The identification of polymerized and oxidized alpha-1 antitrypsins (ATs) induced by cigarette smoke as proinflammatory factors in the pathogenesis of emphysema

Zhenjun Li

Student ID 0921900

Department of Life Sciences

Anglia Ruskin University

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My special thanks go to my collaborators (a) Dr Sam Alam, (b) Dr Jichun Wang, (c) Dr Carl Atkinson, (d) Professor Sabina Janciauskiene. For clarification of their contributions, I have identified them with supercripts a-d within the text of this thesis, wherever appropriate.

I wish also to thank my family for their support over the last 12 years.

Abbreviations

AP-1:	activator protein 1
AT:	alpha1-antitrypsin
BALF:	bronchoalveolar lavage fluid
CCR2:	C-C chemokine receptor type 2
CCR49:	C-C chemokine receptor type 49
Cl-AT:	cleaved alpha1-antitrypsin
COPD:	chronic obstructive pulmonary disease
CS:	cigarette smoking
CSE:	cigarette smoke extract
ELISA:	Enzyme-linked immunosorbent assay
FCS:	fetal calf serum
IL-8:	interleukin-8
IL-1β:	interleukin-1 beta
IHC:	immunohistochemistry
JNK:	c-Jun N-terminal kinase
LH:	lung homogenates
LP-AT:	long-chain AT polymers
LPS:	lipopolysaccharide (Endotoxin)
MAPK:	p38 mitogen-activated protein kinases
MCP-1:	monocyte chemotactic protein-1
MPO:	myeloperoxidase
Murine JE:	equal to human MCP-1
Murine KC:	equal to human IL-8
NAC:	*N*-acetyl cysteine
N-AT:	native M-AT
NF-kB:	nuclear factor kB
NHBE cell:	Normal Human Bronchial /Tracheal Epithelial Cells
NO:	nitric oxide
NE:	neutrophil elastase
Ox-AT:	oxidized antitrypsin
PBS/T:	phosphate buffered saline with Tween 20 or Triton X-100
PMSF:	phenylmethanesulfonyl fluoride
RNS:	reactive nitrogen species
ROS:	reactive oxygen species
RT:	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLPI:	secretory leukoproteinase inhibitor
SNP	single nucleotide polymorphisms
SP-AT:	short-chain polymers
TNF-α:	tumor necrosis factor-alpha

Abstract

The molecular mechanisms that cause emphysema are complex but most theories suggest that an excess of proteinases is a crucial requirement. I find that the excess of neutrophils in Z alpha(1)-antitrypsin mutation individuals and the demonstration that polymers cause an influx of neutrophils when instilled into murine lungs. Polymers exert their effect directly on neutrophils rather than via inflammatory cytokines. I also find that Ox-AT promotes release of human monocyte chemoattractant protein-1 (MCP-1) and IL-8 from human lung type epithelial cells (A549) and normal human bronchial epithelial (NHBE) cells. These findings were supported by the fact that instillation of Ox-AT into murine lungs resulted in an increase in JE (mouse MCP-1) and increased macrophage numbers in the bronchoalveolar lavage fluid. The effect of Ox-AT was dependent on NF-kB and activator protein-1 (AP-1)/JNK. These demonstrate that both polymer AT and Ox-AT not only reduce the antielastase lung protection, but also converts AT into a proinflammatory stimulus. More experiments show that mice transgenic for Z-AT develop a significant increase in pulmonary polymers after acute CS exposure ($P = 0.001$). Oxidation of human plasma Z-AT by CS or N-chlorosuccinimide greatly accelerates polymerization ($P = 0.004$), which could be abrogated by antioxidants ($P = 0.359$ versus Z control). CS accelerates polymerization of Z-AT by oxidative modification, which, in so doing, further reduces pulmonary defense and increases neutrophil influx into the lungs. These novel findings provide a molecular explanation for the striking observation of emphysema in ZZ homozygotes who smoke and smoking individuals with emphysema without AT deficiency. Further work is required to assess whether antioxidant therapy may be beneficial in Z-AT-related COPD.

CONTENT

Index：**Paper 1**: Ogawa N, Li P ,**Li Z,** Takada Y, Sugai S. Involvement of the interferon-r-induced T cell attracting chemokines, interferon-r-inducible 10-kd protein (CXCL10) and monokine induced by interferon-r (CXCL9), in the salivary gland lesions of patients with Sjogren's Syndrome. **Arthritis Rheum** 2002; 46(10): 2730-2741.

Paper 2: Wen L, Peng J, **Li Z**, Wong FS The effect of innate immunityonautoimmune diabetes and the expression of Toll-like receptors on pancreatic islets. **J Immunol** 2004 Mar 1; 172 (5): 3173-80.

Paper 3 Mahadeva R, Atkinson Carl, **Li Z**, Stewart S, Janciauskiene S, Kelley DG, Parmar J, Pitman R, Shapiro SD, Lomas DA. Polymers of Z $_1$-Antitrypsin Co-Localize with Neutrophils in Emphysematous Alveoli and Are Chemotactic *in Vivo.* **Am J Pathol** 2005; 166: 377-386.

Paper 4 Li Z, Alam S, Wang J, Sandstrom CS, Janciauskiene S, Mahadeva R. Oxidized alpha-1 antitrypsin stimulates the release of monocyte chemotactic Protein-1 from lung epithelial cells: potential role in emphysema. **Am J Physiol Lung Cell Mol Physiol** 2009 Aug; 297(2): L388-400.

Paper 5 Alam S, **Li Z**, Janciauskiene S and Mahadeva R. Oxidation of Z {alpha}1-antitrypsin by Cigarette Smoke Induces Polymerization: A Novel Mechanism of Early-onset Emphysema. **Am J respir cell mol boil** 2011 Aug; 45(2):261-9).

Paper 6 Abstract for 2009 BTS (British thoracic Society)：**Polymers of Z a1-antitrypsin are induced by pulmonary inflammation**

Paper 7 Abstract for 2011 BTS (British thoracic Society): **Formation of oxidised alpha-1 antitrypsin induces inflammatory response in human bronchial epithelial cells**

Paper 8 Abstract for 2012 ERS (European Respiratory Society): **Z antitrypsin polymerization is associated with enhanced pulmonary inflammation**

Paper 9 EMBO Abstract 2013 (submitted): **Z alpha-1 antitrypsin confers a pro-inflammatory phenotype: relevance to COPD**

Career progression

2003-2012
Alpha-1 Antitrypsin
University of Cambridge (4,5,6)

2000-2003
Type I Diabetes
University of Bristol (3)

2000
Sjogren's Syndrome
Japan (2)

1993-1994
Rheumatoid arthritis
China (1)

Publications:

1 **Li Z**, Liu Y, Xu C. The role of substance P in the pathogenesis of adjuvant arthritis rats. **Basic Medicine and Clinic** 2001; 21(2):185-186.

2 Ogawa N, Li P ,**Li Z,** Takada Y, Sugai S. Involvement of the interferon-r-induced T cell attracting chemokines, interferon-r-inducible 10-kd protein (CXCL10) and monokine induced by interferon-r (CXCL9), in the salivary gland lesions of patients with Sjogren's Syndrome. **Arthritis Rheum** 2002; 46(10): 2730-2741.

3 Wen L, Peng J, **Li Z**, Wong FS The effect of innate immunity on autoimmune diabetes and the expression of Toll-like receptors on pancreatic islets. **J Immunol** 2004 Mar 1; 172 (5): 3173-80.

4: Mahadeva R, Atkinson Carl, **Li Z**, Stewart S, Janciauskiene S, Kelley DG, Parmar J, Pitman R, Shapiro SD, Lomas DA. Polymers of Z $_1$-Antitrypsin Co-Localize with Neutrophils in Emphysematous Alveoli and Are Chemotactic *in Vivo.* **Am J Pathol** 2005; 166: 377-386.

5 **Li Z**, Alam S, Wang J, Sandstrom CS, Janciauskiene S, Mahadeva R. Oxidized alpha-1 antitrypsin stimulates the release of monocyte chemotactic Protein-1 from lung epithelial cells: potential role in emphysema. **Am J Physiol Lung Cell Mol Physiol** 2009 Aug; 297(2): L388-400.

6. Alam S, **Li Z**, Janciauskiene S and Mahadeva R. Oxidation of Z {alpha}1-antitrypsin by Cigarette Smoke Induces Polymerization: A Novel Mechanism of Early-onset Emphysema. **Am J respir cell mol boil** 2011 Aug; 45(2):261-9).

Figure 1. Research experience and publications

Technique development

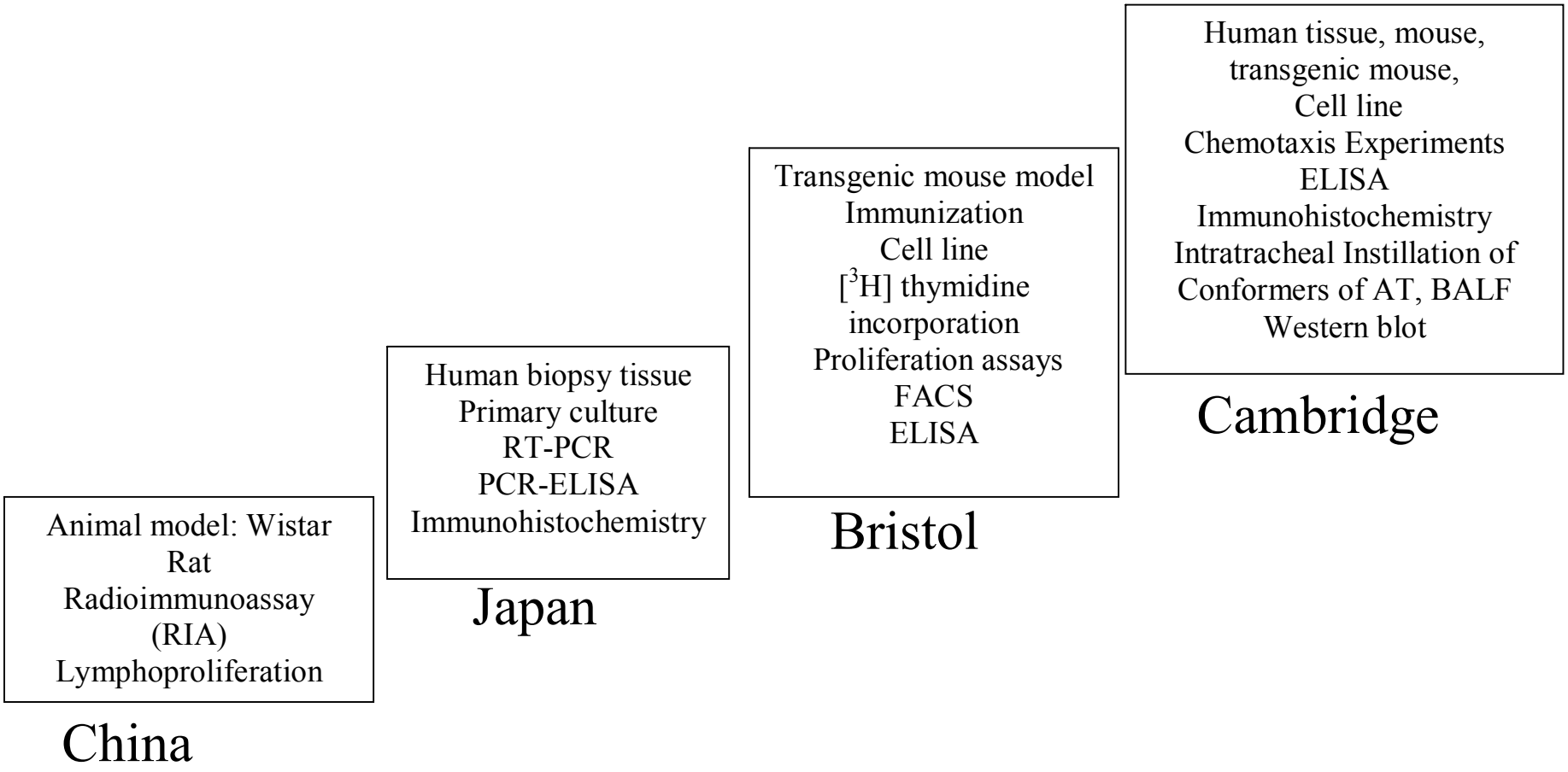

Figure 2. Technique development. In China and Japan, I only experienced short periods of training in research techniques. However, in Bristol and Cambridge Universities, I was intensively trained in most techniques in basic life science research, including some of the main ones involved in the research shown in this thesis.

