University Of Southampton

The role of Toll-like receptors in human lung tissue

David Howell

Supervisor: Dr J A Warner
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ABSTRACT

SCHOOL OF MEDICINE

Viral exacerbations of COPD play a major role in disease pathogenesis. The specific mechanisms through which viruses invoke exacerbations, however, remain unclear. To date, there is little information on the inflammatory response induced by many different viruses and likewise the kinetics and dose dependency of this response. To better understand the innate immune response of human lung tissue to viral infections, this study investigated the role of TLR3 in response to the TLR3 agonist Poly I:C and killed influenza virus. These agonist responses were compared to that of a known inflammatory stimulus, LPS, a well recognized TLR4 activator. Using a tissue explant model system, it has been shown that ligands for TLR3 and TLR4 can initiate responses in human lung tissue of mild to moderate COPD patients. It is apparent however, that the responses between the ligands for these receptor subtypes differ markedly based upon the cytokines produced and their kinetic profiles. It has been demonstrated that different classes of the chemokine family may contribute significantly to the inflammatory response for Poly I:C, killed virus and LPS. Interestingly, however, there appears to be marked inter-patient variability for chemokine production with Poly I:C. It has also been shown that Th_2 cytokines do not appear to be significantly elevated by Poly I:C or killed virus, suggesting the mechanisms of viral exacerbation are likely to be a Th_1 response. Interestingly, it was also observed that pro-inflammatory cytokines, TNFα, IL-1β and IL-6 were not significantly elevated by Poly I:C or killed virus, which was in complete contrast to the response seen with LPS. Overall, this work provides an insight into the contribution of cytokines in the acute inflammatory response to viral and bacterial infection.

Abbreviations

BAL	Bronchoalveolar lavage
COPD	Chronic obstructive pulmonary disease
DC	Dendritic cell
DsRNA	Double stranded RNA
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbant assay
ENA-78	Epithelial derived neutrophil chemoattractant-78
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GOLD	Global initiative for chronic obstructive pulmonary disease
HRP	Horse radish peroxidase
HSA	Human serum albumin
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP–10	Inducible protein-10
IRAK	IL-1 receptor associated kinases
IRF	Interferon regulatory factor
LPS	Lipopolysaccharide
MIP-1β	Macrophage inflammatory protein 1-beta
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
NF-κB	nuclear factor-kappa B
NK	Natural killer cell
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PGN	Peptidoglycan
Poly I:C	Polyinosinic:polycytidylic acid
PPR	Pattern-recognition receptor
RNA	Ribonucleic acid
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TMB	3, 3', 5, 5' tetramethyl benzedine
TNF	Tumour necrosis factor
TRAF	TNF receptor associated factors

Chapter 1:

Introduction

1. Introduction

1.1 Chronic obstructive pulmonary disease (COPD)

COPD is a slowly progressive chronic airways disorder, characterized by unalleviated inflammation that perpetuates and accumulates locally, resulting in gradual decline of lung function (MacNee et al 2005). It represents the fourth leading cause of death in the U.K and is projected to be the third major cause of death worldwide by the year 2020 (Johnston et al 2005). COPD is defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as 'a disease state characterised by airflow limitation that is not fully reversible' (Barnes 2006). Airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases. In particular, the GOLD guidelines take into account the importance of cigarette smoking and airways inflammation. GOLD ranks disease in four stages: 0 = at risk; stage I = mild COPD; stage II = moderate COPD; stage III = severe COPD. COPD is used as an umbrella term for the clinical diagnosis of chronic bronchitis, chronic obstructive bronchitis, and emphysema, or more commonly, overlapping combinations of these conditions (Johnston et al 2005). The degree of overlap varies between individuals and leads to a wide disease heterogeneity. COPD rarely affects patients under the age of 25; isolated adolescent cases can be related to genetic or extreme environmental abnormalities. The most important risk factor for COPD is cigarette smoking. Evidence suggests that active cigarette smoking accounts for 85-90% of total COPD cases worldwide (Snider et al, 1989). Pipe, cigar, other types of tobacco smoking, and passive exposure to cigarette smoke are all significant risk factors. It is still not clear, however, why many life long smokers never develop the disease.

Persistent exposure to the complex mixtures of particulates, chemicals and free radicals present within cigarette smoke, lead to pulmonary infiltration of activated macrophages, neutrophils and $CD8^+$ T-lymphocytes (MacNee et al 2005). It is believed that recruitment of these inflammatory cells, together with a combined response from the peripheral tissues, is the cause or effect of increased local expression of numerous cytokines, chemokines, and adhesion molecules. Over time, the bronchioles become irreversibly obstructed with mucus and inflammatory exudate. In addition, factors released from macrophages and neutrophils cause inactivation of anti-proteinases and oxidative injury. Together, these factors ultimately lead to tissue destruction and airway wall remodelling. COPD causes pathological changes in all conducting airways of the lung (Wilkinson et al 2003). There is hypertrophy of the mucus glands in the large central conducting airways (chronic bronchitis), inflammatory exudate obstruction and fibrosis of the bronchioles (bronchiolitis) and permanent enlargement of the airspaces in the alveoli (emphysema). Studies have shown that there are indeed structural abnormalities in the small airways of smokers with and without COPD (Barnes et al 2006). Furthermore, there is also a relationship between the severity of COPD and the extent of occlusion of the airway lumen by inflammatory mucous exudates. During disease progression, afflicted individuals increasingly lose their ability to respire. Annual decline of FEV_1 typically increases to around 80mls per year, compared with only 30mls for non-smokers (Donaldson et al 2003). Furthermore, COPD patients are subject to periodic exacerbation of their disease most often precipitated by bacterial or viral infections and exposure to airway pollutants (*figure 1.1*).

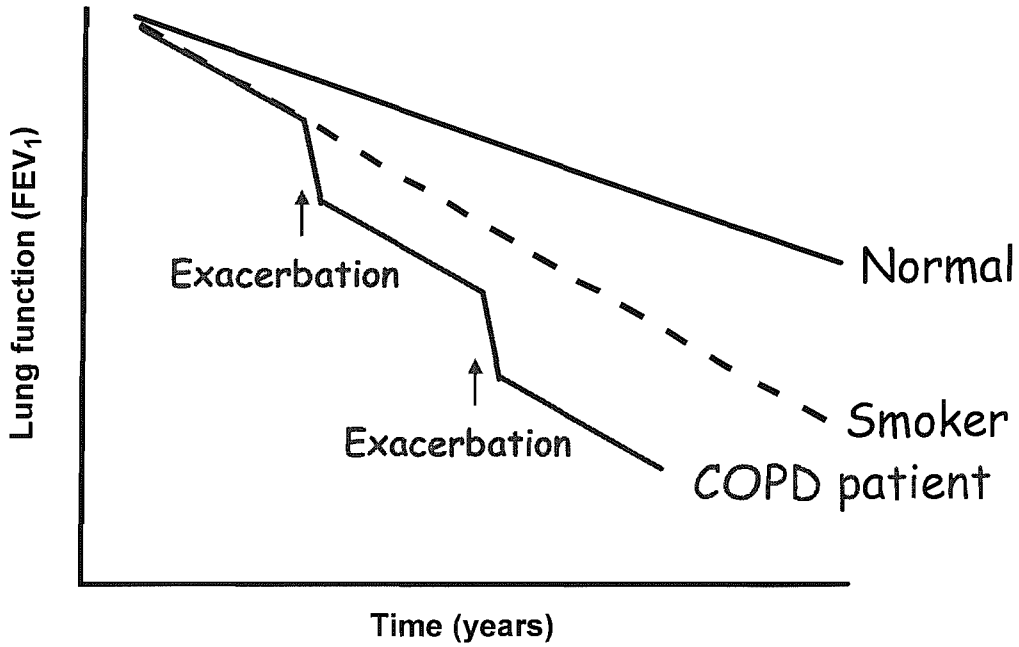

Figure 1.1: The effect of exacerbations of COPD on lung function. This schematic diagram demonstrates the accelerated decline in lung function observed in patients with acute exacerbations of COPD. This has been compared to lung function decline in healthy smokers and in natural lung function decline with age. Lung function lost during exacerbations of COPD are never fully recovered.

1.2 Exacerbations

Exacerbations are an important determinant of health-related quality of life, morbidity and mortality in COPD (Wedzicha et al 2002). Conners et al (1996), found that approximately half of patients admitted to hospital for an exacerbation of COPD, died within the following two years. Acute exacerbations of COPD are defined as 'a sustained worsening of the patients condition that is acute in onset, and necessitates a change in regular medication in a patient with underlying COPD' (Rodriguez-Roisin et al 2000). Exacerbations are characterised by acute worsening of symptoms, increased airway inflammation and deterioration of lung function. Occasionally, this occurs to the extent where hospitalisation

maybe required and is a major component of the 'winter bed crisis'. Exacerbations clinically manifest as dyspnoea, cough, increased sputum volume and purulence, and non-specific symptoms such as fatigue and malaise. Due to the vast heterogeneity of COPD, classification to define the severity of exacerbations has proven difficult. In 1987, Anthonisen et al proposed a simple classification method based upon clinical manifestations of the disease. In Type-1 exacerbations, patients presented with all the major symptoms of increased dyspnoea, sputum volume, and sputum purulence. Type-2 patients presented with only two of the latter symptoms and Type-3 with only one symptom combined with mild cough and wheeze. Dyspnoea is recognised as a key symptom of exacerbation and is often preceded by bacterial or viral infection. On average, COPD patients experience a median of 2.5 exacerbations per year; however their frequency increases with disease severity (Seemungal et al, 1998). A study of 5887 patients in the Lung Health Study showed that COPD smokers with chronic bronchitis had exacerbations 1.6-1.9 times more frequently than those without (Kanner et al 2001). This suggests factors associated with smoking and chronic bronchitis predispose patients to exacerbation, possibly due to reduced mucociliary clearance (Mossberg et al 1986), facilitating bacterial or viral infection of the mucosa (Wilson et al 1985, Potts et al 2002). These studies emphasise the importance of targeting COPD exacerbations, with aims to significantly reduce the impact of disease progression and the huge economic burden to society.

1.3 Evidence for bacterial induced exacerbation

Historically, bacteria have been considered the main infectious cause of exacerbation. Whilst many studies support this view, the precise mechanisms of bacterial induced

exacerbation remain controversial. In a large Manchester COPD cohort, BAL fluid was obtained from patients hospitalised during a period of severe exacerbation. Of those, around 50-60% of patients were found to have high levels of bacterial infection (Johnston et al 2005). The most common bacteria isolated were non-typeable *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae*. These species of bacteria are also implicated in the pathogenesis of many other respiratory and epithelial tract infections (Sethi et al 2001). In contrast, however, bacteria can also be detected in potentially pathogenic quantities in around 30% of patients with stable COPD (Murphy et al 2000). Furthermore, it has been suggested that if bacteria are an important cause of exacerbation, antibiotics should show dramatic benefits over placebo in randomized double-blind trials. However, the results such studies have been inconsistent and on the whole, demonstrate little to no benefit (Donaldson et al 2003). These findings suggest more research is required to fully elucidate the role bacteria play in exacerbations of COPD.

