 5

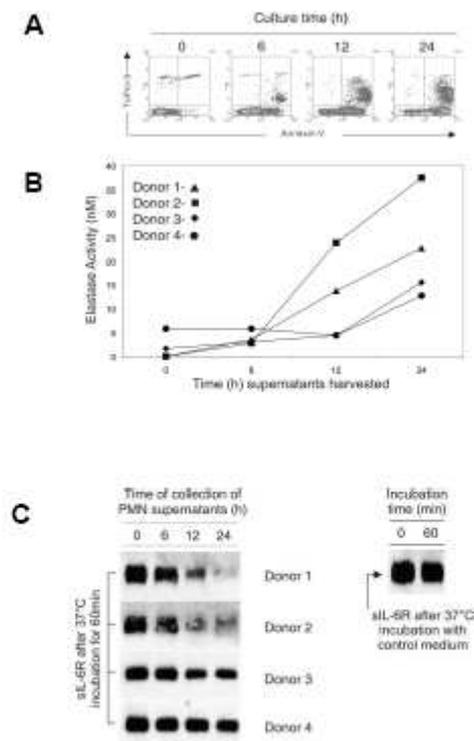


Figure 6

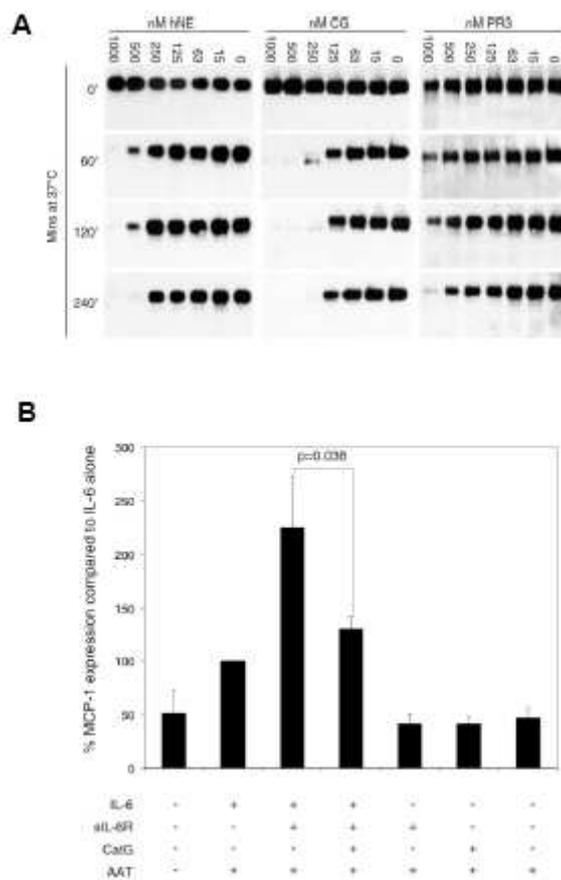


Figure 7

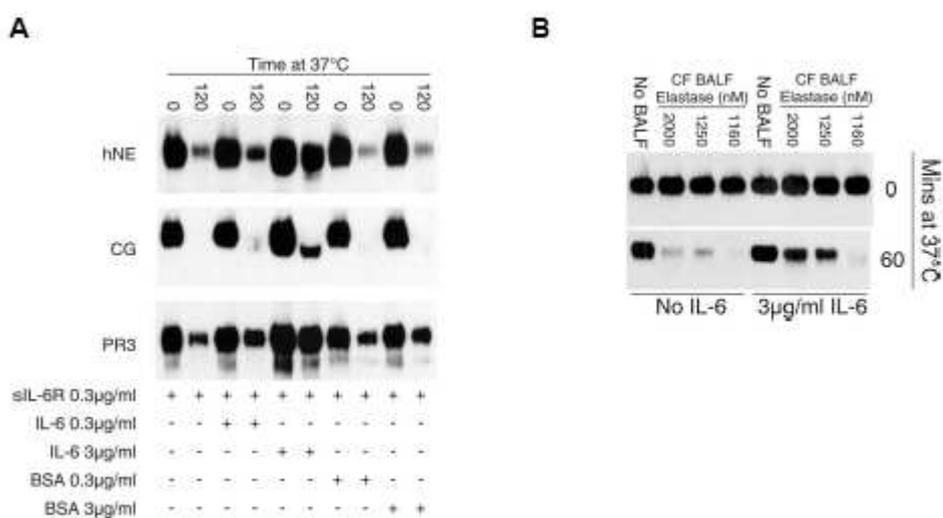


Figure 8