Summary

Chronic Obstructive Pulmonary Disease (COPD) is a chronic inflammatory disease, characterized by progressive and largely irreversible airflow limitation due to alveolar destruction (emphysema), small airway narrowing, and chronic bronchitis. It is one of the leading causes of morbidity and mortality worldwide and in the UK, it may affect approximately 1.5 per cent of the population; and up to one in eight emergency admissions may be due to COPD, corresponding to over one million bed days, with some 24160 people in the UK dying as a result of COPD in 2005 (Burden of Lung Disease 2nd Edition, British Thoracic Society 2006).

Most cases of COPD are triggered by chronic inhalation of cigarette smoke. However, some people do not suffer from COPD even if they smoke for many years. COPD cannot be cured, and patients usually live with poor life quality. Treatments include giving up smoking, medication and oxygen therapy.

Genetic factors contribute to the development of COPD. In Northern Europe, Z-AT homozygotes (342Glu→Lys) develop emphysema in their third or forth decade. One explanation is AT deficiency because they form inactive polymers. However, this cannot explain why bronchoalveolar lavage fluid (BALF) from Z-AT homozygotes with emphysema contains more neutrophils than BALF from individuals with emphysema and normal AT (M-AT). Inhaling pollutants which include smoking (cigarettes, pipes, cigars, etc.) and other fumes such as those found in many industrial work environments probably also plays a role in an individual's development of COPD.

Previously, it has been shown that the polymeric conformer of AT is present in BALF from Z-AT homozygotes and that it is a chemoattractant for neutrophils *in vitro* (Parmar JS, 2002). These findings have been confirmed by others (Mulgrew AT, 2004). However, it is unknown where the polymers form and if

they are chemotactic *in vivo*. My colleague Dr Carl Atkison[†] showed that polymers of Z α_1-AT are present in the alveolar wall of Z-AT homozygotes with emphysema, which accounts for 20% of the total AT from lung homogenates. These Z-AT individuals also have an excess of neutrophils in the alveolar wall compared with M-AT homozygotes. Furthermore, neutrophils and polymeric AT co-localize in the alveolar wall (Mahadeva R, 2005). To investigate whether there was a direct relationship between polymers of Z-AT and the excess neutrophils, polymers of AT were instilled into the lungs of wild-type mice (Mahadeva R, 2005). This produced a significant increase in neutrophil influx into the lungs compared with instillation of the native protein. Examination of the time course demonstrated that the influx of neutrophils was closely linked to the presence of polymeric AT. The mechanism of neutrophil recruitment in this mouse model was subsequently shown to be a direct chemotactic effect rather than stimulation of IL-8 homologues or other CXC chemokines.

Oxidized AT (Ox-AT) promotes release of human monocyte chemoattractant protein-1 (MCP-1) and IL-8 from human lung type epithelial cells (A549) and normal human bronchial epithelial (NHBE) cells. Native, cleaved, polymeric AT and secretory leukoproteinase inhibitor (SLPI) and oxidized conformations of cleaved, polymeric AT and SLPI did not have any significant effect on MCP-1 and IL-8 secretion. These findings were supported by the fact that instillation of Ox-AT into murine lungs resulted in an increase in JE (mouse MCP-1) and increased macrophage numbers in the bronchoalveolar lavage fluid. The effect of Ox-AT was dependent on NF-κB and activator protein-1 (AP-1)/JNK. These findings have important implications. They demonstrate that the oxidation of methionines in AT by oxidants released by cigarette smoke or inflammatory cells not only reduces the anti-elastase lung protection, but also converts AT into a proinflammatory stimulus. Ox-AT generated in the airway

[†] My colleagues' contributions are acknowledged in future text where appropriate by the following superscripts: (a) Dr Sam Alam, (b) Dr Jichun Wang, (c) Dr Carl Atkinson, (d) Dr Sabina Janciauskiene.

interacts directly with epithelial cells to release chemokines IL-8 and MCP-1, which in turn attracts macrophages and neutrophils into the airways. The release of oxidants by these inflammatory cells oxidizes AT, perpetuating the cycle, potentially contributing to the pathogenesis of COPD. Furthermore, this demonstrates that molecules such as oxidants, anti-proteinases, and chemokines, rather than acting independently, collectively interact to cause emphysema (Li Z, 2009).

To investigate the molecular basis for the interaction between Z-AT and Ox-AT associated with cigarette smoking, female mice transgenic for normal (M-AT) or Z-AT on CBA background were exposed to cigarette smoke (CS). Transgenic mice for Z-AT developed a significant increase in pulmonary polymers following acute CS exposure. Increased levels of neutrophils in CS-Z lungs were tightly correlated with polymer concentrations. Oxidation of human plasma Z-AT by CS or N-chlorosuccinimide greatly accelerated polymerization, which could be abrogated by antioxidants. The results showed that cigarette smoke accelerated polymerization of Z-AT by oxidative modification, which in so doing further reduced pulmonary defense and increased neutrophil influx into the lungs. These novel findings provided a molecular explanation for the striking observation of premature emphysema in ZZ homozygote smokers, and raised the prospect of anti-oxidant therapy in Z-AT related COPD (Alam S, 2011).

Chapter 1

Initial characterization of different conformations of AT

1.1. Research Training

My initial research training (Fig 1.) began as a medical student in 1993 and had an immunological focus, specifically relating to studies on adjuvant arthritis at Peking Union Medical College in Beijing. In 2000, I extended my immunological training through the study of Sjogren 's Syndrome in Japan for six months. Then, I moved to Bristol (UK) and spent nearly another 3 years studying the role of CD8+ in Type I diabetes. Through development of research strategy on cell culture, animal models and finally human samples, using biomedical techniques which included ELISA, IHC. Over this period I became sufficiently confident to design experiments, write papers, apply for grants, and to present my data to my peers at conference. This provided the basis for me following my move to Cambridge to develop my research interests into inflammatory aspects of oxidative injury in emphysema (presented herein) and I believe this has set a solid foundation for the future development of my research into new therapies for emphysema (Fig 1 and Fig 2).

1.2. Introduction to α_1-Antitrypsin (AT)

AT is a single-chain glycoprotein consisting of 394 amino acids in the mature form and exhibits a number of glycoforms, it is a serine proteinase inhibitor produced primarily by hepatocytes, macrophages, and bronchial epithelial cells. The normal protein is termed M-AT according to its migration on isoelectric focusing. It is the most abundant proteinase inhibitor (serpin) within the lung whose main physiological target is neutrophil elastase. It protects tissues from enzymes of inflammatory cells, especially elastase, and has a reference range in blood of 1.5 - 3.5 gram/liter, but the concentration can rise many folds upon acute inflammation (Kushner,1993). The crystal structure of

AT is characterized by a dominant β-sheet A, and an exposed reactive centre loop (Silverman GA, 2001) (Fig 3A). The inhibitory mechanism of AT relies on cleavage of the methionine-serine P1-P1' by neutrophil elastase. This initiates a conformational change in AT whereby the elastase molecule is transferred to the lower pole of the AT molecule and inactivated (Huntington JA, 2000).

In addition to acting as an anti-proteinase, AT plays important role in modulating inflammation. It may inhibit immune responses, and fibroblast-proliferation and fibroblast procollagen production thereby contributing to repair and matrix production (Dabbagh, 2001), and have antibacterial activities (Hadzic, 2006), and also blocks the cytotoxic and stimulatory activity of defensins (Hiemstra, 1998). AT also has direct and indirect anti-apoptotic properties by inhibiting caspase-3 or NE mediated apoptosis, respectively (Petrache, 2006). AT is also involved in calcium-induced activation mechanisms; AT inactivates calpain I (μ-calpain), induces a rapid cell polarization and random migration of neutrophils

The AT gene is located on chromosome 14q31-32.1, and is co-dominantly expressed (Schroeder, 1985; Bull World Health Organ, 1997; Brantly,1988). The gene is 12 kb in length and contains seven exons (Ia, b, c and II–V) and six introns. Exon I contains the 3’ untranslated promoter sequences: Ia and Ib contains the promoter sequence for macrophage-specific, and Ic for hepatocyte-specific transcription, respectively. The coding regions (Exons II-V) are 1434 base-pairs (bp) in length and the reactive centre is within Exon V (Long, 1984). Aside from the promoter elements, there are other regulatory sequences including an enhancer element in the 5' and 3' flanking sequences of exonic regions of the AT gene. A polymorphism in the 3’ flanking region is associated with susceptibility to COPD (Kalsheker, 1987; Morgan, 1992). A map of single nucleotide polymorphisms (SNPs) in the 5' and 3' flanking regions showed that among the 15 SNPs, five SNPs increased the risk of COPD by 6- to 50-fold (Chappell, 2006). These polymorphisms within regulatory sequences are associated with normal basal plasma levels, but can result in reduced levels of AT transcription in response to stimulation *in vitro*, which is postulated to relate to the susceptibility to COPD (Henry, 2001;

Chappell, 2006). However, this has not been proven *in vivo* (Mahadeva, 1998; de Faria, 2005; Courtney, 2006; Brennan, 2007).

Over 100 different variants of α_1-antitrypsin have been described in various populations. The plasma level of the principal AT phenotypes are MM (20-39 µmol/L), MS (19-35 µmol/L), SS (14-20 µmol/L), MZ (13-23 µmol/L), SZ (9-15 µmol/L), ZZ (2-8 µmol/L). One in two thousand North Europeans are homozygous for the Z (342Glu→Lys) variant of AT. This variant accumulates in the hepatocyte by a process of loop-sheet polymerization whereby the reactive loop of one molecule inserts into β-sheet A of a second and so on to form chains of polymers (Fig 3B). Polymerization in the liver is associated with cirrhosis, and the accompanying plasma deficiency leaves the lungs exposed to neutrophil elastase resulting in premature emphysema (Eriksson S, 1986; Perlmutter DH, 2002).

AT can be cleaved at the reactive center loop resulting in a profound conformational rearrangement in which the loop is fully inserted into the A sheet of the molecule (Fig 3A). The cleaved species is inactive, has increased thermostability, and is more resistant to unfolding with denaturants (Powell LM, 1992) when compared to the native protein. It is clear that the sequence of events involved in proteinase inhibition requires the loop to be mobile and to adopt a variety of conformations. Oxidation of the P1 methionine (methionine 358) or methionine 351 to methionine sulfoxide significantly reduces the ability of AT to inhibit neutrophil elastase. Hydrogen peroxide in cigarette smoke and N-chloroamines and hypochlorous acid in neutrophils can oxidize and inactivate AT (Scott LJ 1999; Taggart C, 2000). AT can exist *in vivo* in different conformational forms; native, reactive center loop-cleaved, oxidized, complexed with neutrophil elastase and Z-AT can be found as polymers (Fig 3A, Fig3B).

1.3. Chemical modification of α_1-Antitrypsin (AT)

My initial experiments focussed on the preparation and characterization of conformations of AT. Native M-AT (N-AT) was purified from human plasma by ammonium sulfate fractionation followed by glutathione and anion exchange

Serpin Structure

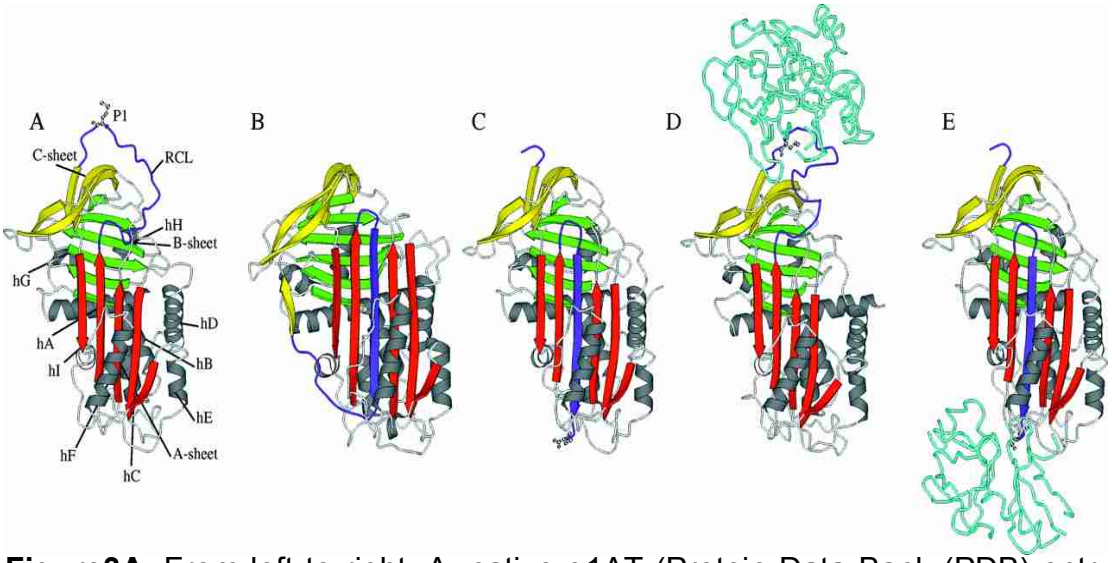

Figure3A: From left to right: A, native α1AT (Protein Data Bank (PDB) entry 1QLP); B, latent ATIII (PDB entry 2ANT); C, cleaved α1AT (PDB entry 7API); D, Michaelis complex between Serpin 1 (Alaserpin from Manduca sexta) and trypsin (PDB entry 1I99(S. Ye, A. Cech, R. Belmares, E. J. Goldsmith, R. Bergstrom, D. Corey, and M. Kanost, submitted for publication)); and E, covalent complex between α1AT and trypsin (PDB entry1ezx). In all structures the A-sheet is in red, the B-sheet is in green, the C-sheet is in yellow, and the RSL(RCL) is in purple. The helices are in gray and are labeled on the structure of native α1AT. Trypsin is shown as a cyan coil.