It has been proposed that lower airway bacterial colonisation can result in activation of the host defences leading to the release of inflammatory cytokines and leukocyte recruitment, mucus hyper-secretion, impaired mucociliary clearance and respiratory epithelial cell damage (Alder et al, 1986, Wilson et al 1985). Early research into airway colonisation uncovered that bacteria was associated with significant inflammatory changes in the peripheral tissues. Sethi et al (2001) showed that airway infection with H. *influenzae* was associated with significantly higher levels of airway inflammatory mediators including TNFα, IL-6 and IL-8. Wilson et al (1985) demonstrated that *Streptococcus pneumoniae* was capable of stimulating pro-inflammatory cytokines TNFα and IL-8 from bronchial

epithelial cells. Bacteria can also stimulate the influx of numerous pro-inflammatory cell types. Soler and colleagues (2006) demonstrated that the presence of *H. influenzae* in BAL fluid from COPD patients obtained during bronchoscopy, also correlated with a higher degree of neutrophilic and activated macrophage influx. Rodriguez-Roisin et al (2000), showed elevated airway bacterial load in quantitative sputum cultures from patients with COPD. Furthermore, a proportion of patients with COPD also have increased numbers of bronchial eosinophils, which may contribute to inflammation (Pesci et al, 1998). Interestingly, although one may expect an exacerbation to result from acutely increased bacterial colonisation, only a minority of studies have confirmed this. Jadwiga et al (2002) reported that *P. aeruginosa* count was related to the degree of airflow obstruction and exacerbation frequency. The majority of research however, fails to provide such a direct link and requires further investigation. It has been recently suggested that changes in strains of bacteria, rather than bacterial load, maybe responsible for the cause of exacerbations, however this theory remains to be proven.

1.4 Evidence for viruses as a cause of exacerbation

More recent clinical investigations have suggested that viruses play a more prominent role in the aetiology of exacerbations. In a report from an East London COPD cohort, a respiratory virus was identified in 39% of outpatients treated for mild to moderate exacerbations, with rhinoviruses accounting for 58% of total virus present (Mallia et al 2006). Two separate studies in patients with more severe exacerbations requiring hospital admission, detected a respiratory virus in 56% and 64% of patients respectively (Wilkinson et al 2004, Mallia et al 2006). Collectively, these studies suggest that as many as 40 to 60%

of acute exacerbations of COPD are associated with respiratory viral infection. In addition, epidemiology has provided many links for virus as a cause of exacerbation. An association between the onset of cold prior to an exacerbation has long been recognized. It has also been observed that exacerbation frequency in COPD patients is increased during the winter months when there are more respiratory viral infections present in the community. Furthermore vaccination, especially against influenza virus has been shown to significantly benefit patients with COPD (Kristin et al, 1999). Despite these observations and current research publications, the cellular and molecular mechanisms by which viruses cause exacerbations are poorly understood.

The prevailing view for many years was that viruses could not survive in the lower airways, based on evidence that the optimal temperature for the growth of many viruses was 33°C. Papadopoulos et al (1999), however, demonstrated that both influenza and rhinovirus can replicate in the conditions of the lower airway epithelium. It is now widely accepted that viral replication and damage to the lower airway epithelium does occur and is likely to contribute to COPD exacerbations. It is believed that an exacerbation following respiratory viral infection is a consequence of both the direct cytotoxic effects of the virus itself and of localised inflammation. It is well established that viral exacerbations are associated with more severe inflammatory changes in the lung than non-viral pathogens. This supports clinical evidence that viral exacerbations are accompanied with greater symptom severity and prolonged recovery. Johnston et al (2005) demonstrated that viral exacerbations generate significantly higher levels of the pro-inflammatory cytokine IL-6, than any other known cause of exacerbation. In addition, *in vitro* infection of airway epithelial cells with

rhinovirus induces the secretion of a host of pro- and anti-inflammatory mediators (Hennan et al, 2001). This also occurs *in vivo* in both experimental and naturally acquired viral infections (Sethi et al, 2006). In the lower respiratory tract, increases in IL-6, IL-8, and the chemokines regulated on activation, normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein 1 beta (MIP-1β) and epithelial derived neutrophil chemoattractant 78 (ENA-78) have been documented in the sputum of COPD patients (Johnston et al 2005). The production of chemokines by epithelial cells in response to a viral infection leads to an influx of activated macrophages, eosinophils and neutrophils into the airways (Harris et al 2005). The release of inflammatory cell products such as neutrophil elastase from neutrophils, major basic protein and eosinophil cationic protein from eosinophils, and reactive oxygen species can cause tissue damage and fibrosis. Experimental rhinovirus infection studies have reported increased numbers of neutrophils in BAL fluid samples but not in sputum samples (Van Reeth et al 2000). In contrast to bacteria, there is definitive proof that viral concentrations in the lung are directly associated with the onset of exacerbation. Smith and co-workers (2005) were able to show greater numbers of viral isolates during exacerbations when compared to clinically stable periods of the disease Bandi et al 2003). The contribution of viruses to acute exacerbations of COPD still requires further investigation.

Overall, in order to develop more successful strategies to treat and prevent exacerbations, it will be important to elucidate the aetiology of exacerbation and further understand the complex interactions of the innate immune system.

1.5 The Innate immune system

The innate immune system represents the first line of defence against microbial infections in the lung. It is composed of many cells, including the epithelial barriers, phagocytes and natural killer cells. The major role of the innate immune system is to detect and respond rapidly to infectious agents, with the initiation of an inflammatory response and to shape subsequent adaptive immune responses. An integral part of the innate immune system are a set of germline-encoded receptors referred to as pattern-recognition receptors (PRRs) (Janeway et al 2002). PRRs are important for the recognition of lipid, carbohydrate, peptide and nucleic-acid structures that are broadly expressed by different groups of microorganisms, collectively termed pathogen-associated molecular patterns or PAMPs (Trinchieri et al 2007). Characterized PAMPs include bacterial cell wall constituents such as lipopolysaccharide (LPS) or peptidoglycan (PGN), but also include flagellin, imidazoquinolines, single or double stranded viral RNA (Poly I:C), and unmethylated CpG DNA (Brunn et al 2004). Stimulation of PRRs by microbial products lead to the activation of signalling pathways, that ultimately result in the induction of antimicrobial genes and inflammatory cytokines. Among the cells that bear germline-recognition receptors are macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, and the NK cells. One of the major classes of PRRs is the Toll-like receptor superfamily.

1.5.1 Toll-like receptors and innate immunity

Mammalian Toll-like receptors (TLRs) are a family of 12 transmembrane proteins that play a major role in PAMP recognition and initiation of pro-inflammatory and immune responses. Each TLR differs in ligand specificity, expression patterns and the target genes

they induce. Most TLRs are expressed at the cell surface, whereas some are expressed on the membrane of endocytic vesicles or other intracellular organelles (Takedal et al 2003). TLRs trigger innate immune responses through nuclear factor-κB (NF-κB)-dependent or interferon regulatory factor (IRF)-dependent signalling pathways, *figure 1.2*. The exact nature of TLR mediated signalling, however, is complex and still being elucidated. A number of factors are believed to modulate the downstream events of TLR signalling cascades, resulting in a multitude of *in vivo* responses. These factors include adapter molecules such as myeloid differentiation primary response gene 88 (MyD88), toll-like receptor adaptor molecule 1 (TICAM1), IL-1 receptor associated kinases (IRAKs), transforming growth factor kinase (TAK-1), TRAFs (TNF receptor associated factors), and IκB kinases (IKKs) (Brunn et al 2006, Valenza et al 2006). Furthermore, there has been some recent evidence that has suggested TLRs interact with one another to further modulate the immune response (Mogensen et al 2001).

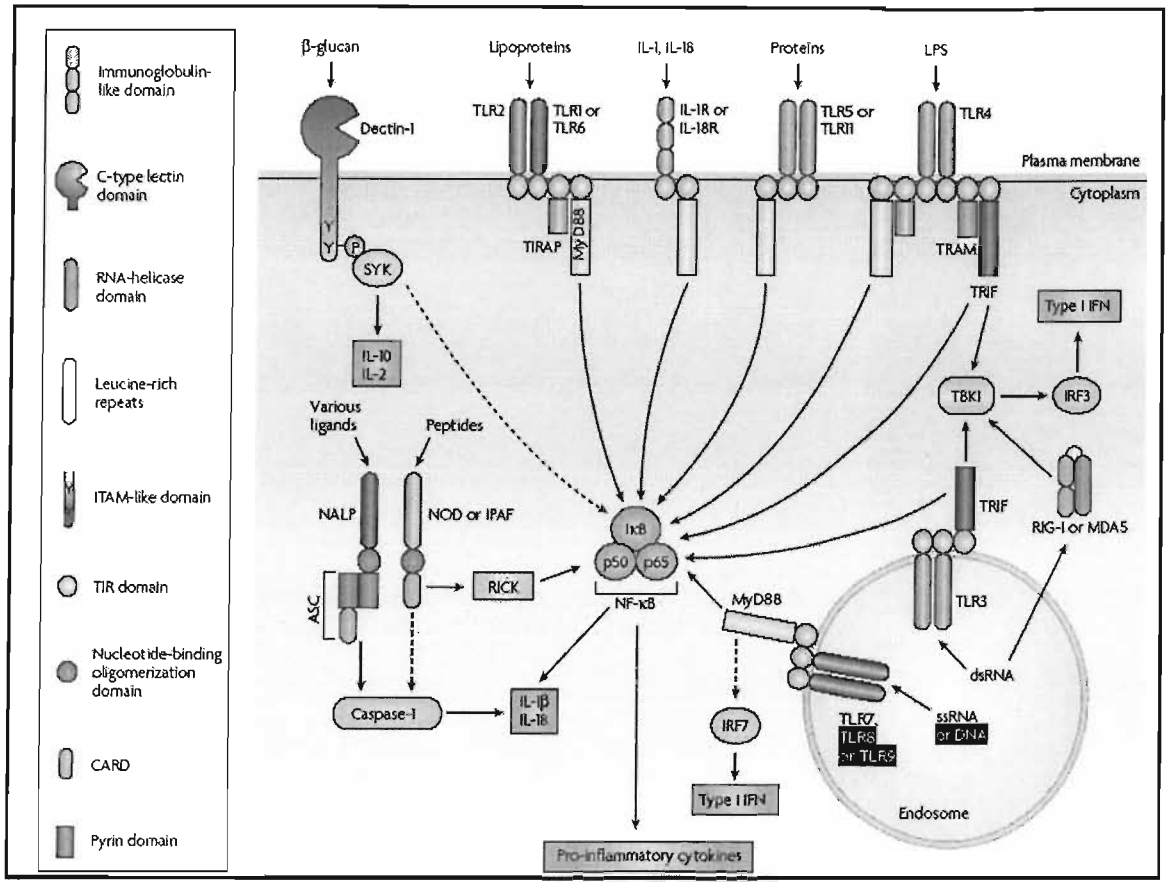

Figure 1.2: Overview of the complex pathways involved following toll-like receptor activation. TLRs mediate inflammatory actions through the NFκB or IFN signalling pathways respectively. Many adaptor molecules are known to be involved in the process (e.g. MyD88 and TRAM). In both cases, the result is increased transcription of pro-inflammatory cytokines and other antimicrobial genes. Figure taken from *Nature reviews Immunology,* 2007.

Perhaps two of the most important TLRs involved in COPD exacerbations are TLR3 and TLR4 (Seitz et al 2003). TLR4 is expressed in high concentration on the cell surface of predominantly macrophages, neutrophils and T and B-cells. It is stimulated by subnanomolar concentrations of LPS, a component of the cell wall of Gram-negative bacteria. For this reason, it is believed to have clinical relevance to bacterial induced inflammation of COPD. TLR4 is thought to be the major mammalian receptor involved in *H. influenzae* dependent signaling (Brunn et al 2006). Lorenz et al (2006) used knockout

mice to demonstrate that TLR4 function is essential for TNFα induction, neutrophilic influx, and ultimately bacterial clearance in response to *H. influenzae* aerosol inhalation. Furthermore, experimental stimulation of TLR4 present on human macrophages has been found to be pro-inflammatory for a wide species of bacteria, inducing predominantly IL-8 and TNFα production (Valenza et al 2006).

TLR3 is constitutively expressed in human alveolar and bronchial epithelial cells as well as dendritic cells (DCs). TLR3 plays an important role in antiviral responses through recognition of double-stranded viral RNA produced during viral replication within cells. For this reason, TLR3 is associated with viral inflammation in COPD. TLR_3 is expressed predominantly on the membranes of intracellular organelles or within the cell cytosol (Takedal et al 2003, Doyle et al 2003). These are predicted to be the same regions where viruses gain access to the intracellular compartments of cells during infection and replication. Unlike most TLRs, it is rare that TLR3 is ever expressed on the cell surface. In contrast to TLR4, TLR3 expression decreases rapidly following exposure to viral products, the relevance of which remains unclear (Johnston et al 2005). The function of TLR3 is to detect viral pathogens and induce responses that block their replication. Alexopoulou et al. (2001) demonstrated that TLR3 deficient mice show reduced inflammatory response to polyinosine-polycytidylic acid (Poly I:C), a synthetic viral dsRNA analog. Under closer inspection, it was found that TLR3 knockout mice had significantly reduced IL-6, IL-12, and TNFα responses when compared to wildtype (Khair et al 2003).

1.5.2 TLR signaling pathways

Both TLR3 and TLR4 mediate signalling predominantly through the NFκB pathway. In unstimulated cells, NF-κB dimers are sequestered in the cytoplasm by a family of inhibitors, called IκBs (Valenca et al 2006). Upon TLR activation, the adaptor molecule MyD88 bound to the intracellular structure of the receptor dissociates, binding to an IL-1 receptor associated kinase (IRAK) (Takedal et al 2003). Following a cascade of events, IκB kinase (IKK) is activated and proceeds to phosphorylate IκB. Phosphorylation of IκB induces conformational changes that ultimately result in dissociation from complexed NFκB. Phosphorylated IκB is subsequently targeted for degradation through the ubiquitin-dependent 26S proteasome pathway. NFκB translocates into the nucleus where upon it functions as a transcription factor, binding to DNA and upregulating protein translation of antimicrobial and inflammatory genes (Trinchieri et al 2007). Although the NFκB pathway is common to both TLR3 and TLR4, the influence of downstream factors (e.g. growth factor kinases, TAK-1 and TRAFs) combined with the interaction between different TLRs, significantly changes the overall cytokines produced by both these receptors (Trinchieri et al 2007, Brunn et al 2006).

1.6 Cytokine stimulation by bacteria and virus

Cytokines are important inflammatory mediators, synthesised and released by a variety of host cells including granular lymphocytes, mononuclear phagocytes, mast cells and epithelial cells (Chung et al 2001). Following their release, they play a pivotal role in neutrophil and macrophage influx, cellular differentiation and activation of phagocytosis.

Phagocytosis is particularly important during exacerbations when the tissue is battling the invasion of bacterial or viral pathogens.

There has been a vast amount of research into TLR4 activation and its functional roles during exacerbation. Many studies using LPS to stimulate TLR4 in bronchial biopsies, have found that there is increased local production of numerous cytokines including IL-1β, -6, -8, -10, and TNFα (Khair et al 1994, Lorenz et al 2005, Message et al 2004). These same cytokines have also been found to be elevated in sputum (Donaldson et al 2006) and BAL fluid (Wilkinson et al 2004) when obtained from COPD patients during a bacterial exacerbation. At present, the exact structure and sequential release of these cytokines remains unclear and requires further investigation. Furthermore, these cytokines are by no means comprehensive and are likely to encompass many more inflammatory mediators and chemokines that are, as of yet, unknown. Nevertheless, most of our knowledge about the role of TLR4 has come from our understanding of some of the cytokines it induces. TNFα is a major pro-inflammatory cytokine induced by TLR4 activation, the main sources of which are alveolar macrophages (Nash et al 1991). TNFα has multiple pro-inflammatory actions including neutrophil degranulation and stimulation of the respiratory burst, upregulation of adhesion molecules, stimulation of IL-8 production, and induction of airway hyperresponsiveness (Seemungal et al 2000, Roland et al 2001). In view of its effect upon the recruitment and activation of neutrophils, TNFα is believed to be a central mediator of neutrophilic inflammation in COPD. IL-6 and IL-10 are classified lymphokines; soluble factors produced by activated lymphocytes and $CD4^+$ cells. IL-6 is a pro-inflammatory cytokine, synthesised and released by macrophages, T cells, fibroblasts

and some B cells, of which the main targets are T and B cells (Bhowmilk et al 2003). It has important roles in T and B cell activation and differentiation, as well as functioning as a regulatory factor for TNFα production. In contrast, IL-10 is an anti-inflammatory cytokine synthesised by $CD8^+$ T-cells, monocytes and macrophages (Takanashi et al 1994, Cassatella et al 1993). Among its many other functions, IL-10 is believed to inhibit production of IL-1, IL-6 and TNFα, thus regulating the overall inflammatory response. IL-8 is a CXC chemokine that is chemoattractant for neutrophils and $CD8^+$ T lymphocytes (Barnes et al 2004). It is secreted by numerous cell types including bronchial epithelial cells, macrophages, and neutrophils. Its expression is upregulated in direct response to cigarette smoke and bacterial infection (Van Reeth et al 2000). Interestingly, patients with COPD who have more frequent exacerbations have higher sputum levels of IL-8 even when stable (Contoli et al 2007). This suggests that there may be pro-inflammatory effects of exacerbations that persist long after the acute episode has resolved. In support of this, many studies have shown that cytokines such as TNFα, IL-10 and IL-6 are chronically elevated in patients with COPD (Chung et al 2000). Clearly, a better understanding of the cytokine patterns involved during TLR4 activation will help us to gain an insight into the aetiology of bacterial induced exacerbations.

TLR3 receptors induce many different cytokines and chemokines compared to that of bacterial induced TLR4 response. Some of the early studies into TLR3 activation demonstrated that rhinoviral infection of bronchial epithelial cells and PBMCs induced the expression of several pro-inflammatory genes, including IL-8, GROα, ENA-78 and RANTES (Jadwiga et al 2002, Doyle et al 2002). For many viruses, however, it has become

apparent that cytokine patterns differ markedly dependent upon strain and/or multiplicity of infection (MOI). For example, clinical studies of patients with lower respiratory tract infections with respiratory syncytial virus (RSV) showed that there was enhanced expression of a variety of mediators including IL–1, IL-6, IL-10, TNFα as well as the chemokines interferon-gamma (IFNγ), inducible protein-10 (IP–10), and macrophage inflammatory protein (MIP-1β) (Guerrero-Plata et al 2005, Spurrell et al 2005). In contrast, many *in vitro* studies with influenza virus, have suggested that TNFα and IP-10 are not significantly elevated (Chen et al 2006, Donaldson et al 2006). These cytokine differences maybe explained, in part, due to viral binding proteins in the cell cytosol, prior to interactions with TLRs. The CXCL5 chemokine, ENA-78, has been found to elevate significantly in many different types of viral infections including rhinovirus, RSV, influenza and Poly I:C. ENA-78 is an epithelial-derived neutrophil attractant, and as its name suggests, is chemoattractant for neutrophils. It is believed that ENA-78 and IL-8 are the major chemokines involved in neutrophilic influx during viral associated COPD, however their roles in exacerbation are poorly understood. Another important component of the antiviral TLR3 response is the production of MIP-1β by Th_1 and/or CD8+ T cells (Heer et al 2007). MIP-1β has a number of antiviral functions, which include activation of alveolar macrophages and interaction with other antiviral cytokines such as TNFα, bringing about airway epithelial cell apoptosis and thus favoring efficient viral elimination (Saetta et al 2002). In conclusion, the vast heterogeneity of the host inflammatory response to virus, combined with a better understanding of the mechanisms of viral induced exacerbations, is required for future therapeutic advances in COPD.

1.7 Methods for studying COPD exacerbations

It is unethical to obtain bronchoalveolar lavage (BAL) samples from COPD patients during an exacerbation. Therefore, current investigations to study the inflammatory status of exacerbations have been to measure inflammatory mediators in sputum and blood (Lovel et al 2005). Unfortunately, due to the nature of sputum samples, it is impossible to define the location or kinetic release of such mediators. Furthermore, sputum samples can also be contaminated by salivary enzymes such as MMPs, which are capable of degrading inflammatory proteins (Fern et al 2001). Blood samples can be routinely taken over the course of an exacerbation but these can only provide detailed information into the systemic response to lung inflammation. Animal models have provided a good insight into the mechanisms of lung injury and inflammation, however, their relevance to humans remains controversial. Single cell cultures are also useful in eliciting local cell specific responses that may occur in within tissue however this does not allow us to understand the complex interactions of the tissue as a whole. One of the most effective ways to study COPD exacerbations, therefore, is to use a tissue explant model system (Bochner et al, 1989). Tissue fragments of lung can be stimulated in a controlled manner by bacteria and virus or stimuli that mimic both these types of pathogens and production of inflammatory mediators measured. This model is a very versatile system and allows for identification of cytokine kinetics and also dose response investigations.

1.8 Stimuli for mimicking exacerbations

1.8.1 Lipopolysaccharide (LPS)

Bacterial lipopolysaccharide (LPS) is a structural component of the outer cell wall of gram-negative bacteria. It is a powerful endotoxin and a potent initiator of the acute inflammatory response. LPS consists of three structural components; a lipid A moiety, a core (R) antigen and somatic (O) antigen or O polysaccharides (*figure 1.3*). The Lipid A contains unusual fatty acids (*e.g.* hydroxy-myristic acid) that are embedded in the outer membrane of the bacterium while the rest of the structure projects from its surface. It is this region of LPS that has endotoxic properties. Attaching both the O-specific chain(s) and the Lipid A is a long chain central core that extends out from the cell surface. O-specific chains are short length polysaccharides, varying dramatically in composition between different sub-types and species of bacteria. This makes LPS a very diverse structure, serving to prevent immune targeting with the body. It is generally the O-specific chains of LPS that are recognised through antibody association, thus generating a host immune response *in vivo*.

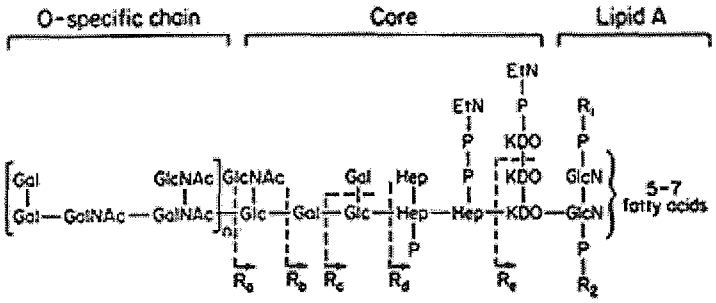

Figure 1.3: General structure of the components of LPS derived from E *coli*.