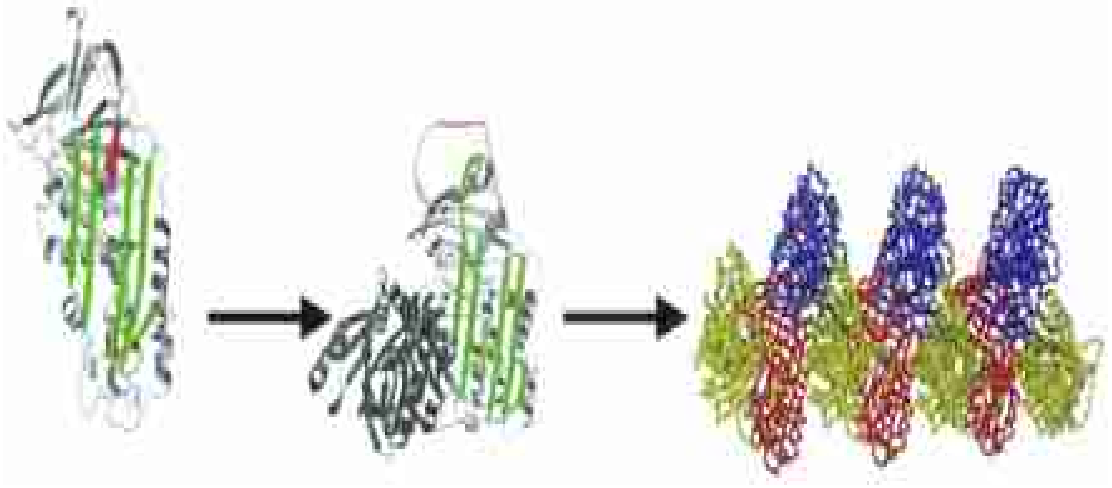

Figure3B: Demonstration of the partially loop-inserted Z AT (red) that opens up ß-sheet A (green) to favour insertion of the reactive loop of another molecule to form an AT dimer (centre) and polymers (right) (From Mahadeva R, 2005).

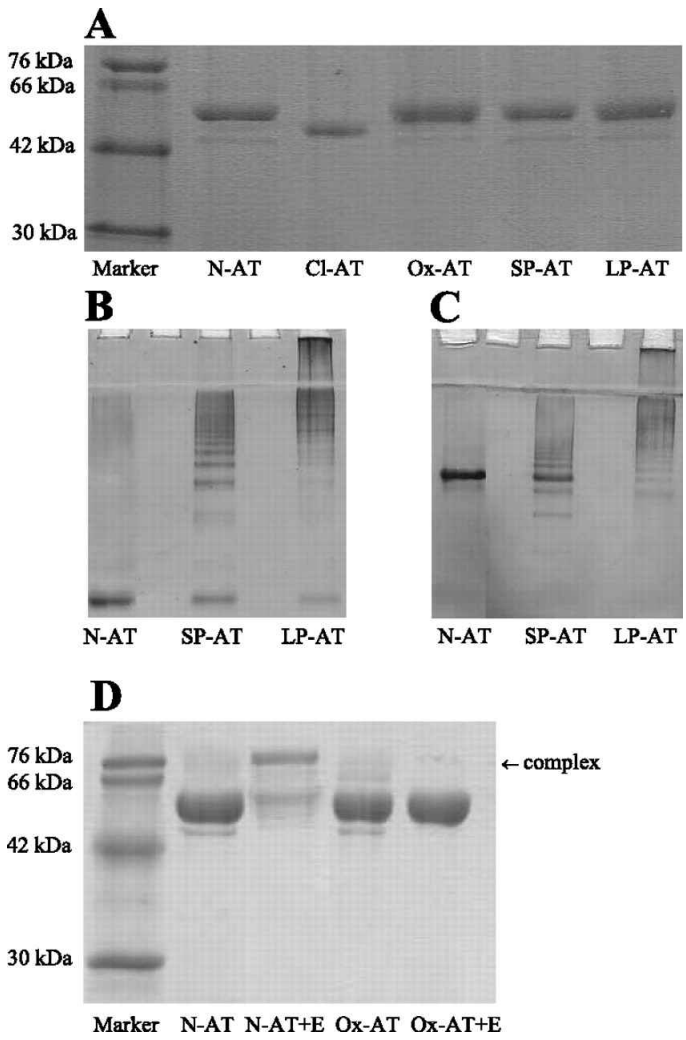

Figure 4 Characterization of conformations of α_1-antitrypsin (AT).

A: 12% (wt/vol) SDS-PAGE. Two micrograms of native AT (N-AT, 52KD), *Staphylococcus aureus* V8 protease reactive loop-cleaved AT (Cl-AT), oxidized AT (Ox-AT), short-chain polymers (SP-AT), and long-chain AT polymers (LP-AT), were loaded onto 12% SDS-PAGE, 7.5% (wt/vol) non-denaturing PAGE with (B) and without (C) 8 M urea. A showed N-AT, Ox-AT, SP-AT, LP-AT are similar molecular weight, except Cl-AT. B and C showed different structure among N-AT, SP-AT, and LP-AT. Aggregates are apparent in LP-AT. D: 12% (wt/vol) SDS-PAGE demonstrating the difference in N-AT and Ox-AT. N-AT could form an SDS-stable complex with human neutrophil elastase, unlike Ox-AT.

(from page 8) chromatography by Dr Mahadeva. N-AT migrated as a single band on 7.5% nondenaturing and 12% SDS-PAGE and showed a normal unfolding transition on transverse urea gel PAGE. Its molecular weight is 52KD (Fig 4A). It was 70–80% active when assessed by titration against bovine α-chymotrypsin. The native protein was then used to prepare the other AT conformations. Reactive loop-cleaved antitrypsin (Cl-AT) was generated by incubation with *Staphylococcus aureus* V8 protease (Sigma, UK) at 37°C for 2 h. The protease was then removed by anion exchange chromatography using a mono-Q column. This is distinct from the cleaved fragment; in this form COOH-terminal segment remains firmly attached to the core of cleaved AT.

Long- and short-chain polymeric AT (LP-AT and SP-AT, respectively) were formed by heating native M-AT (2 mg/ml) at 55°C for 40 and 3 h, respectively. These polymers are predicted to have the same loop sheet linkage as Z-AT polymers, and thus the same tertiary structure. Polymeric AT was assessed on 7.5% non-denaturing PAGE with and without 8 M urea (Fig 4B, C). Native, cleaved, and polymeric AT, secretory leukocyte protease inhibitor (SLPI) (purified recombinant SLPI was obtained from the Dept. of Otolaryngology and Head and Neck Surgery, Malmoe University Hospital, Sweden). Material was oxidized as follows: Briefly, N-chlorosuccinimide (Sigma, UK) was incubated in a 25 M excess to AT in 0.1 M Tris·HCl (pH 8.0) at room temperature for 30 min. Excess N-chlorosuccinimide was removed and the buffer was exchanged with PBS, using a centrifugal microconcentrator (Centricon YM30). Ox-AT was tested for the ability to form an SDS-stable complex with neutrophil elastase (Calbiochem). Samples of Ox-AT or N-AT were incubated with neutrophil elastase at a 2:1 molar ratio for 15 min at 37°C. The reaction was stopped by adding SDS sample buffer and boiling. Mixtures were analyzed by 12% SDS-PAGE and stained with Coomassie blue. Ox-AT was confirmed and distinguished from N-AT by its inability to form an SDS-stable complex with human neutrophil elastase (Fig 4D).

The results show that N-AT, Ox-AT, SP-AT, LP-AT are similar molecular weights, 52KD in SDS-PAGE, Cl-AT has lower molecular weight. Ox-AT loses

elastase binding capacity, compared with N-AT. LP-AT and appears aggregated in 7.5% (wt/vol) non-denaturing PAGE with and without 8 M urea. Further characteristics of polymeric AT are described in subsequent chapters 2-7.

Chapter 2

Polymers of Z α_1-Antitrypsin has proinflammatory properties in a model of emphysema (Mahadeva R, 2005)

This work was designed to elucidate the role of polymeric AT on the pathogenesis of emphysema in Z-AT homozygotes. Emphysema is thought to arise mainly from deficiency of the proteinase inhibitor. Instillation of proteinases with elastolytic properties into mammalian lungs, and the association of genetic deficiency of AT with emphysema together have formed a central pillar of the anti-proteinase-proteinase hypothesis of chronic obstructive pulmonary disease. There are however many other mechanisms and factors that are important in emphysema (Calverley, 2003). Importantly, there are several differences between emphysema with normal and sub-normal levels of AT. The emphysema in Z-AT homozygotes develops earlier in life, initially at least predominantly affecting the basal areas and is of the panacinar rather than the centriacinar variety. One striking observation is that bronchoalveolar lavage fluid (BALF) from Z-AT homozygotes with emphysema contains more neutrophils than BALF from individuals with emphysema and M-AT (Morrison, 1987). The reasons for this remain unclear, but may in part be because of an excess of interleukin-8 or leukotriene B4 in the BALF. However, one additional explanation may be the presence of polymers of AT in the lungs of Z-AT homozygotes. Preliminary studies indicated that polymers of AT can be detected in BALF from Z-AT homozygotes, and, *in vitro*, polymers of AT are chemotactic to neutrophils (Elliott, 1997; Parmar, 2002). This observation raised the novel hypothesis that Z-AT undergoes a conformational transition to polymers within the lungs that further depletes the local anti-proteinase protection. This would transform AT into a proinflammatory stimulus, thus possibly exacerbating the lung disease.

To test the hypothesis, co-localization of Z-AT polymers with neutrophils in the alveoli of individuals with Z-AT by immunhistochemstry (IHC) was investigated. (I was assisted by my colleague[c], Dr Atkinson). The following work

demonstrated that polymers were proinflammatory in cell and mouse models of disease.

2.1 Polymer AT exists in human lung confirmed by ELISA

Frozen emphysematous lung tissue from five Z-AT and six M-AT individuals (samples from lung transplant surgery in Papworth Hospital) were homogenized using Tissue Tearor (Biospec Products) in cell lysis buffer (CellLytic; Sigma, St. Louis, MO) on ice. The lung homogenates were centrifuged and proteinase inhibitor cocktail (Sigma, Poole, UK). The supernatants were kept on ice, then immediately assessed with an in-house enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of total α_1-AT and for polymers of AT.

Briefly, immunoplates (Nunc, Denmark) were coated with rabbit anti-human AT (Sigma) primary antibody overnight. This antibody detects all conformations of AT. Unbound sites were blocked with PBS containing 1% (w/v) bovine serum albumin. Lung homogenates and polymer standards were added and incubated at room temperature for 1 hour. A goat anti-human AT antibody labelled with horseradish peroxidase (Abcam, UK) was added followed by the substrate 2, 2'-azino-bis (3-ethybeniazoline-6-sulfonic acid) (Chemicon, Temecula, CA). The plate was read at OD 405 nm and the concentration of AT in the samples was calculated from the standard curve. The concentrations of AT recovered from the M-AT and the Z-AT lungs were compared using the Student's *t*-test.