Due to its antigenic properties, LPS can be used as a mimic for bacterial infection within the lung. LPS initially complexes with a soluble acute phase protein called Lipopolysaccharide-Binding Protein (LBP). LBP is important in presenting LPS to cell surface PRRs. In vitro, LPS/LBP complex binds to a CD14/TLR4/MD-2 receptor complex, promoting the secretion of numerous pro-inflammatory cytokines and chemokines via the NF-κB pathway (Hirano et al, 1997). Koay et al (2002) were one of the first to define alveolar macrophages as a prominent cell type for NF-κB initiation by LPS. More recently, however, studies have shown that the epithelium may also play a large role in response to LPS. Guillot et al (2002) demonstrated by reverse transcription-PCR that TLR4 and the accessory molecule MD2 are constitutively expressed in distinct human alveolar and bronchial epithelial cells. Subsequent infection of the epithelium with *H.* influenzae resulted in a wide variety of inflammatory cell mediators being synthesized. Overall, the mechanisms underlying LPS induced inflammation have been well documented and shown to be mediated by pro-inflammatory cytokines, the most important being IL-1β, IL-6, and TNFα (Gullingham et al, 2001).

1.8.2 Polyinosinic:polycytidylic acid (poly I:C)

Poly I:C is a synthetic, double-stranded RNA (dsRNA) molecule that can be used to mimic viral infection during its replicative phase. Most viruses produce double-stranded RNA at some point during their replication within tissue cells, which proceed to stimulate the antiviral properties of the innate immune system via TLR3 present on the endosomal compartments (Kumar et al 2006). Advantages of using Poly I:C over live viruses include safety, convenience, but more importantly reproducibility and control over dose and time of

challenge administration. Although *in vitro* studies have demonstrated that stimulation of lung epithelial cells with Poly I:C elicited the secretion of multiple cytokines, chemokines and the induction of transcription factors and increased expression of TLRs, the physiological relevance of such events remain unclear (Ieki et al, 2004). Studies have shown that peripheral administration of Poly I:C in humans causes plasma IL-6 concentration to increase significantly, later found to be a TNFα and IL-1β dependent cascade (Fortier et al 2004). Furthermore, it has also been reported that Poly I:C stimulates the production of IL-6, IL-12, and MIP-1β in human and mouse leucocyte cultures (Krutzik et al, 2001). Overall, however, despite many attempts to discover the underlying mechanisms of the Poly I:C response, little it still known about the cytokines produced and the kinetics of the response within human lung tissue.

1.9.1 Hypothesis

Ligands for TLR3 and TLR4 will initiate inflammatory responses in human lung tissue, but will differ for the cytokines produced and their kinetic profiles.

1.9.2 Aims

Throughout this chapter, it has been highlighted that acute exacerbations of COPD are of great important to disease progression and are, as of yet, an unmet therapeutic target. A better understanding of the mechanisms of viral-induced exacerbations are required. It is the aims of this study to assess the contribution of cytokines in the acute inflammatory response to viral infection and to furthermore, compare this response to that of a known bacterial inflammatory stimulus, LPS.

Chapter 2:

Methods

2.1 Materials

RPMI-1640 medium was supplemented with penicillin, streptomycin and gentamycin (5000units/ml, 5mg/ml and 10mg/ml respectively). Tyrode's salts contained 1.8mM $CaCl_2$, 1mM $MgCl_2$, 2.7mM KCl, 137mM NaCl, 0.42mM NaH_2PO_4, 5.5mM D-glucose with 12mM $NaHCO_3$. Phosphate buffered saline (PBS) contained 0.1M NaCl, 2.7mM KCl, 1.8mM KH_2PO_4 and 10mM Na_2HPO_4; PBS Tween contained 0.1% Tween 20 in PBS; coating buffer for TNFα, MIP-1beta, IL-5, IL-6, IL-8 and IL-10 ELISAs containing 0.05mM Na_2CO_3 and 0.05mM $NaHCO_3$, pH 9.4; reagent diluent for TNFα, MIP-1beta, IL-6, IL-8, and IL-10 ELISAs containing 0.14mM NaCl, 0.01mM Na_2HPO_4, 1.47mM KH_2PO_4, 2.68mM KCl and 5.0g/l bovine serum albumin (BSA); reagent diluent for IL-1β, ENA-78 and IL-13 ELISAs containing 1% BSA in PBS.

2.2 Other reagents

Polyinosinic:polycytidylic acid (Poly I:C) (Sigma, UK); Lipopolysaccharide of gram-negative bacteria *E. coli* 0111:b4 (LPS) (Sigma, UK); ELISA Duoset kits for TNFα, MIP-1beta, IL-5, IL-6, IL-8 and IL-10 from Biosource (Europe, SA); ELISA kits for IL-1β, ENA-78 and IL-13 from R&D Systems (Minneapolis, MN); 3,3',5,5' tetramethylbenzidine (TMB); 1M H_2SO_4

2.3 The tissue explant model system

Human lung tissue was collected with ethical consent from patients undergoing resection for lung cancer, from the Asthma and Allergy Research Department at Guy's Hospital in

London. Tissue received was from the non-cancerous margin surrounding the tumour. Patient data such as smoking history, age, gender and lung function were obtained at the same time as sample collection. In all cases patients had some degree of chronic airways obstruction which was characterised by their GOLD status.

Tissue was placed immediately into saline following surgical removal. Hereafter, lung tissue was finely chopped into fragments approximately $1mm^2$, during several washes with Tyrode's buffer containing 0.1% sodium bicarbonate. Processing of lung explants followed a 16-hour overnight incubation in RPMI-1640 medium containing 1% penicillin/streptomycin and gentamycin (PSG) at 37°C in 5% carbon dioxide/air. Tissue fragments were aerated in a gas culture dome for two minutes prior to sealing, ensuring complete removal of airborne contaminants and an even distribution of 5% carbon dioxide/air. The following day, approximately 25mg of tissue was placed into fresh RPMI-1640/PSG and stimulated with buffer control, LPS, Poly I:C or killed virus. Lung fragments were harvested at the appropriate time points from the wells using tweezers. Tissue was carefully blotted onto paper towel so that a semi dry tissue weight could be obtained. Tissue fragments were then weighed in Eppendorf tubes of a pre-calculated weight thus allowing the actual semi dry tissue mass to be determined. At the same time, supernatants from each well were aliquoted and stored at -80°C until analysis. Initial experiments using protease inhibitors were found to make no difference to percentage cytokine recovery from spiked aliquots and therefore were excluded from the following study. However, the time taken between tissue culture and performing ELISAs were always kept to a minimum and generally did not exceed more than one week. Methods,

concentrations and time courses for individual tissue culture experiments can be found in the appropriate chapters.

2.4 TNFα, MIP-1beta, IL-6, IL-8, IL-10 ELISAs

TNFα, MIP-1beta, IL-5, IL-6, IL-8 and IL-10 were all measured in human lung supernatant using commercially available ELISA Duosets from Biosource (Europe, SA). The limits of detection for TNFα, MIP-1beta, IL-5, IL-6, IL-8 and IL-10 were 0.26pg/ml, 0.29pg/ml, 0.26pg/ml, 0.28pg/ml, 0.26pg/ml and 0.25pg/ml respectively. ELISAs were carried out according to the manufacturer's instructions and recommended reagents used throughout. Briefly, a 96-well plate was coated with the appropriate coating antibody at a working concentration of 1μg/ml in coating buffer, 100μl per well and left at 4°C overnight. The next morning the plates were washed four times with PBS Tween and excess liquid completely removed. Hereafter, 200μl reagent diluent was added per well to block unspecific binding and left for 2hrs at room temperature on a rocker to guarantee continual agitation. Following this, the plate was washed as before and samples added at the appropriate dilution. In addition, a standard curve for each cytokine was added in duplicate for later comparison. To each well 50μl of the correct detection antibody was added at a concentration of 0.4μg/ml for IL-6, IL-5, TNFα and IL-10 and 100ng/ml for IL-8 and MIP-1β and left for 2hrs at room temperature. Following another washing and aspiration step, streptavidin-HRP conjugate was added at a dilution of 1:2500 and left for 30mins at room temperature. After this, TMB was added and the reaction duly terminated with 1M H_2SO_4. The optical density was obtained at a wavelength of 450nm

and a log-log graph drawn from the standard curve to obtain the concentration of cytokine present (*example figure 2.1*).

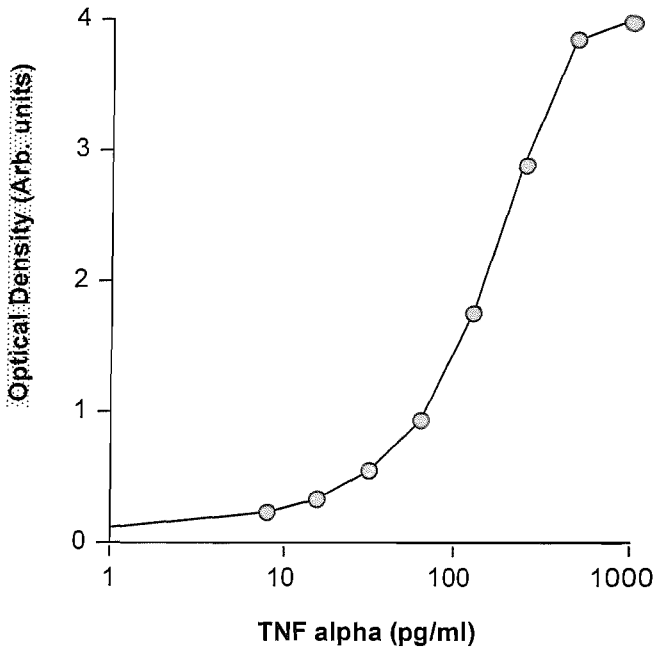

Figure 2.1: Typical standard curve obtained for a TNFα ELISA. This curve can be used to determine the concentration of the protein within the sample by using the optical density detected by the assay at a wavelength of 450nm. The top standard for this ELISA is 1000pg/ml and the samples were analysed at the appropriate dilution.

2.5 IL-1β, ENA-78, IL-13 and IFNα/β ELISAs.

ENA-78, IL-13 and IFNα/β levels were measured in human lung supernatant using commercially available ELISAs from RnD Systems (Minneapolis, MN). The limit of detection for the ENA-78 assay was 0.31pg/ml, 0.65pg/ml for IFNα, 0.5units/ml for IFNβ and 0.5pg/ml for IL-13. These assays were carried out according to the manufacturer's instructions. The optical density was obtained at a wavelength of 450nm

and a log-log graph drawn from the standard curve to obtain the concentration of cytokine present.

2.6 Statistics

Statistical analyses were performed using Statview^{TM}. All results were normalised using the semi-dry weight of tissue and are expressed as the mean ± SEM. Non-parametric analysis was used to examine the effect of agonist stimulation for all cytokines using the Wilcoxon signed rank test. Correlations between parameters were examined for statistical significance by Spearman's correlation. The results were considered statistically significant if $P<0.05$.

Chapter 3:

The acute inflammatory response to Poly I:C and LPS

3.1 Introduction

LPS is widely used as a model for studying bacterial infection and inflammation. The mechanisms underlying LPS induced inflammation have been well characterized and shown to be mediated by pro-inflammatory cytokines, the most important being IL-1β, IL-6, and TNFα (Fortier et al 2004). Unlike bacterial infection, little is known about the inflammatory response to viral infection. As most viruses produce double-stranded RNA (dsRNA) at some point during their replication, a synthetic viral-like dsRNA that stimulates antiviral activities of the innate immune system, polyinosinic:polycytidylic acid (Poly I:C), can be used to mimic viral infection within the lung. Advantages of using Poly I:C over live viruses include safety, convenience, but more importantly reproducibility and control over dose and time of administration. Despite many attempts to discover the underlying mechanisms of the Poly I:C response, little it still known about the cytokines produced and the kinetics of the response within human lung tissue.

Current investigations to study the inflammatory status of patients during exacerbation have been to measure inflammatory mediators in sputum and blood. Unfortunately, due to the nature of sputum samples, it is impossible to define the location or kinetic release of mediators. Furthermore, sputum samples can also be contaminated by salivary enzymes such as MMPs, which are capable of degrading inflammatory proteins. Blood samples can be routinely taken over the course of an exacerbation but these can only provide detailed information into the systemic response to lung inflammation. This chapter aims to contribute to the kinetic profile of inflammatory mediators released during an inflammatory

response in human lung tissue. Within this chapter we shall analyse the inflammatory response of Poly I:C and compare this to the better described LPS response.

3.2 Methods

Human lung tissue was excised from patients undergoing resection for cancer. The tissue used was from the non-cancerous margin surrounding the tumour. Data was collected regarding patient age, gender, smoking status and lung function at the time of sample collection. Tissue fragments were processed as previously described (*Methods 2.3*) and prepared for primary cell culture.

3.2.1 Dose response

Tissue fragments from 16 patients with mild to moderate COPD were cultured in RPMI-1640/PSG and stimulated with either a buffer control, 100ng/ml LPS, or 100, 10 or 1μg/ml Poly I:C. A fixed time point of 24hrs was chosen based upon background research and the slow nature of the viral response. After 24hrs, tissue fragments were harvested and weighed and lung supernatants aliquoted and stored at -80°C prior to analysis. Patient characteristics relating to age, gender, lung function and smoking status are shown in *table 3.1* below. On average patients in this cohort were typically classed as GOLD II.

No. Subjects	n=16
Age	68.3±1.7
Gender	10 Males 6 Females
Lung function (FEV_1/FVC)	0.67±0.08
Smoking Status	Ex =11 Current =5
Pack years	50.9±5.25

Table 3.1: Patient characteristics of subjects prior to removal of human lung tissue.

3.2.2 Kinetics response

Tissue fragments from 14 patients with mild to moderate COPD were cultured in RPMI-1640/PSG and stimulated with either a buffer control, 100ng/ml LPS or 100μg/ml Poly I:C. Tissue fragments were harvested and weighed at 1, 2, 4, 6, 24 and 48hrs and lung supernatants aliquoted and stored at -80°C prior to analysis. Patient characteristics relating

to age, gender, lung function and smoking status are shown in *table 3.2*. Again patients in this cohort were typically classed as having stage II disease.

No. Subjects	n=14
Age	66.7±2.1
Gender	9 Males 5 Females
Lung function (FEV_1/FVC)	0.64±0.05
Smoking Status	Ex =10 Current =3 Unknown=1
Pack years	47.9±5.25

Table 3.2 Patient characteristics of subjects prior to removal of human lung tissue.

In both sets of experiments, five patients overlapped between the two groups. Where different groups of patients were used for individual experiments, patient characteristics were controlled to ensure the clinical details of the whole group were represented.

3.3 Results: Poly I:C dose response

3.3.1 Inflammatory cytokines

The concentration of three inflammatory cytokines in culture supernatant were investigated using ELISA (*figure 3.1*). These were TNFα (*left*), IL-1β (*centre*) and IL-6 (*right*). Both the production of pro-inflammatory cytokines TNFα and IL-1β, were statistically elevated ($P>0.05$) in the LPS stimulated tissue at 24hrs compared to buffer control (TNFα Mean±SEM=58.5±18.4 vs 1.9±0.05 pg/mg tissue; IL-1β =10.5±2.3 vs 1.2±0.02 pg/mg tissue). IL-6 production was not significantly increased in LPS stimulated tissue at 24hrs. Interestingly, dose dependent concentrations of Poly I:C did not generate a significant response for any of the three inflammatory cytokines, when compared to appropriate controls at 24hrs.

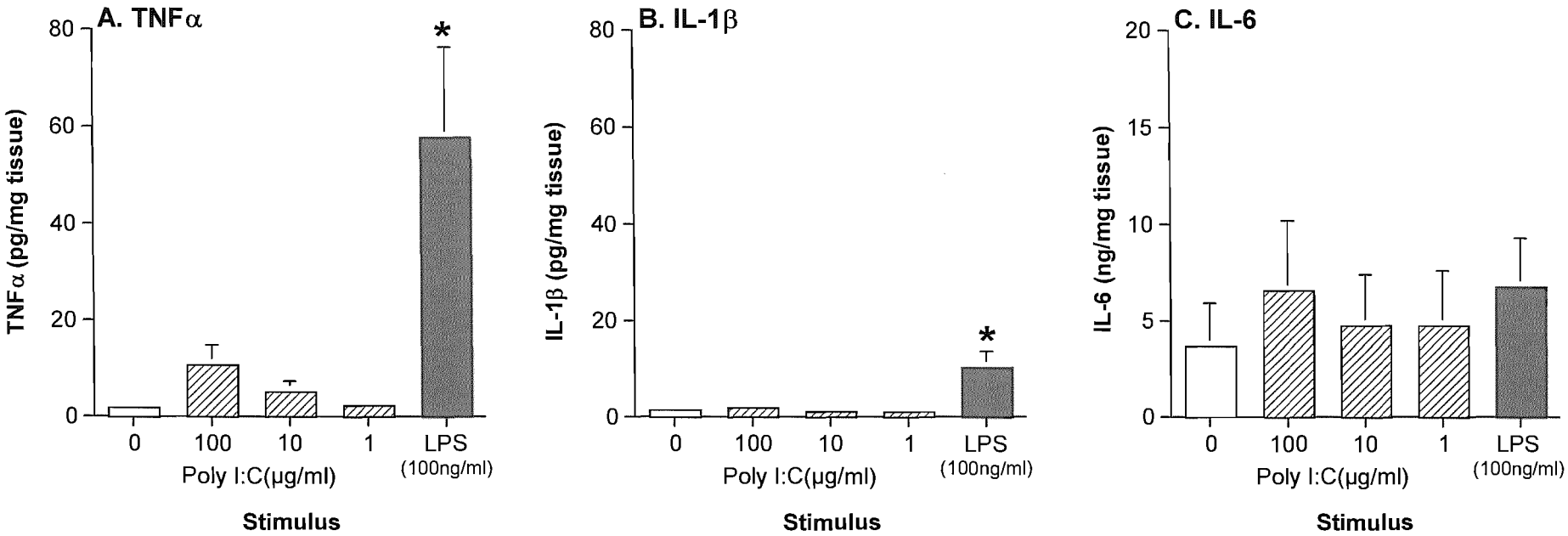

Figure 3.1: TNFα, IL-1β and IL-6 levels in supernatant from human lung tissue at 24hrs. Human lung tissue (n=16) was stimulated with 100ng/ml LPS, 100, 10 or 1µg/ml Poly I:C or buffer control. The release of cytokines were measured using ELISA. Values shown are the mean ± SEM and are expressed as pg/mg tissue for TNFα and IL-1β, and ng/mg tissue for IL-6. LPS and Poly I:C were compared to controls using Wilcoxon signed rank; * indicates a P value <0.05.

3.3.2 Chemokines

Chemokines are a large family of chemotactic cytokines involved in the migration and activation of cells, especially phagocytic cells and lymphocytes. They play a pivotal role in the innate inflammatory response to bacterial and viral infection. A group of three chemokines were investigated as shown in *figure 3.2*. Interestingly, we can see that for both MIP-1β (*left*) and IL-8 (*centre*), poly I:C is having significant effects upon chemokine production. Looking firstly at MIP-1β (CCL4), we can see that 10μg/ml and 100μg/ml Poly I:C generated significant production of MIP-1β above control (109.2±52.9pg/mg vs 19.5±1.22pg/mg and 101.5±24.2pg/mg vs 19.5±1.22pg/mg respectively). No significant difference was found between these two doses of Poly I:C. In addition, there was a large and significant LPS response for MIP-1β production at 24hrs.

IL-8 is a CXC chemokine that can signal through CXCR1 and CXCR2 receptors. It is a potent chemoattractant and activator of neutrophils. Levels of IL-8 detected in the supernatant were found to be much greater in concentration than other chemokines and cytokines analysed. IL-8 was found to be significantly elevated in tissue stimulated with 100μg/ml Poly I:C compared to control (17.5±4.8ng/mg tissue vs 8.5±3.3ng/mg tissue). Intriguingly, this Poly I:C response matched the response of the LPS stimulated tissue, which was also significant at 24hrs (17.5±5.2ng/mg tissue vs 8.5±3.3ng/mg tissue). Neither of the lower two doses of poly I:C significantly elevated IL-8 production.

Epithelial neutrophil activating peptide 78 (ENA-78) is a CXC chemokine that signals through the CXCR2 receptor. It is widely expressed in monocytes, platelets, endothelial cells and mast cells. It is apparent from the results *figure 3.2* (*right*), that none of the concentrations of poly I:C significantly elevated ENA-78 production, although there was a significant LPS response when compared to control (99.8±18.9pg/mg tissue vs 41.2±9.9pg/mg tissue). Interestingly, results for 100μg/ml Poly I:C stimulated tissue demonstrated a high degree of inter-patient variability and teetered in and out of statistical significance (P=0.078).

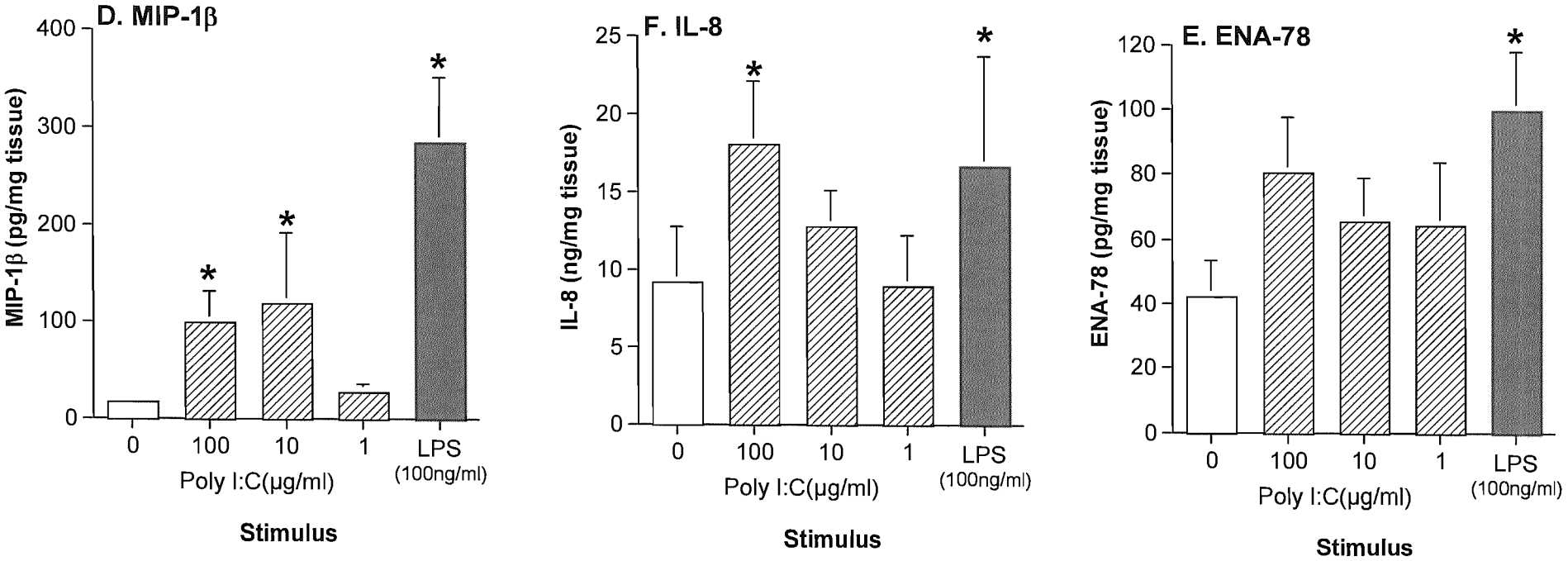

Figure 3.2: MIP-1β, IL-8 and ENA-78 levels in supernatant from human lung tissue at 24hrs. Human lung tissue (n=16) was stimulated with 100ng/ml LPS, 100, 10 or 1μg/ml Poly I:C or buffer control. The release of cytokines were measured using ELISA. Values shown are the mean ± SEM and are expressed as pg/mg tissue. LPS and Poly I:C were compared to controls using Wilcoxon signed rank; * indicates a P value <0.05.