For the quantification of polymers, the above method was followed, only differing in that the primary coating antibody used was a monoclonal antibody, ATZII (referred to as the anti-polymer antibody) with polymer standards and lung homogenates. The latter antibody recognizes polymeric AT and the AT-elastase complex, but not the cleaved, or native form of AT. Homogenization of monomeric Z-AT in cell lysis buffer did not cause the Z-AT to polymerize.

ELISA for total AT revealed a 2.5-fold increase in the amount of AT recovered from M-AT lungs compared to Z-AT lungs. The mean (SD) concentration of

AT was 59.6 (7.0) μg/ml and 23.8 (6.9) μg/ml for M-AT and Z-AT lungs, respectively (*P*= 0.007). Polymeric AT was detected in the Z-AT emphysematous lung homogenates mean 4.7 (SD 2.6) μg/ml, but not in the M-AT lung homogenates. Polymers constituted on average 20% (SD, 8%) of the total AT recovered from Z-AT lungs.

2.2 Polymers are proinflammatory in mouse experiments

KC and MIP-2 are the murine homologues of human IL-8, main neutrophil and T cell chemoattractants. To assess whether the effect of polymeric AT was mediated via chemokines, the BALF content for KC and MIP-2 were assessed by specific ELISA (R&D Systems, Minneapolis, MN). BALF was collected from C57BL/6J 4, 24, and 72 hours after intratracheal instillation of conformers of AT into Murine Lungs. Intratracheal instillation is a delicate procedure. The most technically difficult step is setting a cannula into the mouse trachea. This work was taught to me by Dr Mahadeva as there were problems with other staff being unable to master the technique and so I subsequently took charge of this procedure for subsequent experiments.

Instillation of polymeric AT produced a significant neutrophil influx into the BALF at 4 hours and 24 hours after instillation compared with PBS and native AT controls; $P < 0.01$. By 72 hours neutrophil counts had returned to baseline (Figure 5A). There was no significant difference in the neutrophil influx after instillation of native AT compared with LPS control. Native and polymer samples of AT had a contaminating dose of 10 ng/ml of LPS that equates to 0.8 ng in 40 μl. Instillation of LPS control produced a small increase in neutrophil numbers compared with PBS control. To verify these results, neutrophil elastase activity was assessed after lysis of cells recovered from BALF using a specific substrate for neutrophil elastase-MeOSuc-Ala-Ala-Pro-Val-AFC. Neutrophil elastase levels were detectable only after polymer instillation at 24 hours, but not after intrapulmonary instillation of native AT or

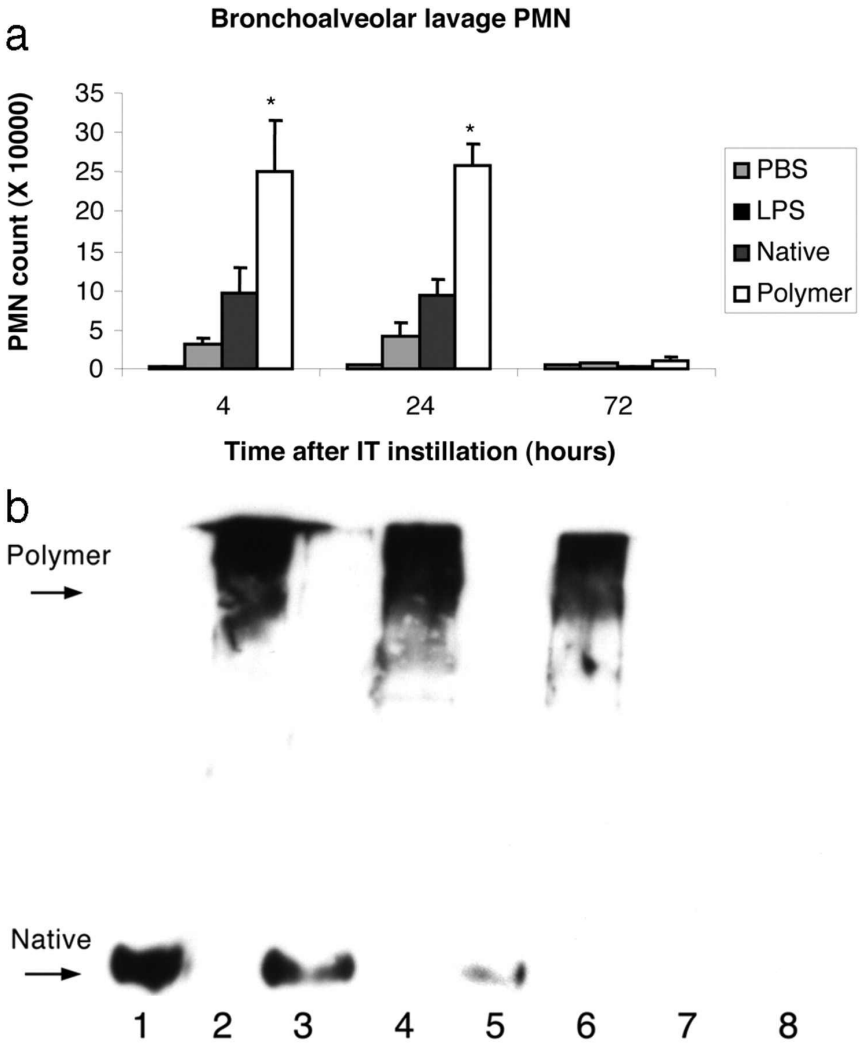

Figure 5. a: Graph demonstrating the effect of intratracheal instillation of native and polymeric 1-AT on polymorphonuclear leukocyte (PMN) numbers in BALF from C57BL/6J mice. *,*P* < 0.01 for polymeric AT compared with native AT. **b:** C57BL/6J mice were anesthetized and intubated. Native or polymeric -AT in 40 µl of PBS was instilled via the intratracheal route. At 4, 24, and 72 hours after instillation, BALF was performed and aliquots were assessed on a 7.5% (w/v) non-denaturing PAGE followed by Western blot analysis for AT using a polyclonal antibody that recognizes all forms of AT. **Lane 1**: Native AT, starting material 0.1 µg; **lane 2**: polymeric AT, starting material 0.1 µg; **lane 3**: BALF 4 hours after native AT instillation; **lane 4**: BALF 4 hours after instillation of polymeric AT; **lane 5**: BAL fluid 24 hours after native AT instillation; **lane 6**: BAL fluid 24 hours after instillation of polymeric AT; **lane 7**: BALF 72 hours after native AT instillation; **lane 8**: BALF 72 hours after instillation of polymeric AT.

PBS. There were no differences in the numbers of alveolar macrophages seen compared with controls.

To assess the relationship between polymers and neutrophil influx, Western blot analysis for AT in BALF after instillation of AT was performed on 7.5% (w/v) non-denaturing PAGE. Polymers and native AT were detected at similar times after instillation. They were both present at 4 and 24 hours after instillation with a suggestion that there was more polymeric AT remaining at 24 hours. Both native and polymeric AT were undetectable at 72 hours (Figure 5b). Thus, the time course of neutrophil influx paralleled the presence of polymers in the BALF. Furthermore, there was no change in the conformation of native AT, which did not spontaneously form polymers within the murine lung.

Disappointingly, concentrations of these cytokines were extremely low. There was no significant difference between the concentrations of KC and MIP-2 as assessed by ELISA of BALF after instillation of either native or polymeric AT, P = 0.14 and 0.22, respectively. There was no detectable KC or MIP-2 at 24 and 72 hours after instillation of native or polymeric AT. Thus, polymer instillation *in vivo* produced a neutrophil influx that did not appear to be related to CXC chemokines. *In vitro* neutrophil chemotaxis experiments[d] showed that neutrophil recruitment was a direct effect of polymers. In retrospect if I were to repeat this experiment again, I would check the KC and MIP-2 expression level in the lung by RT-PCR, as the detection sensitivity of the RT-PCR technique is much greater than that of ELISA.

On the other hand, the reason for low concentration of cytokines may be that the animal models were not sufficiently sensitive to polymers *in vivo*, However, A549 lung epithelial cell line (American Type Culture Collection) proved reactive to polymers and selective conformations of AT *in vitro* and subsequently exciting results were obtained (see chapter 3).

Chapter 3

Ox-AT induced production of IL-8 and MCP-1 from A549 cells and NHBE cells (Li Z, 2009)

IL-8 in humans is produced by numerous cell types, particularly monocyte /macrophages, epithelial cells, neutrophils, fibroblasts, and endothelial cells. This occurs upon infection by bacteria, or stimulation by bacterial products (i.e., pathogen-associated molecular patterns), or stimulation by cytokines such as IL-1β and TNF-α. IL-8 is mainly active on neutrophils, promoting their recruitment and also their strong activation which is characterized by activation of the leukotriene pathway, by the release of their granular content (i.e., elastase, lactoferrin, and MPO), and their increased adherence to endothelial cells, as well as NO activation. IL-8 is also a chemoattractant for other cell types such as basophils, T lymphocytes, and NK cells, and also enhances permeability of endothelial cells (Singer M, 2004).

The monocyte chemoattractant protein-1 (MCP-1/ CCL2) is a member of the C-C chemokine family, and a potent chemotactic factor for monocytes. Human MCP-1 is composed of 76 amino acids and is 13 kDa in size. MCP-1 is produced by many cell types, including endothelial, fibroblasts, epithelial, smooth muscle, mesangial, astrocytic, monocytic and microglial cells. The cell surface receptors that bind MCP-1 are CCR2 and CCR49 (Deshmane SL, 2009).

Oxidation of the P1 methionine (methionine 358) or methionine 351 to methionine sulfoxide significantly reduces the ability of AT to inhibit neutrophil elastase. Hydrogen peroxide in cigarette smoke and N-chloroamines and hypochlorous acid in neutrophils can oxidize and inactivate AT (Ossanna PJ, 1986; Scott LJ, 1999; Taggart C, 2000). Thus, the oxidation of AT by cigarette smoke or free radicals *in vivo* could lead to a relative deficiency of elastase inhibitors and has been suggested as a mechanism contributing to the development of emphysema in M-AT individuals (Gadek JE, 1979). Oxidant-mediated inactivation of AT has also been suggested as playing a role in other

lung diseases such as cystic fibrosis, adult respiratory distress syndrome, and bronchiectasis (McElvaney NG, 1991). In this study, the effect of Ox-AT on the production of monocyte chemotactic protein-1 (MCP-1) and IL-8 was assessed.

The group of experiments described in sections 3.1-3.3 took nearly three years to finish between 2004 and 2007. It was known that cigarette smoke extract and chronic exposure of mice to cigarette smoke augmented the generation of IL8 (Vassallo, 2008). However, no studies of the effects of the different conformers of AT on MCP-1 were apparent in the literature. To clarify this omission, I designed the following experiments to test the effect of N-AT, Ox-AT, Cl-AT, LP-AT, SP-AT on the production of MCP-1. Another inhibitor SLP1 was also tested for comparison. SLPI consists of 107 amino acids (11.7 kDa) organized in a duplicated, boomerang-shaped whey acidic protein four-disulfide core (WFDC) domain. It is found in various secretory fluids including parotid secretions, bronchial, nasal, cervical mucous and seminal fluid. SLPI is a potent inhibitor of many proteolytic enzymes including the neutrophil proteases, elastase, cathepsin G, chymotrypsin, and trypsin. In addition to its antiprotease function, SLPI has an anti-inflammatory function through the modulation of nuclear factor- B acting intracellularly, especially in macrophages.

3.1 Ox-AT induced production of IL-8 and MCP-1 from A549 cells

A549 lung epithelial cell line (American Type Culture Collection) was first developed in 1972 through the removal and culturing of cancerous lung tissue from the explanted tumor of 58-year-old caucausian male. It is hypotriploid with a modal chromosome number of 66, which occurs in 24% of cells. It is used as an *in vitro* model for a type II pulmonary epithelial cell model for lung disease research (Standiford T J, 1990). The cells were grown in DMEM supplemented with 4,500 mg of glucose/l, L-glutamine, $NaHCO_3$, and pyridoxine HCL (Sigma), with 5% FCS and 100 units of penicillin and 0.1 mg/ml of streptomycin (Sigma), at 37°C, 5% carbon dioxide (CO_2) until ~80%

confluent. Cells were then trypsinized, and $1x10^5$ cells/100 µl media/well were cultured in 96-well plates overnight. The following morning, the media was aspirated and replaced with 100 µl of fresh media for 30 min followed by addition of 15 µl of either N-AT, Ox-AT, SLPI, Ox-SLPI, Cl-AT, Ox-Cl-AT, LP-AT, or Ox-LP-AT to give a final concentration of 0.03, 0.1, or 0.3 mg/ml. TNFα was used as a positive control, and LPS (0.01 µg/ml) was used as a negative control. The cells were cultured for 4, 10, and 24 h. The culture supernatant was collected at each time point and levels of IL-8, MCP-1, and TNFα were measured by ELISA using human IL-8, MCP-1/CCL2, TNF α DuoSet, according to the manufacturer's instructions (R&D Systems). The LPS dose was chosen as the conformations of AT had a contaminating dose of less than 1 ng/ml LPS.