3.3.3 Th_2 cytokines

Interleukins 5, 13 and 10 are cytokines associated with a Th_2 response (*figure 3.3*). IL-5 is an interleukin produced by T helper-2 cells (Th2) and mast cells. Its functions are to stimulate B cell growth and increase in immunoglobulin secretion in response to presented antigens. IL-13 is also secreted from Th2 cells. It is implicated as a central mediator of the physiological changes induced by allergic inflammation in many tissues, especially within the lungs. IL-13 production can promote airway hyperresponsiveness, goblet cell metaplasia and mucus hypersecretion, all of which contribute to airway obstruction. In addition, IL-13 can stimulate matrix matalloproteinases (MMPs) to degrade the lung parenchyma as part of a mechanism that protects against excessive allergic inflammation that predisposes to asphyxiation. IL-10 is an interleukin produced primarily by monocytes and to a lesser extent by lymphocytes. This cytokine has pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th_1 cytokines, MHC class II expression, and costimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. IL-10 can also block NF-κB activity, making it of particular interest to TLR signalling.

No significant difference in the release of IL-5 (*left*) or IL-13 (*centre*) in poly I:C or LPS stimulated tissue was observed when compared to control. Intriguingly, *figure 3.3* shows that the anti-inflammatory cytokine IL-10 (*right*) was significantly elevated by both 10μg/ml and 100μg/ml Poly I:C compared to control (2.4±0.33pg/mg vs 0.4±0.01pg/mg and 7.0±2.7pg/mg vs 0.4±0.01pg/mg respectively). Furthermore, the response of the tissue for IL-10 production with Poly I:C appears to demonstrate a dose dependent nature.

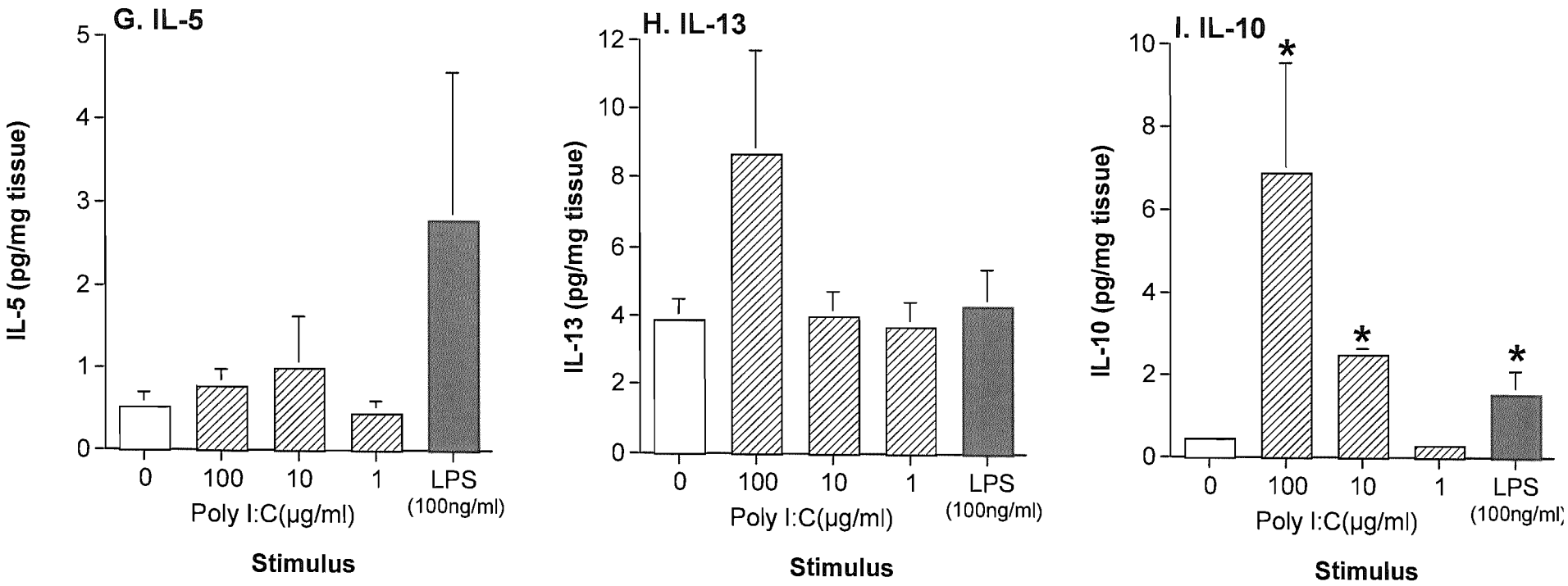

Figure 3.3: IL-5, IL-13 and IL-10 levels in supernatant from human lung tissue at 24hrs. Human lung tissue (n=16) was stimulated with 100ng/ml LPS, 100, 10 or 1µg/ml Poly I:C or buffer control. The release of cytokines were measured using ELISA. Values shown are the mean ± SEM and are expressed as pg/mg tissue. LPS and Poly I:C were compared to controls using Wilcoxon signed rank; * indicates a P value <0.05.

3.4 Results: Poly I:C and LPS kinetics

3.4.1 Inflammatory cytokines

Kinetic profiles of the three inflammatory cytokines TNFα (*left*), IL-1β (*centre*) and IL-6 (*right*) were observed over 48hrs, *figure 3.4.* Release of TNFα was elevated in the LPS stimulated tissue, becoming significant from 6hrs (Mean±SEM=23.1±10.9pg/mg tissue) and continuing to rise and peak at 24hrs (77.2±12.1pg/mg tissue) compared to buffer controls. Hereafter the response remained elevated until 48hrs. With regards to Poly I:C, no differences were observed over the 48hrs compared to buffer control.

The response of stimulated tissue on IL-1β production can be seen in *figure 3.4 centre*. It was observed that once again there was a significant LPS response, however no statistical differences were observed with Poly I:C over the 48hr duration. The LPS response for IL-1β is markedly different to that observed for the LPS induced TNFα response. IL-1β demonstrates much slower LPS induced kinetics and only becomes significant from 24hrs (4.8±1.2pg/mg tissue), where upon the response appears to continue to increase up to 48hrs (8.5±1.1pg/mg tissue).

The data illustrated by *figure 3.4 right* shows the response of the tissue for IL-6 production. It was observed that neither LPS nor Poly I:C were able to stimulate significant production of IL-6, when compared to buffer controls over the entire 48hr period. It should be observed, however, that buffer control levels of IL-6 are very high and continue to increase over 48hrs without stimulation.

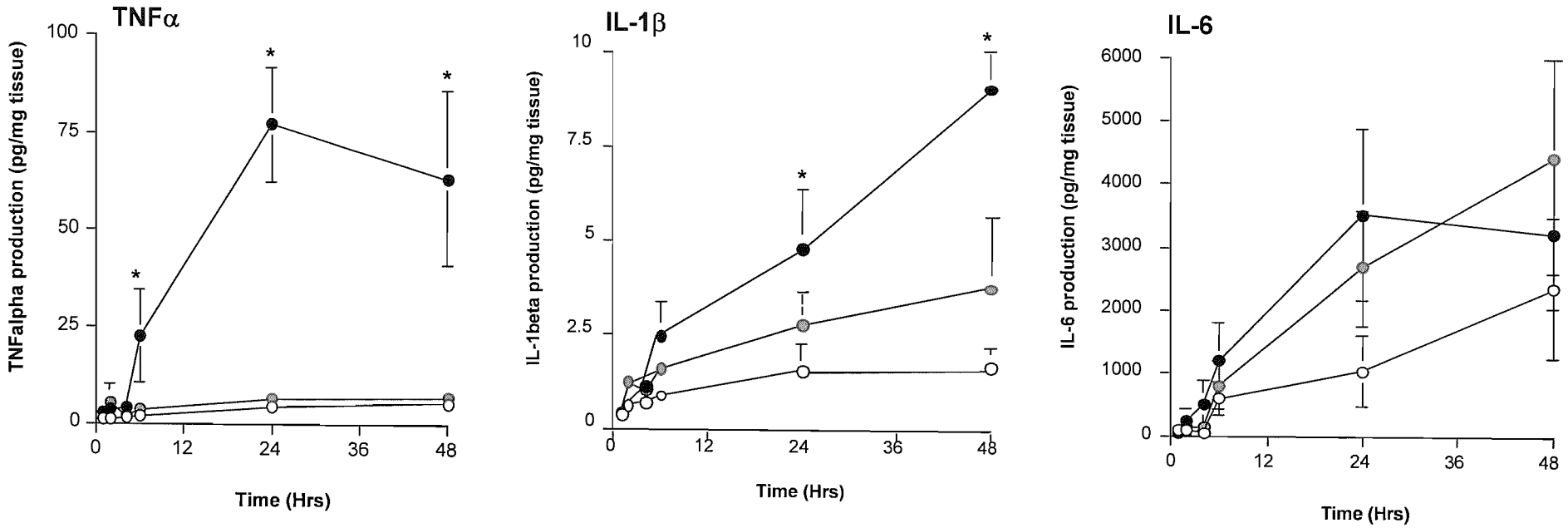

Figure 3.4: TNFα, IL-1β and IL-6 levels in supernatant from human lung tissue. Human lung tissue (n=13) was stimulated with 100ng/ml LPS (filled circles), 100μg/ml Poly I:C (grey circles) or buffer control (open circles). The release of cytokines were measured using ELISA. Values shown are the mean ± SEM and are expressed as pg/mg tissue. Groups were compared to controls using Wilcoxon signed rank; * indicates a P value <0.05.

3.4.2 Chemokines

In contrast to the pro-inflammatory cytokines, Poly I:C once again induced significant production of the three chemokines MIP-1β (*left*), IL-8 (*centre*) and ENA-78 (*right*), *figure 3.5*. It can clearly be observed that there is a large LPS response for MIP-1β production, significant from control at 6hrs (15.7±4.1pg/mg tissue) and peaking and plateauing from 24 to 48hrs (57±12.7pg/mg tissue). Poly I:C stimulated tissue also induced a significant MIP-1β response, however the kinetic profile for Poly I:C is a lot slower compared to that of the LPS response. MIP-1β production following Poly I:C stimulation was significant from 24hrs (22.6±3.0pg/mg tissue) and continued to increase up to 48hrs with no signs of peaking or plateauing. It was interesting to observe that at 48hrs, Poly I:C was as effective a stimulus for MIP-1β production as LPS.

Figure 3.5 centre illustrates the results obtained for IL-8 production. It was interesting to observe that both the kinetic profiles for LPS and Poly I:C over the 48hrs shared similar characteristics. IL-8 production was significant from 24hrs for Poly I:C (2301.3±531pg/mg tissue) and LPS (2866±529ng/mg tissue) and in both cases continued to increase up to 48hrs (~3000pg/mg tissue for both stimuli). The release of chemokine ENA-78 followed a similar pattern to IL-8 release with a maximum response for both LPS and Poly I:C at 48hrs (413±41.6pg/mg tissue and 467±38.7pg/mg tissue respectively). Kinetics profiles for the two stimuli followed almost identical patterns for ENA-78 production, both LPS and poly I:C only significant from 24hrs (~170pg/mg tissue for both stimuli). Interestingly although Poly I:C was in this instance significant at 24hrs, there was a large range between individual patient results. Therefore results always bordered on statistical significance compared to control with Wilcoxon signed rank test ($p=0.0497$).

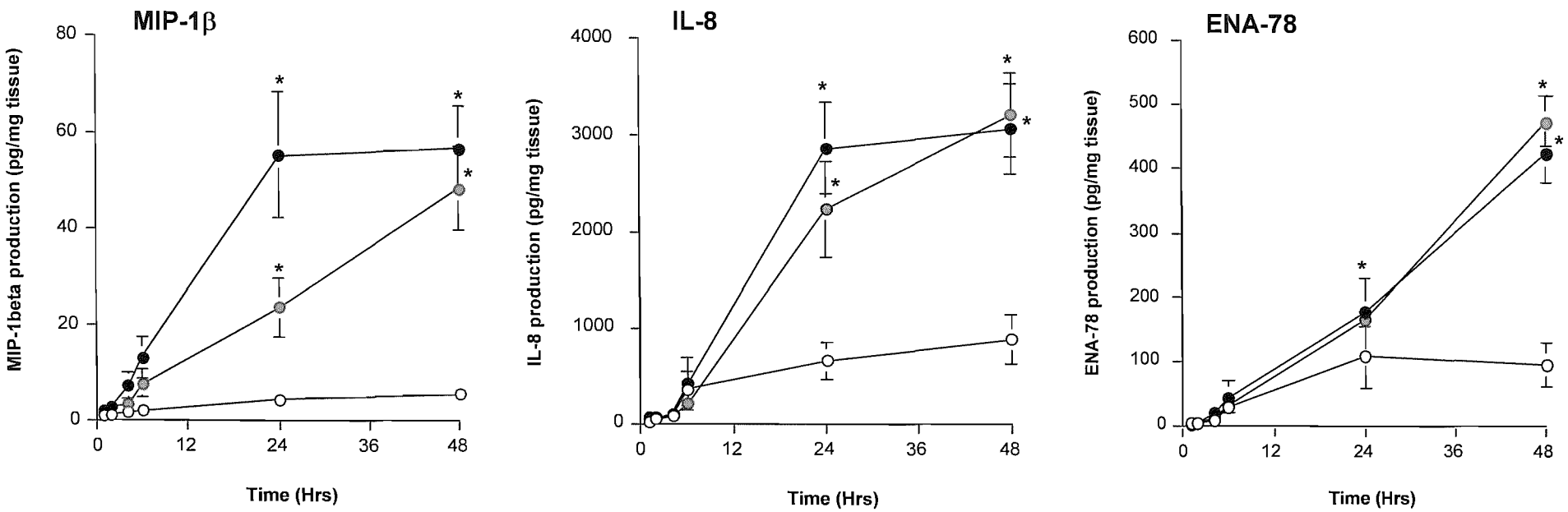

Figure 3.5: MIP-1β, IL-8 and ENA-78 levels in supernatant from human lung tissue. Human lung tissue (n=13) was stimulated with 100ng/ml LPS (filled circles), 100μg/ml Poly I:C (grey circles) or buffer control (open circles). The release of cytokines were measured using ELISA. Values shown are the mean ± SEM and are expressed as pg/mg tissue. Groups were compared to controls using Wilcoxon signed rank; * indicates a P value <0.05.

3.4.3 Th_2 cytokines

Kinetic profiles of the Th_2 cytokines were next observed, *figure 3.6*. As previously observed for interleukins 5 and 13 (*left* and *centre* respectively), both LPS and Poly I:C demonstrated no significant increase in cytokine production over 48hrs. Levels of IL-5 and IL-13 in all conditions were just above the detectable limit.

Figure 3.6 illustrates the data obtained for the anti-inflammatory IL-10 (*right*). In contrast to both IL-5 and IL-13, both LPS and Poly I:C significantly elevated IL-10 production above buffer control. The LPS induced response for IL-10 was significant from 6hrs (2.45±0.94pg/mg tissue) and peaked at 24hrs (13.2±2.33pg/mg tissue) before the response tailed off at 48hrs (11.6±1.46pg/mg tissue). A Poly I:C response was also observed, again much slower than the LPS response, significant from control at 24hrs (4.11±0.61pg/mg tissue) and continuing to increase up to 48hrs (6.87±0.50pg/mg tissue).

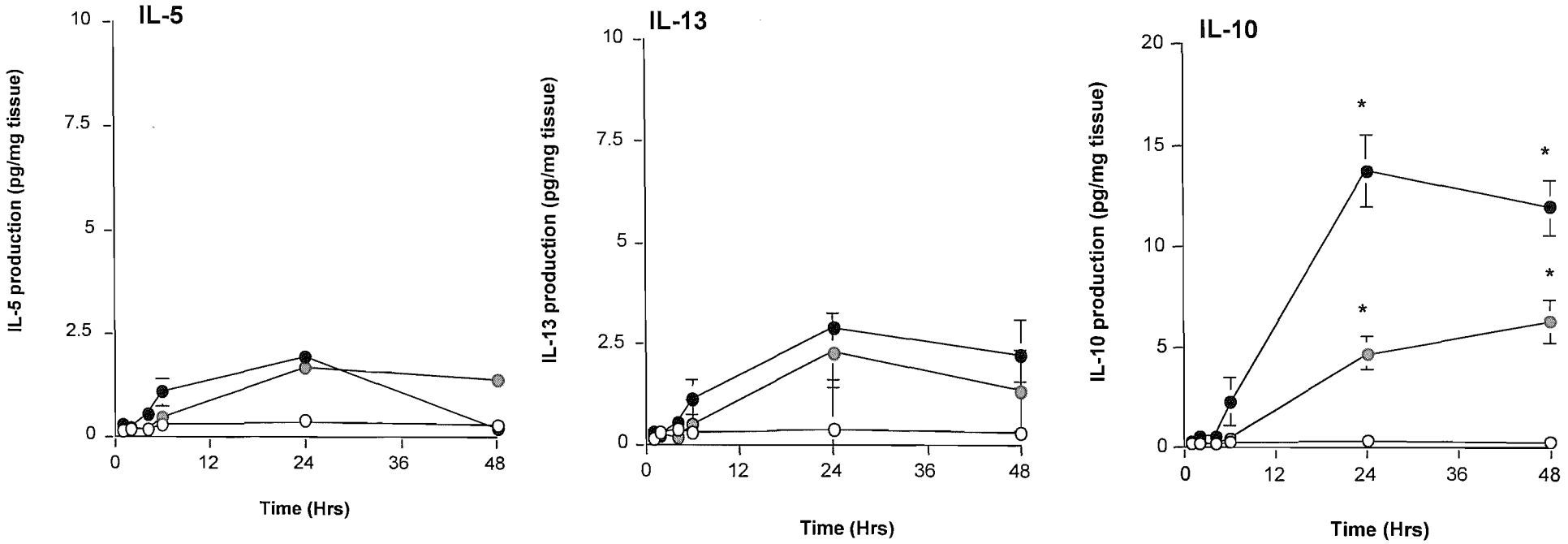

Figure 3.6: IL-5, IL-13 and IL-10 levels in supernatant from human lung tissue. Human lung tissue (n=13) was stimulated with 100ng/ml LPS (filled circles), 100μg/ml Poly I:C (grey circles) or buffer control (open circles). The release of cytokines were measured using ELISA. Values shown are the mean ± SEM and are expressed as pg/mg tissue. Groups were compared to controls using Wilcoxon signed rank; * indicates a P value <0.05.

3.4.4 Cytokine variability

It was noted that there was a high degree of variability amongst the individual patient data for chemokine expression. This was most apparent for ENA-78 production where results for Poly I:C bordered constantly on statistical significance at 24hrs. In contrast, other Poly I:C elevated cytokines such as IL-10 were found to have relatively small differences between patients. The results composite *figure 3.7* illustrates the individual patient differences in ENA-78, MIP-1β and IL-8 production at control and following Poly I:C stimulation at 24hrs. It is clear to see that although some patients respond well to Poly I:C stimulation, others demonstrate little to no chemokine expression above that of control. Furthermore, it is apparent that different chemokines are up regulated in different patients. In an attempt to try and explain the differences between responders and non-responders, patients were split up according to several patient characteristics including gender, smoking status, GOLD status and recent respiratory infection, in order to find any statistical variations among the groups. Unfortunately, no statistical significances were found in all variables analysed and therefore requires further investigation.

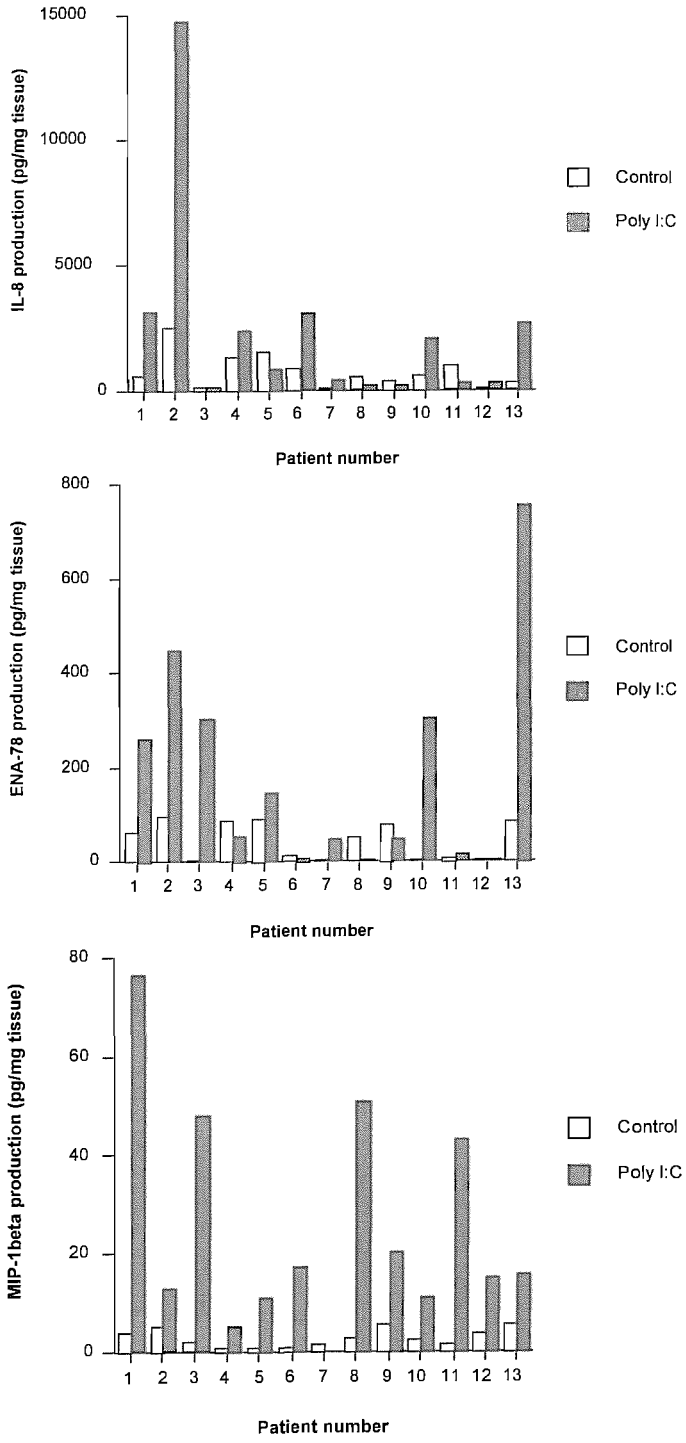

Figure 3.7: Composite illustrating the individual patient differences (n=13) in ENA-78, MIP-1β and IL-8 production at control and following 24hrs Poly I:C stimulation in human lung tissue. Results highlight large inter-patient variability in chemokine production.