Ox-AT significantly induced the production of IL-8 in A549 cells in a time- and dose-dependent manner (Ox-AT0.1, 24 h, IL-8 (means SE) =424(14) pg/ml vs. PBS 24 h,IL-8 =110(8) pg/ml, *P* = 0.029; Ox-AT 0.3, 10 h, IL-8 =418(26) pg/ml vs. PBS 10 h, IL-8 =43(3) pg/ml, *P* =0.049;Ox-AT 0.3, 24 h, IL-8 =1,168(9) pg/ml vs. PBS 24 h, *P* =0.008); Ox-AT also significantly induced the production of MCP-1(Ox-AT 0.03, 24 h, MCP-1 = 8,725(894) pg/ml vs. PBS 24 h, MCP-1 = 4,225(470) pg/ml, *P* = 0.041;Ox-AT 0.1, 24 h, MCP-1 = 9,167(1,229) pg/ml vs. PBS24 h, *P* = 0.035; Ox-AT 0.3, 4 h, MCP-1 = 3,925(711)pg/ml vs. PBS 4 h, MCP-1 = 1,900(424) pg/ml, *P* = 0.044;Ox-AT 0.3, 10 h, MCP-1 = 8,608(519) pg/ml vs. PBS 10 h,MCP-1 = 3,075(459) pg/ml, *P* = 0.028; Ox-AT 0.3, 24 h,MCP-1 = 14,500(424) pg/ml vs. PBS 24 h, *P* = 0.005). MCP-1 and IL-8 can be produced in response to TNFα; therefore, this cytokine was also measured in the supernatant. No significant TNFα secretion was detected in response to Ox-AT.

3.2 Ox-AT induced production of MCP-1 from NHBE cells

To further confirm these findings in A549 adenocarcinoma epithelial *cell* line, normal human bronchial epithelial (NHBE) cells (CC-2540, Lonza) were cultured in supplemented DMEM at 37°C and 5% CO_2 until ~80% confluent. Using a similar experimental procedure to that described in section 3.1. Again,

Ox-AT significantly induced MCP-1 and IL-8 production in NHBE cells in a time- and dose-dependent manner compared with N-AT (0.3 mg/ml) at 4 and 24 h. There was no significant increase in MCP-1 production with 0.03 mg/ml Ox-AT compared with 0.03 mg/ml N-AT at 4 or 24 h.

3.3 Only Ox-AT, (no other oxidized proteins) induced production of IL-8 and MCP-1 from A549 cells

To assess whether the effects seen with Ox-AT were specific, or merely a function of oxidized proteins in general, SLPI, Cl-AT, and LP-AT were chosen for oxidation (see section 1.3.). Oxidised SLPI, Ox-Cl-AT, Ox-LP-AT at final concentrations of 0.03, 0.1, or 0.3 mg/ml were added to A549 cells. The supernatant was removed at 4, 10, and 24 h and quantified by ELISA. None of these oxidized proteins had any significant effect on the production of IL-8 or MCP-1[a].

After this paper was submitted, my reviewer suggested that I further assay the effects of oxidised SLPI, Ox-Cl-AT, and Ox-LP-AT on the system described above. Dr Alam subsequently finished this experiment. I started to practise medicine from end of September, 2008. Since then I have only worked in the lab in some of weekend. Dr Alam has worked in the lab from May, 2008.

Chapter 4

Signalling pathway for production of IL-8 and MCP-1 induced by Ox-AT in A549 cells[b]

4.1 Effect of Ox-AT on NF-κB p50 activation in A549 cells

The expression of many of chemokines such as IL-8/CXCL8 and cytokines such as TNFα is regulated by transcription factor, NF-κB a pleiotropic regulator of inflammatory processes. NF-κB is in macrophages and epithelial cells of COPD patients. Szulkaowski examined the proteosomal degradation of NF-κB DNA binding activity in the human lung in response to cigarette smoking. NF-κB DNA binding was significantly increased in healthy smokers and current smokers with moderate COPD, compared with healthy non-smokers (Szulkaowski P, 2006). Analysis of transcription factor binding sites occurring in the promoter regions of differentially-expressed genes revealed that NF-κB sties were overrepresented among those genes that were downregulated by smoking (relative to nonsmokers) and upregulated by COPD (relative to healthy smokers) (Pierrou S, 2007).These observations, taken together, suggest that modulation of NF-κB activity may represent a potential target in COPD therapy. The effect(s) of Ox-AT on NF-κB p50 activation in A549 cells were therefore examined.

Nuclear protein extraction was performed according to the manufacturer's instructions (Nuclear Extract Kit, Active Motif, Belgium); the protein levels were measured using the RC DC protein kit (Bio-Rad). NF-κB activation was measured by ELISA using the TransAM NF-κB kit according to the manufacturer's instructions (Active Motif, Belgium). Briefly, in the TransAM NF-κB kit, an oligonucleotide that contained the NF-κB consensus site (5'-gggattttcc-3') has been immobilized onto a 96-well plate. The active form of NF-κB can bind to this site. The NF-κB active site on nuclear cell extract (4 µg) from cultured cells and homogenized lung tissue was detected through use of NF-κB antibody p50 (1:1,000) followed by addition of a secondary antibody

conjugated to HRP (1:1,000). Active NF-κB was quantified by reading absorbance at 450 nm (model 680, Bio-Rad). The positive control in this case were Jurkat cells which express high endogenous NF-κB p50 activity.

The results of these experiments showed that Ox-AT increased the activity of NF-κB significantly in a concentration-dependent manner compared with PBS (P = 0.014). N-AT had no effect on the activity of NF-κB.

4.2 Effect of Ox-AT on AP-1 activation in A549 cells

The activator protein 1 (AP-1) is a transcription factor which is a heterodimeric protein composed of proteins belonging to the c-Fos, c-Jun, ATF and JDP families. It regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stress, and bacterial and viral infections. AP-1 in turn controls a number of cellular processes including differentiation, proliferation, and apoptosis (Ameyar M, 2003). AP-1 activation was assessed using the TransAM AP-1 c-Jun kit according to the manufacturer's instructions (Active Motif), similar to NF-κB assay.

Ox-AT increased the activity of AP-1 significantly in a concentration-dependent manner compared with PBS (0.03 mg/ml Ox-AT,P = 0.024; 0.1 mg/ml Ox-AT, P< 0.001; 0.3 mg/ml Ox-AT,P = 0.005). N-AT had no significant effect on the activity of AP-1. The positive control was K562 cells with high endogenous AP-1 activity.

4.3 Effect of SP-600125 (JNK inhibitor) on the activation of JNK in A549 cells treated with Ox-AT

C-Jun N-terminal kinases (JNKs), originally identified as kinases that bind and phosphorylate c-Jun on Ser63 and Ser73 within its transcriptional activation domain, are mitogen-activated protein kinases which are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in T cell differentiation and apoptosis. JNK, by phosphorylation, modifies the activity of numerous proteins that reside at the

mitochondria or act in the nucleus. This way, JNK activity regulates several important cellular functions.

SP-600125 (10 μM), an inhibitor of JNK, was added to the cell culture media of A549 cells 20 min before treatment with 0.3 mg/ml Ox-AT. The stimulated cells in a 96-well plate were directly fixed in situ for the JNK assay 1 h after treatment with Ox-AT. Antibody to phosphorylated JNK and antibody to total JNK (FACE JNK kit, Active Motif) were used separately for the detection of activated JNK and total JNK. A standardized JNK level was obtained by subtracting from background (PBS) and expressed as optical density (OD) 450 nm (JNK level)/OD 595 nm (cell number) (model 680, Bio-Rad).

Ox-AT significantly increased the level of activated JNK (P = 0.001) but had no effect on total JNK (P = 0.7). The ability of Ox-AT to induce JNK activation was significantly blocked by the JNK inhibitor SP-600125: OD450/OD595 = 0.049(0.016), P = 0.016 vs. Ox-AT. SP-600125 had no effect on the total level of JNK in cells of both PBS group and Ox-AT group (P = 0.109 and 0.171, respectively).

4.4 Effect of inhibitors of NF-κB, p38 MAPK, and JNK on the induction of IL-8 and MCP-1 in A549 cells treated with Ox-AT

Bay11-7082, an inhibitor of NF-κB, SB-203580, an inhibitor of p38 MAPK, and SP-600125, an inhibitor of JNK, were dissolved in 10% DMSO and added to cells at a final concentration of 10 μM. Previously published reports indicated that 10–50 μM inhibitor do not have any significant cytotoxic effects. The cytotoxic effect of each inhibitor was also evaluated using A549 cells at 4, 10, and 24 h of culture using Trypan blue assay. There was no significant cytotoxic effect of any of the inhibitors (10 μM) on cell proliferation. They were separately added to the cell culture media of A549 cells 20 min before treatment with 0.3 mg/ml Ox-AT.

Ox-AT-inducedIL-8 production [IL-8 = 1526.133(270.03)] was significantly

reduced in the presence of the NF-κB inhibitor [Ox-AT + Bay11-7082, IL-8 =231(33) pg/ml vs. Ox-AT (*P* = 0.023)] and the JNK inhibitor [Ox-AT + SP-600125, IL-8 = 311(47) pg/ml vs. Ox-AT (*P* = 0.027)]. Ox-AT-induced MCP-1 production [MCP-1 = 11,188(780)] was significantly reduced in the presence of the NF-κB inhibitor [Ox-AT + Bay11-7082, MCP-1 = 7,502(524) pg/ml vs. Ox-AT, *P* = 0.049] and the JNK inhibitor [Ox-AT + SP-600125, MCP-1 = 4,658(727) pg/ml vs. Ox-AT, *P* =0.003]. There was no significant reduction in Ox-AT-induced IL-8 and MCP-1 production in the presence of the MAPK inhibitor.

4.5 Effect of inhibitors of NF-κB and JNK on Ox-AT-induced AP-1 and NF-κB activity

To examine whether NF-κB and JNK work independently, JNK inhibitor SP-600125 was used to study Ox-AT-induced AP-1 activity. Ox-AT induced AP-1 activity in a dose-dependent manner. The Ox-AT-induced AP-1 activity was significantly inhibited in the presence of SP-600125 (0.03 mg/ml Ox-AT, *P* = 0.007; 0.1 mg/ml Ox-AT, *P* < 0.001; and 0.3 mg/ml Ox-AT, *P* = 0.006). However, Bay11-7082 had no effect on AP-1 activity. Ox-AT induced NF-κB activity in a dose-dependent manner. Bay11-7082 significantly inhibited Ox-AT-induced NF-κB activity (0.03 mg/ml Ox-AT, *P* < 0.001; 0.1 mg/ml Ox-AT, *P* = 0.018; and 0.3 mg/ml Ox-AT, *P* = 0.007). There was no significant difference in NF-κB activity after addition of the JNK (AP-1 pathway inhibitor) inhibitor. These findings suggest that NF-κB and JNK work independently.

4.6 Relative effect of NF-κB and JNK inhibitors on the induction of IL-8 and MCP-1 treated with Ox-AT.

To check whether NF-κB and JNK work equally, A549 cells (4 x 10^5cells/well) were cultured in a 24-well plate overnight. The following morning, cells were incubated in fresh media for 20 min. Cells were pre-incubated for 20 min with either Bay11-7082 (10 μM) or SP-600125 (10 μM) or in combination of Bay11-7082 (10 μM) and SP-600125 (10 μM). Ox-AT (0.3 mg/ml) was added 20 min

later. 24 hours cultured supernatant was collected and quantified for IL-8 and MCP-1 production, as detailed previously.

Ox-AT-induced IL-8 and MCP-1 production was significantly reduced in the presence of SP-600125 (10 μM) alone and Bay11-7082 (10 μM) alone. Ox-AT-induced IL-8 and MCP-1 production was almost completely abolished in the presence of both SP-600125 and Bay11-7082. These data show that the combination of both inhibitors (SP-600125 and Bay11-7082) acts additively to reduce IL-8 and MCP-1 production.

All these results demonstrated that Ox-AT increased NF-κB and AP-1 activity and promoted activation of JNK. Furthermore, inhibition of the NF-κB and the JNK/AP-1 pathways inhibited production of IL-8 and MCP-1. Both pathways appear to act independently and both appeared to mediate the effects of Ox-AT.

Chapter 5

Ox-AT is pro-inflammatory in animal models

5.1 Intratracheal instillation of N-AT and Ox-AT into murine lungs

After identifying that Ox-AT is pro-inflammatory in cell lines, I sought to further confirm the finding in animal models. All experiments were approved by the Home Office. Female C57BL/6 mice, 8 weeks of age, were anaesthetized and incubated with a nonpyrogenic 22-gauge cannula. Mice received either 40 µl of 2 mg/ml of Ox-AT or N-AT diluted in PBS. A separate group of mice received 40 µl of PBS alone. After 4, 24, and 72 h, cohorts of C57BL/6J mice (at least 6 in each group) were sacrificed. Bronchoalveolar lavage (BAL) was performed with eight aliquots of 0.5 ml of PBS. The BAL samples were centrifuged, and BAL fluid (supernatant) was aliquoted. Protease inhibitor cocktail (Sigma) and PMSF (1 mmol/l) were added, and the samples were stored at –80°C until use. The cell pellet was resuspended, and red blood cells were lysed in 0.15 mol/l NH_4Cl, 0.01 mol/l $KHCO_3$, and 1 µmol/l disodium ethylene diaminetetra acetic acid (pH 7.2) for 30 s followed by a 5-min wash (200 g) in 5 ml of PBS. The cell pellet was resuspended in 1 ml of PBS, and the total number of cells was quantified by the mean of two hemocytometer counts. To determine inflammatory cell types in BAL fluid, cytospins were prepared (350 rpm, 3 min, Cytospin 3, Shandon) from the BAL cellular fraction and were stained with Hema 3 (Fisher, Pittsburgh, PA). The proportions of different inflammatory cells were determined by counting at least 200 cells.

Frozen whole lung tissue from mice was homogenized using Tissue Tearor (Biospec Products) in cell lysis buffer (CelLytic, Sigma) on ice. The lung homogenates were centrifuged, and proteinase inhibitor cocktail (Sigma), PMSF (1 mmol/l), and 1 and 10 phenanthroline (1 mmol/l) were added to the supernatants. The supernatants were kept on ice and immediately assessed for the total protein (RC DC protein kit, Bio-Rad).

5.2 Effect of intratracheal instillation of Ox-AT and N-AT in murine lungs

Intratracheal instillation of Ox-AT resulted in increased JE (homologue of human MCP-1) in the airway and in the lung tissue. In the BAL fluid, 4 h after Ox-AT, there was a significant increase in JE/total protein 16.2(3.6) pg/mg vs. N-AT 2.9(1.9) pg/mg, *P* = 0.037. Concentrations of JE had returned to baseline by 24 h, and there was no significant difference.

There was also a significant increase in JE in homogenized lung tissue 4 h after instillation of Ox-AT. JE/total protein was 24.7(4.1) pg/mg vs. N-AT 10.1(2) pg/mg, *P* = 0.05. After 24-h Ox-AT treatment, there was no significant change.

There was a trend towards increased KC production in the airway and in lung tissue, which was not statistically significant. In the BAL fluid, after 4 h, Ox-AT KC/total protein 11.8(2.1) pg/mg vs. N-AT 7.1(1) pg/mg, *P* = 0.11; 24 h, Ox-AT 7.2(1.7) pg/mg vs. N-AT 6.8 (1.5) pg/mg, *P* = 0.43. In the homogenized lung tissue after 4 h, Ox-AT KC/total protein 48.9 (10) pg/mg vs. N-AT 32.5(5.8) pg/mg, *P* = 0.10; 24 h, Ox-AT 24.7(4) pg/mg vs. N-AT 28.2(2.7) pg/mg, *P* = 0.31. There was no effect of Ox-AT on secretion of TNF$_{\alpha}$ in BAL fluid or lung tissue.

For cells in BALF, intratracheal instillation of Ox-AT was found to significantly induce macrophage influx after 24 h; Ox-AT 23.6(2.8) x 104 vs. N-AT 16.3(0.7) x 104, P = 0.03. Ox-AT did not have any significant effect on the number of neutrophils.

5.3 Effect of Ox-AT on NF-$_{\kappa}$B p50 and AP-1 activity in lung tissue

Following intratracheal instillation of Ox-AT, NF-$_{\kappa}$B was found to be significantly increased in lung homogenates in a time-dependent manner

compared with PBS (4 h, P = 0.028; 24 h, P = 0.029; and 72 h, P = 0.037). Intratracheal N-AT had no significant effect on the activity of NF-κB in lung homogenates

Intratracheal Ox-AT resulted in a significantly increased activity of AP-1 in lung homogenates in a time-dependent manner compared with PBS (4 h, P = 0.019; 24 h, P = 0.033; and 72 h, P = 0.049). Intratracheal N-AT had no significant effect on the activity of AP-1 in lung homogenates.

5.4 Western blot analysis of lung tissue for JNK

Homogenized lung tissue was electrophoresed on a 12% SDS-PAGE and then electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Biosciences). The blots were blocked with PBS/T containing 5% nonfat dry milk for 1 h at room temperature. Western blot for activated or phosphorylated JNK (phospho-JNK) was performed by incubating with a primary antibody, phospho-JNK (1 mg/ml, that detects endogenous human, mouse, and rat Thr183/Tyr185 of JNK1), and phosphorylated and nonphosphorylated JNK (total-JNK, 0.2 mg/ml, R&D Systems) at 1:500 in PBS/T with 5% nonfat dry milk for 1 h at room temperature. The blots were washed three times with PBS/T and incubated with HRP-conjugated swine anti-rabbit IgG at 1:1,000 (Dako). The signals were visualized by an enhanced chemiluminescence (ECL) system (Amersham). Densitometric semiquantitative analysis of Western blot bands was performed using Image Processing and Analysis in Java (ImageJ), version 1.41j. The density of each protein band was corrected for background and expressed in arbitrary units. Western blot analysis of homogenized lung tissue demonstrated that phospho-JNK was induced by Ox-AT compared with N-AT at 4 h, 24 h, and 72 h. Densitometric analysis using ImageJ software[a] showed that the level of phospho-JNK was significantly higher in lung homogenates from Ox-AT-treated mice, peaking at 24 h compared with lung homogenates from N-AT-treated mice (4 h, P = 0.031; 24 h, P = 0.012; and 72 h, P = 0.023). There was no significant difference between the amount of total JNK detected from lung homogenates from Ox-AT- and N-AT-treated mice at 4, 24, and 72 h.

The results from animal experiments further confirmed Ox-AT was proinflammatory and Ox-AT increased NF-κB and AP-1 activity and promoted activation of JNK.

Chapter 6

Acute cigarette smoke exposure promoted polymerization of Z-AT *via* the generation of Ox-AT (Alam S, 2011)

6.1 Cigarette smoke exposure induces polymers of AT and increases pulmonary neutrophils in transgenic Z-AT mice

After identifying that polymerised AT and Ox-AT are proinflammatory, I wished to investigate the relationship between them. The susceptibility of Z-AT individuals who smoke to develop COPD is in part related to the combination of the severe anti-elastase deficiency arising from an absolute and functional reduction in neutrophil elastase inhibitory capacity (Stoller, 2005; Ogushi, 1987), and the independent multiple effects of cigarette smoke on inflammatory cells and molecules (Barnes et al., 2003). In order to investigate the molecular basis for the interaction of among Z-AT, Ox-AT and cigarette smoke, heterozygous mice transgenic for human Z-AT and human M-AT on a CBA background (Ali, 1995) was developed. All animals were maintained under specific pathogen-free conditions. Cohorts of 6-10 eight to ten week old female mice transgenic for M-AT or Z-AT were exposed to 4 cigarettes smoke (CS) per day for 5 days as previously described (Hautamaki, 2004)

The effect of cigarette smoke (CS) exposure on formation of polymers of AT was assessed in bronchoalveolar lavage fluid (BALF) and lung homogenates (LH's) of mice transgenic for Z-AT (Z-mice) and M-AT (M-mice). Polymeric AT was undetectable in BALF and LH's from control M and Z and CS exposed M mice. However, following acute CS exposure there was a significant amount of polymeric AT detected in the BALF of Z-mice (Fig 6).

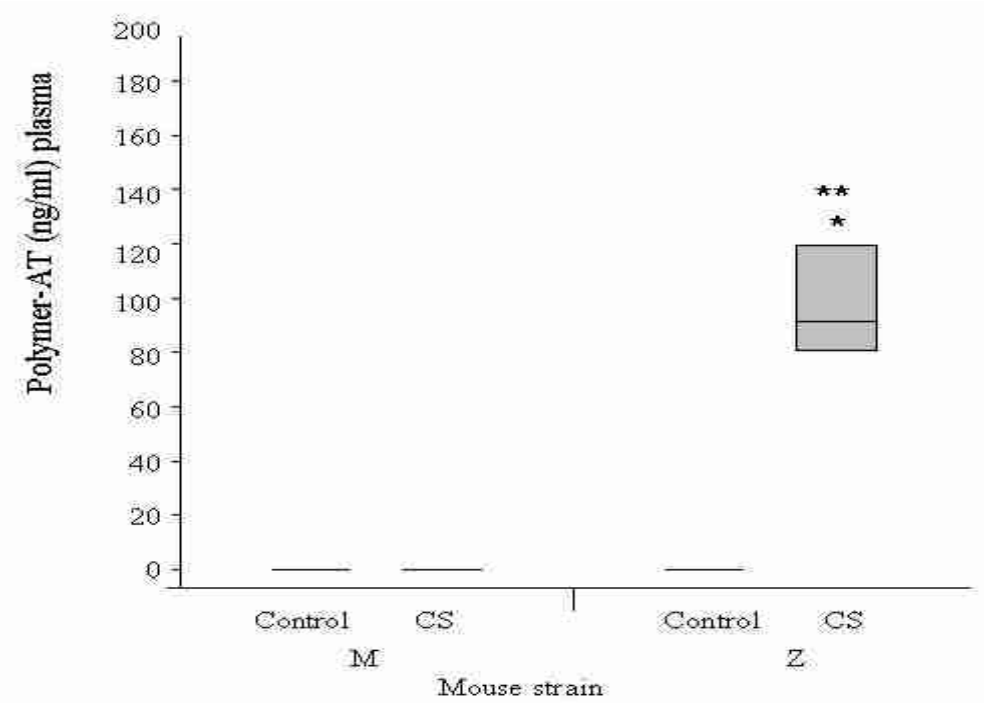

Figure 6 Significant increase in pAT in plasma of CS-Z when compared to control Z mice; p=0.002. Polymeric Z-AT was undetectable in the plasma of control M and Z or in CS-M mice. *, control vs. CS and **, CS-M vs. CS-Z mice.

Acute CS exposure, as expected, also resulted in a significant increase in polymorpholeukocyte numbers in BALF of both M and Z-AT transgenic mice compared to controls, p=0.002 for both. However, CS-Z mice had significantly more neutrophils in BALF compared to CS-M mice, p=0.043. The role of the excess neutrophils in BALF in CS-Z mice was investigated further by assessment of free Neutrophil elastase (NE). CS exposure resulted in a significant increase in free NE in BALF in CS-Z compared with CS-M mice. The amount of pAT in BALF and LH's strongly correlated with both BALF and LH neutrophil numbers; r2=0.93 and 0.88, respectively (Fig 7).

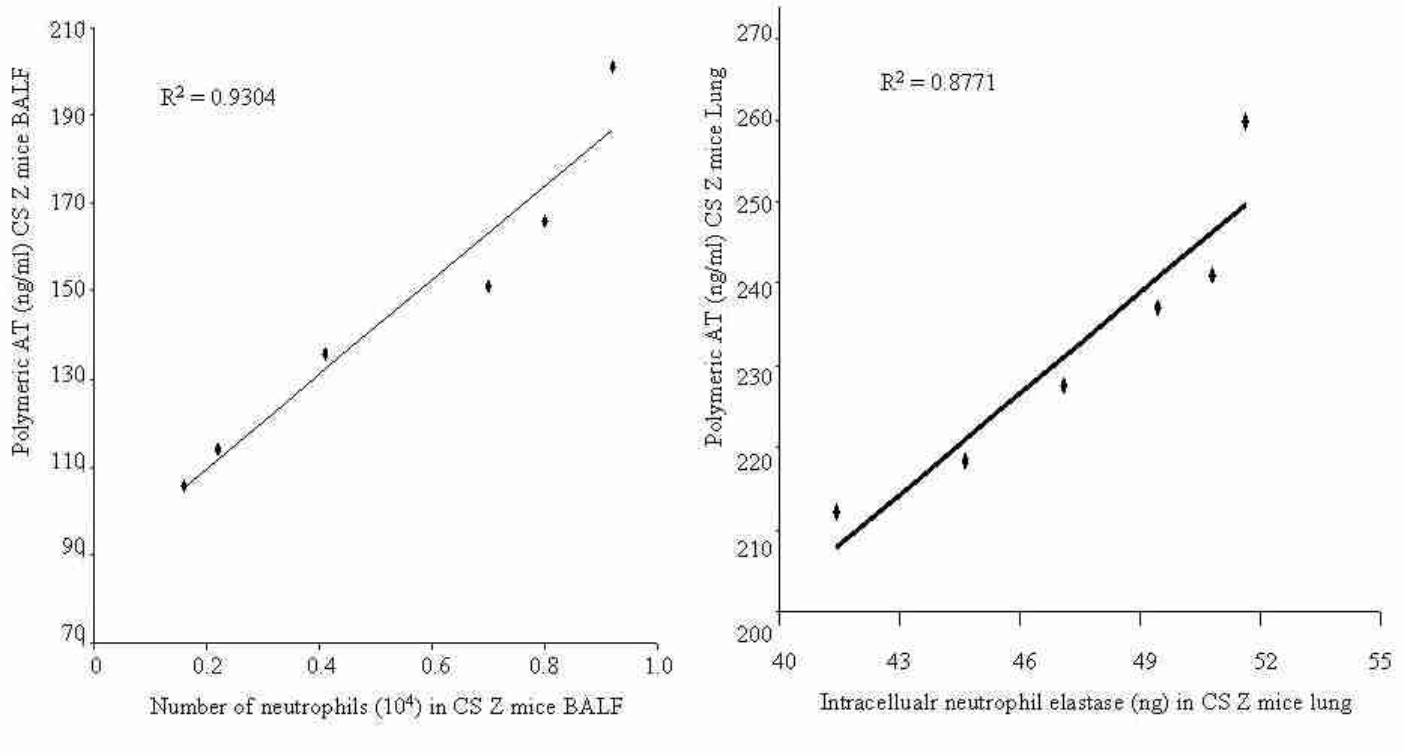

Figure 7 Correlation between pAT and neutrophils in BALF (L) and LH (R). r^2=0.93 for BALF and and 0.88 for LH, respectively. The amount of pAT in BALF and LH's strongly correlated with both BALF and LH neutrophil numbers.

6.2 Cigarette smoke extract induces the formation of oxidized-polymeric Z AT in Z-AT mice[a]

It is known that cigarette smoke contains oxidants that can produce oxidized AT (Ox-AT) *in vitro* (Rahman I, 1999). Therefore, it is hypothesized that oxidative modification of the Z-AT molecule by cigarette smoke could lead to accelerated polymerization of Z-AT.

Acute CS exposure caused production of Ox-AT in the BALF and LH of M-AT and Z-AT mice (Fig 8). Interestingly, there was a significantly greater amount of Ox-AT detected in CS-Z compared with CS-M mice; BALF, p=<0.001 and LH, p=0.029. Acute cigarette smoke exposure also caused significantly

increased production of Ox-AT in the plasma of M-AT and Z-AT mice compared to control mice, p=0.024 and p=0.002,respectively.

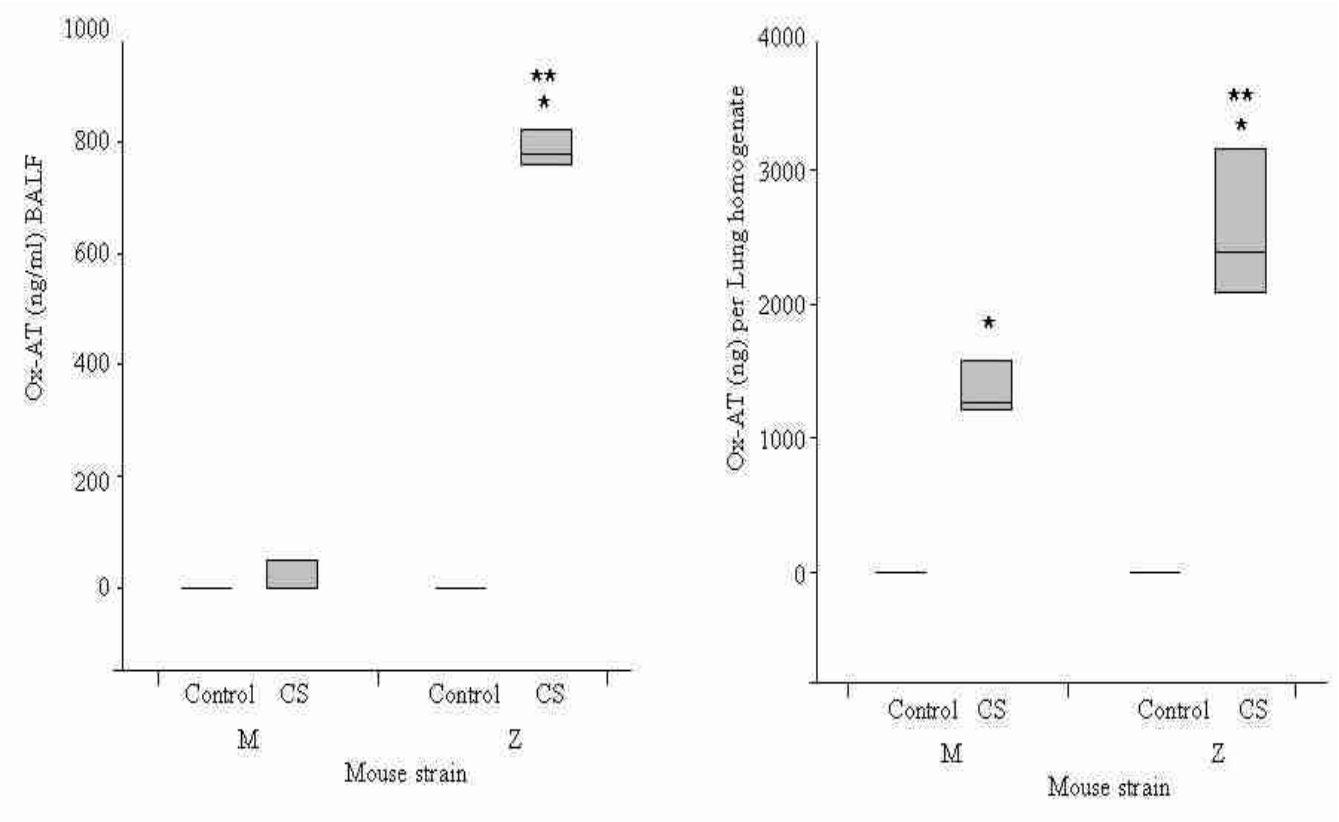

Figure 8 Cigarette smoke extract significantly increase the amount of Ox-AT in BALF and lung homogenates of CS-Z mice when compared to control Z mice.

Although the results of CF exposure promoting polymerization of Z-AT and Ox-AT production were exciting, it was difficult to explain their relationship. Further experiments were on hold for about one year, until a new term “oxidized polymeric AT“, suddenly appeared in the mind. If the hypothesis that acute CS exposure promotes polymerization of Z-AT *via* the generation of Ox-AT is correct, then the production of oxidized polymeric Z-AT was anticipated.

Therefore, a novel assay to detect these conformers was set up[a]. Briefly, 96-well ELISA plates were coated with mouse anti-human Ox-AT overnight at 4oC. Plates were washed initially with water and blocked with 5% milk in PBS for 2 hrs at RT. After standard washes and blocking, the standard ELISA protocol for polymeric-AT was followed by 3 washes with PBS/T and

subsequently BALF or lung homogenate was loaded onto the plates and incubated for 2 hrs at RT. Plates were washed with PBS/T and incubated with a monoclonal anti-polymer antibody. Following 3 washes plates were incubated with a rabbit anti-mouse immunoglobulin for 1 hr and developed with the substrate and quantified by reading absorbance in a microplate reader at wavelength 405 nm. Standards for oxidized-polymeric AT were prepared by heating 50 µl Ox-AT (1 mg/ml) in PBS at 65^{o}C for 45 mins, and polymerization of Ox- AT was confirmed by 7.5% non-denaturing PAGE.

Oxidized polymers were only detected in CS-Z mice BALF, LH and plasma. Whilst there was evidence of the monomeric oxidized species of AT in CS-M mice, there was no detectable oxidized-polymer in the BALF, LH or plasma in these mice. This is consistent with the *in vitro* data that M-AT has a much slower rate of polymerization than ZAT. The production of oxidized-polymer in CS-Z mice was confirmed by Western blot analysis of samples separated on 7.5% non-denaturing PAGE and 12% SDS-PAGE gels. Oxidized AT was only present in the monomeric form in CS-M mice, whereas both oxidized-polymers and oxidized monomers were clearly seen in CS-Z mice.

6.3 Analysis of the Relationship between Oxidation and Polymerization of Z-ATa

Plasma-purified Z-AT was exposed to CSE to assess for the temporal relationship between the oxidized and polymeric conformers.

CSE rapidly induced oxidation of plasma Z-AT by 24 hours compared with control (P = 0.002; Furthermore, CSE resulted in a 10-fold increase in polymers of Z-AT by 24 hours when compared with control Z-AT (P = 0.004). Production of the oxidized polymeric conformation in CSE Z was also increased 10-fold compared with control Z-AT (P = 0.02).

More detailed analysis of the immediate effect of CS exposure revealed that oxidation of Z-AT preceded polymerization of the protein. Oxidation was significantly higher when compared with polymerization (P < 0.001) at the 4- to 24-hour time period.

To assess this further, we incubated plasma-purified Z-AT with the oxidizing agent, *N*-chlorosuccinimide. *N*-chlorosuccinimide also increased the polymerization rate of Z-AT by 3.2-fold compared with control Z-AT at Day 1 (P = 0.004), thus supporting the concept that oxidation of Z-AT promoted polymerization of Z-AT.

6.4 The effect of the anti-oxidant *N*-acetyl cysteine on CS extract induced oxidation and polymerization of Z α1-antitrypsin[a]

To further confirm that acute CS exposure promoted polymerization of Z-AT *via* the generation of Ox-AT, *N*-acetyl cysteine (NAC) was used to abolish the oxidant capacity of CSE. The results showed a reduction in the polymerization rate of Z-AT back to its baseline rate; the rate of polymerization of CSE Z over the first 24 hours was 114.4 nmol/hour, compared with 10.3 nmol/h for control and 13.3 nmol/h for CSE + NAC + Z-AT; p=0.135 .The addition of *N*-acetyl cysteine also resulted in a significant reduction in the rate of formation of oxidized-polymers of Z-AT back to its baseline rate; CSE Z; 40.4 nmol/h compared with 7 nmol/h for control and 4.9 nmol/h for CSE + NAC + Z-AT; p=0.285 .

All these experiments show that CS accelerates polymerization of Z-AT by oxidative modification, which, in so doing, further reduces pulmonary defense and increases neutrophil influx into the lungs.

Chapter 7

Conclusions and future work

7.1 Conclusions

COPD is one of the five leading causes of death worldwide (Mannino D M, 2007). Unfortunately, there is no cure available for COPD. The majority of patients with COPD (80%) are cigarette smokers; however, COPD affects only 15–25% of smokers. It is assumed that cigarette smoke is also the major cause of chronic inflammation of the lungs. This inflammation does not stop after cessation of smoking (Teamoto S, 2007). Pathogenesis of COPD remains incompletely understood, there were three main theories: protease – antiprotese imbalance, oxidative–antioxidative imbalance, and inflammation (Abboud RT, 2008; Mak JCW, 2008; Roth M, 2008).

7.1.1. Protease-antiprotease imbalance in severe AT deficiency

There is strong evidence to support this hypothesis as the main pathogenic mechanism in emphysema associated with severe AT deficiency, as AT is the main inhibitor of neutrophil elastase. My experimental results showed another mechanism leading to a protease-antiprotease imbalance in the lung in severe antitrypsin deficiency (piZZ) is that the abnormal Z antitrypsin polymerizing in the lung acts as an inflammatory factor, leading to neutrophil recruitment in the lung.

A novel mechanism driving the development and progression of emphysema in Z-AT homozygotes has been identified. Cigarette smoke directly accelerates polymerization of Z-AT *via* oxidation of the protein leading to further depletion of the neutrophil elastase protection in the lung and enhanced neutrophil influx. The data uniquely suggests that rather than the major risk factors for COPD namely cigarette smoke and Z-AT deficiency having independent additive effects, they directly interact to create an effect greater than the sum of the individual risks. Furthermore, these observations

link two of the major prevailing hypotheses in COPD namely, oxidants and proteinases in Z-AT related emphysema, and this suggests a role for antioxidants in the treatment of this group of individuals (Fig 9).

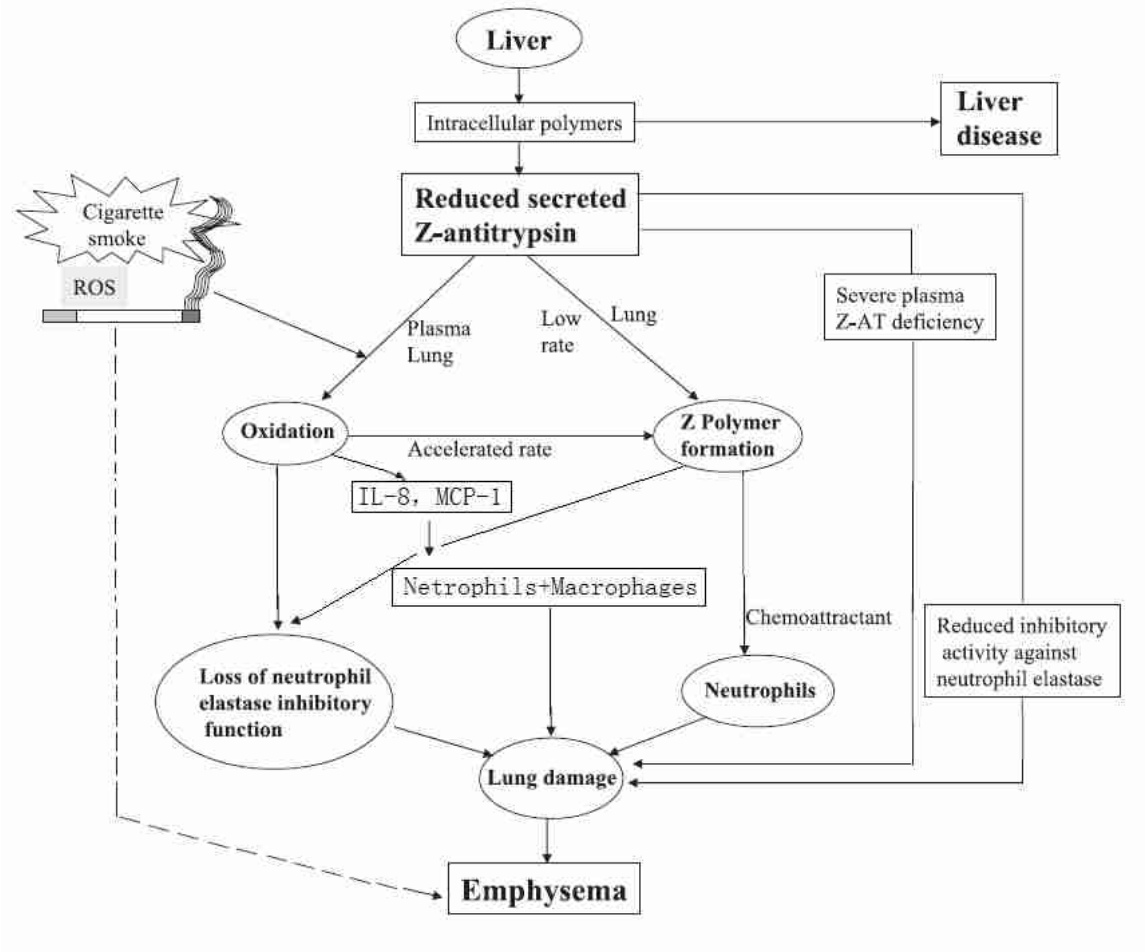

Figure 9. Schematic diagram depicting the role of conformations of AT and the interaction with CS in the development of emphysema.

7.1.2. Protease-antiprotease imbalance in COPD without severe AT deficiency

The importance of oxidative stress in both lungs and systemic circulation is well established in COPD (MacNee W, 2005). Sources of oxidative stress arise from inflammatory cells, such as neutrophils and macrophages, which generate reactive oxygen species (ROS) when activated. Together with inhaled ROS and reactive nitrogen species (RNS), which are abundant in cigarette smoke, these endogenous oxidants may constitute a major oxidative

burden to the lungs. Oxidative stress occurs when the burden of oxidants is not well counterbalanced by the antioxidant defence system.

Hydrogen peroxide in cigarette smoke and N-chloroamines and hypochlorous acid in neutrophils can oxidize and inactivate AT. My results showed oxidized AT (Ox-AT) promotes release of human monocyte chemoattractant protein-1 (MCP-1) and IL-8 from human lung type epithelial cells (A549) and normal human bronchial epithelia (NHBE) cells. These findings were supported by animal experiments. The effect of Ox-AT was dependent on NF-κB and activator protein-1 (AP-1)/JNK. Ox-AT not only lost its characteristic ability to neutralise elastase, but also induced attraction of neutrophils by Il-8 and MCP-1 to produce more elastase, leading to proteolysis of lung connective tissue and emphysema (Fig 9).

7.2. Suggestions for future work

7.2. 1. Novel Inhibitors

Recently, some progress has been made on inhibition of polymerization. It is recognized that polymerization could be prevented by synthetic peptides with homology to strand 4a. However, these peptides are large and also anneal to M-AT and other members of the serpin superfamily such as α 1- antithrombin and therefore were not suitable as therapeutic agents. Understanding the distinct conformation adopted by the Z-AT protein facilitated the design of a shorter six-mer synthetic peptide (Ac-FLEAIG-NH_2). This 6-mer peptide preferentially bound to Z-AT rather than M-AT and other common circulating serpins. However, the specific binding of the 6-mer to pathologic Z-AT renders the Z-AT as inactive, and therefore would not address the predisposition of Pizz homozygotes to develop emphysema (Mahadeva R, 2002).

A shorter four-mer synthetic peptide, Ac-TTAI-NH_2 has been identified that specifically anneals to the pathologic Z-AT by my colleagues (Alam S, 2012). The cell studies showed that it entered the ER of the Z-AT cell without any cytotoxicity, and was able to abrogate Z-AT polymerization in vivo. It was also able to dissociate existing intracellular Z-AT polymers/aggregates. In addition, metabolic labeling demonstrated that in a cell model it was able to significantly

restore normal secretion of Z-AT. Furthermore it reduced the ER overload response as demonstrated by inhibition of PERK-dependant NF-κB activity. These findings uniquely demonstrate the potential of this strategy for prevent of Z-AT polymer formation in the liver, with preservation of inhibitory function in the tissues.

From the experimental results presented here, IL-8, MCP-1 production induced by OX-AT was dependent on NF-kappa B and activator protein-1 (AP-1)/JNK. Abolishing the oxidant capacity of CSE by *N*-acetyl cysteine (NAC) resulted in a reduction in the polymerization rate of Z-AT. These findings imply anti-oxidant could be used to treat, or possibly prevent emphysema. The mouse model could easily be adapted to test this hypothesis.

Imbalance between protease and antiprotease induces emphysema, suggesting that AT could be used to treat and prevent emphysema. Recombinant alpha 1-antitrypsin is not yet commercially available, but is under investigation as a therapy for alpha 1-antitrypsin deficiency. Therapeutic concentrates are prepared from the blood plasma of blood donors. The US FDA has approved the use of three alpha 1-antitrypsin products derived from human plasma: Prolastin, Zemaira, and Aralast. However, these products for intravenous augmentation AT therapy can cost up to $100,000 per year per patient. They are administered intravenously at a dose of 60 mg/kg once a week. Thus, bulk commercial production of recombinant alpha 1-antitrypsin currently seems to be a major barrier to this approach.

Poly (D, L lactide-co glycolide) (PLGA) is a biodegradable and biocompatible polymer approved for sustained controlled release of peptides and proteins. Pirooznia N *at al* (Pirooznia N, 2012) prepared a wide range of particle size as a carrier of protein-loaded nanoparticles to deposit in different parts of the respiratory system especially in the deep lung. The lactic acid to glycolic acid ratio affects the release profile of α1AT. Hence, PLGA with a 50:50 ratios exhibited the ability to release %60 of the drug within 8, but the polymer with a

ratio of 75:25 had a continuous and longer release profile. Cytotoxicity studies showed that nanoparticles do not affect cell growth and were not toxic to cells. α1AT-loaded nanoparticles may be considered as a novel formulation for efficient treatment of many pulmonary diseases.

7.2.2. Aerosol delivery of inhibitors

Aerosolized-augmented AT therapy is currently under study. This involves inhalation of purified human AT into the lungs, so trapping the AT into the lower respiratory tract. This method proves more successful than intravenous-augmented AT therapy because intravenous use of AT results in only 10%-15% of the AT reaching the lower respiratory tract, whereas 25%-45% of AT can reach the lower respiratory tract through inhalation. Ma *et al* assayed levels of Desmosine and isodesmosine (DI), amino acid cross-links in mature elastin, in body fluids which indicated degradation of elastin and can be measured more specifically by mass spectrometry. Administration by aerosol produced statistically significant reductions in levels of DI in BALF(Ma S, 2012).However, inhaled AT may not reach the elastin fibers in the lung where elastase injury actually occurs.

Spencer *et al* observed systemic antibody responses to nonhuman protein that was present in very low concentrations in a sheep-derived transgenic human AAT formulation (Spencer LT, 2005). Strategies to evaluate immune-mediated toxic effects of preparations of transgenic protein should be established to ensure the development of safe and effective therapies. Once sufficient quantities of recombinant alpha 1-antitrypsin become available, further more study should be done in this area.

7.2.3. Stem cell therapy

Finally, stem cells may be used for treatment for end stage emphysema patients. Embryonic and adult stem cells have been suggested as possibilities. Adult stem cells have traditionally been thought of as having limited differentiation ability and to be organ specific. Kim *et al* found impaired colony-forming capacity of circulating endothelial progenitor cells in patients with

emphysema. this may contribute to the development of emphysema(Kim EK, 2012).However, a series of exciting reports over the last 5 to 10 years have suggested that adult bone marrow-derived stem cells may have more plasticity and are able to differentiate into bronchial and alveolar epithelium, vascular endothelium, and interstitial cell types, making them prime candidates for repair (Loebinger MR, 2007). Inflammatory lipid mediators, like leukotriene B4, may control the proliferation of some stem cell populations, again perhaps pointing toward a potentially homeostatic role of some elements of the inflammatory repertoire (Taraseviciene-Stewart L, 2008). Mesenchymal stem cells protect cigarette smoke-damaged lung and pulmonary function partly via VEGF-VEGF receptors, suggesting that MSCs have a therapeutic potential in emphysematous rats by suppressing the inflammatory response, excessive protease expression, and cell apoptosis, as well as up-regulating VEGF, VEGF receptor 2, and TGFβ-1(Guan XJ, 2013).

7.2.4. Recent advances in my research

Since publication of my last paper I have already conducted some work on stem cells. Human cord blood cells were transfused into C57BL/6 mouse lung by intravenous or intratracheal routes. The cord cells persist in the lung for up to 5 days. Large amounts of lymphocytes were also found in BAL fluid, possibly because of rejection response. In future experiments, SCID mouse and emphysema mouse models may be used and the long-term effects will be monitored. If these experiments are successful, stem cells may be a revolution in the development of new therapies for COPD.

However, as I know, there are a lot of patent limitations for the future research or production, from antitrypsin purification using human plasma, expression and production from plant, rice, milk, bacteria, yeast, *et al* to treatment via intravenous or aerosolization.

Although the role of alpha antitrypsin in the pathogenesis of COPD is still far from being fully understood I feel that my work has made a step towards clarification of the mechanism that underpin the pathology of this common

disease. I therefore hope in the future to make further contributions that will build on the work described in this thesis.

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Declaration of my contribution(s) to published work

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