3.4.5 Cytokine correlation

As part of the analysis, we investigated the correlation between cytokine levels for each individual patient. The data illustrated in *figure 3.8 left* was used to correlate the levels of IL-10 with MIP-1β production at 48hrs. Unfortunately, although not statistically significant (P=0.068, Rho=0.399) the results show that there is a general pattern occurring, indicating that IL-10 inhibits the release of MIP-1β from human lung tissue. Conversely, *figure 3.8 right* illustrates the correlation between the two chemokines IL-8 and ENA-78. Although only a weak positive correlation (P=0.077, Rho=0.455) these results indicate that the levels of ENA-78 production increase proportionately to IL-8 release at 48hrs. It is possible that further repeats of these experiments may help to clarify whether these patterns are indeed significant.

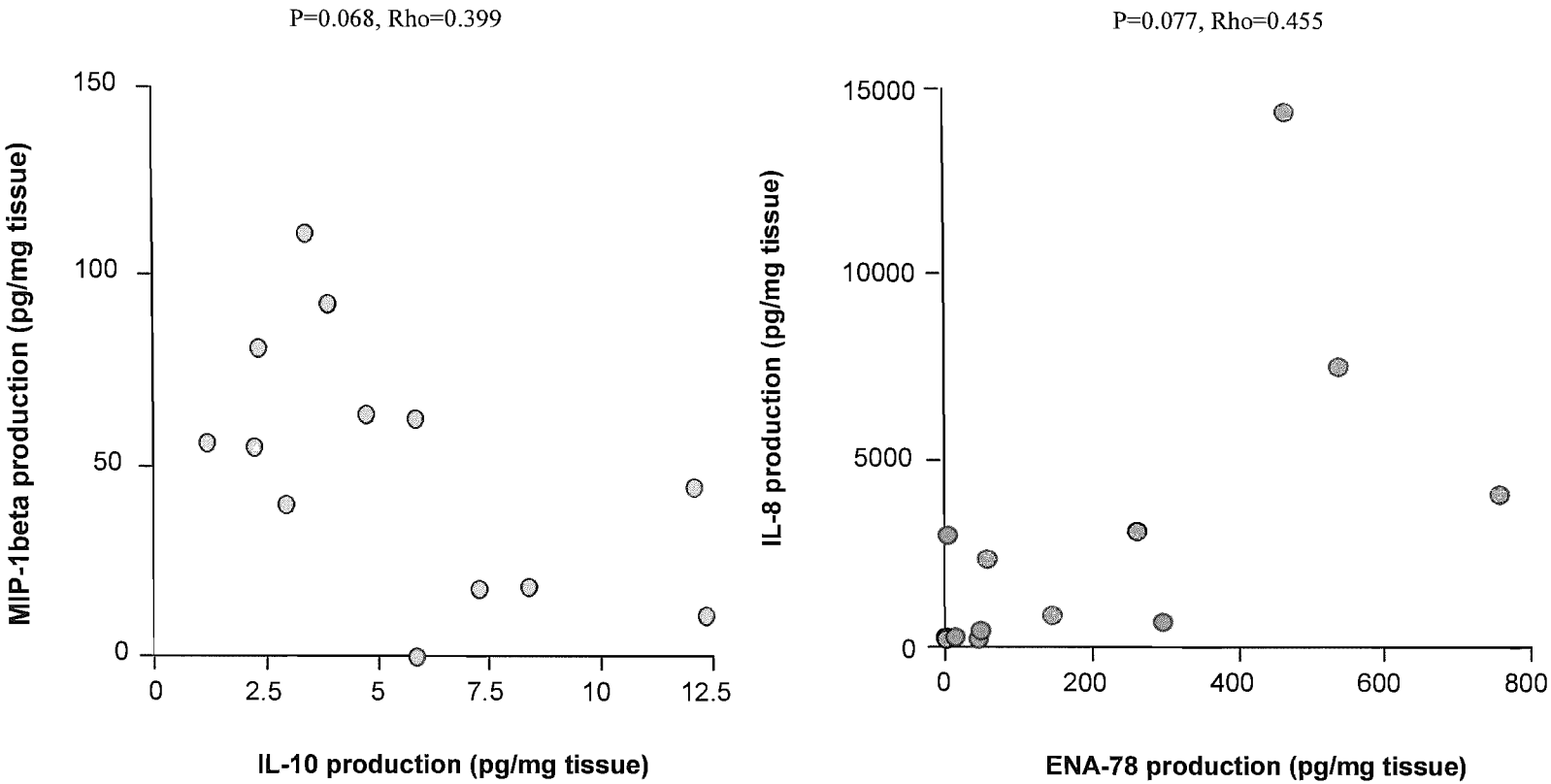

Figure 3.8: Correlation between MIP-1beta and IL-10 production (*left*) and IL-8 and ENA-78 production (*right*). It is apparent that although not statistically significant with n=13, results demonstrate interesting patterns.

3.5 Discussion

Despite the advances made in treatments for COPD, acute exacerbations remain a major cause of morbidity and mortality. Recent years have seen growing epidemiological and scientific evidence linking virus with exacerbations, and now the stage is set for progress in identifying important mechanisms of viral-induced exacerbations of COPD. Acute exacerbations are associated with increased airways inflammation, which may play an important role in the pathogenesis of the COPD (Bhowmilk et al 2000). Unfortunately, there is little information available on the nature and sequential release of inflammatory markers involved in viral exacerbations. A synthetic viral-like dsRNA that stimulates antiviral activities of the innate immune system, Poly I:C, has been used effectively in many studies to mimic viral infection within the lung. Despite many attempts to discover the underlying mechanisms of the Poly I:C response, little is still known about the cytokines produced and the kinetics of the response within human lung tissue. As invasive measures to observe the kinetics of exacerbations *in situ* are unethical, a Poly I:C model of inflammation with human lung tissue could help aid our understanding of these events.

It has been demonstrated using this model system that ligands for TLR3 and TLR4 can initiate responses in human lung tissue of patients with mild to moderate COPD. It is apparent however, that the responses between the ligands for these receptor subtypes differ markedly based upon the cytokines produced and their kinetic profiles. Furthermore, data suggests that there is marked variability in cytokine responses between patients.

It has been shown that 100μg/ml Poly I:C was an effective stimulus for IL-8, ENA-78 and MIP-1β production in human lung tissue fragments. Chemokines are extremely important in the pathogenesis of exacerbations through recruitment of inflammatory cell types (Chung et al 1987). Studies performed in bronchial biopsy specimens (Vachier et al 2004) and in the peripheral airways of COPD patients (Chanez et al 2002) have shown that a mononuclear cell infiltration, consisting predominantly of neutrophils and macrophages is prevalent in patients with moderate to severe COPD. Although the role of neutrophils in the pathogenesis of COPD still has not been fully clarified, it is likely that neutrophil accumulation in the airways of patients with COPD is driven by increased production of IL-8 and ENA-78, exerting a chemotactic effect on these cells (Donner et al 2002). It was demonstrated accordingly in this system that concentrations of IL-8 and ENA-78 increased steadily up to 48hrs with both Poly I:C and LPS stimulation respectively. These results are supported by Mallia et al (2006), who reported an up-regulation in gene expression for several neutrophil chemoattractants in patients intubated during severe viral and bacterial induced exacerbations. In addition, an analysis of BAL (Sanders et al 2000) and sputum samples (Di Stefano et al 2004) have also found increased levels of IL-8 and ENA-78 in patients with mild to moderate COPD, suggesting that migration of neutrophils from the bronchial wall to the lumen could be increased even in patients with the mildest forms of the disease. It has also been shown that Poly I:C exposure triggers MIP-1β in human lung tissue. MIP-1β is a member of the CCL4 chemokine family that is produced by several cell types, particularly epithelial cells and macrophages (Sauty et al 1999). MIP-1β is a ligand for the CCR5 receptor and serves as a selective chemoattractant for monocytes and macrophages. Various TLRs, including TLR3, have been reported to induce the production

of MIP-1β, whose gene is a primary target of the NFkB transcription factor in the antiviral, dsRNA-dependent response (Doyle et al, 2002). Increased expression of MIP-1β has been observed in the airway epithelium following viral infection (Spurrell et al 2005) and also in patients with COPD (Saetta 2002) and asthma (Medoff et al 2002), where levels correlate with increased macrophage numbers. Furthermore, both MIP-1β and RANTES expression (a CC +Tcell chemoattractant) are increased in sputum samples taken from COPD patients during an acute viral-induced exacerbation (Korpi-Steiner et al 2006). Clearly, a better understanding of the mechanisms of chemoattractants, together with identification of the important inflammatory cell types involved in COPD, may allow for novel therapeutic targets in the near future.

Interestingly, not all cytokines were increased in response to Poly I:C. No detectable changes in the pro-inflammatory cytokines TNFα, IL-1β and IL-6 were observed in the model system. In contrast, TNFα production was significantly elevated by LPS stimulation. One possible suggestion for the poor TNFα response by Poly I:C may include the up regulation of specific proteases by the tissue, resulting in the breakdown of this cytokine. Poly I:C however has been shown to stimulate TNFα production from isolated macrophages in patients with mild to moderate COPD (Seemungal et al 2000). Due to the nature of the model, the majority of alveolar macrophages are washed from the system, which may also explain the inconsistiency of these results with this data. Interestingly, although Poly I:C was a poor stimulus for pro-inflammatory cytokine expression, chemokine expression appeared to increase rapidly from 24hrs. This may indicate that there are other important cytokines involved during the Poly I:C response, which are responsible for triggering early chemokine production. Clearly, further work is required to identify

these cytokines and the role they play in the Poly I:C inflammatory response. Neither LPS nor Poly I:C induced significant production of IL-6 from the tissue explant, the main source of which is the lung fibroblasts. Due to the nature of the culturing technique, it is unlikely that there would be many fibroblasts within the system and could therefore provide one possible explanation for this result.

It has also been shown that IL-5 and IL-13 were not up regulated in response to Poly I:C. Interleukins 5 and 13 are associated with a Th_2 type response. Since COPD is believed to be the result of a Th_1 response (Barnes et al 2001), it was therefore unsurprising that these two cytokines should not be elevated. Results from LPS stimulated tissue also demonstrated little to no IL-5 or IL-13 production and provide further evidence that exacerbations are the likely to be the result of a Th_1 type response. IL-5 in particular has been acknowledged for many years as an important mediator of eosinophil intergrin-dependent adhesion. Although eosinophils are not significantly elevated in COPD patients during an acute exacerbation (Bischoff et al 1990), increased influx of eosinophils has been documented in other airways conditions such as Asthma (Chung 2001).

IL-10 is a potent anti-inflammatory cytokine, which resolves pro-inflammatory responses by inhibiting cytokine production in many inflammatory cell types (Takanashi et al 1994, Cassatella et al 1993). Interestingly, although no pro-inflammatory cytokines were significantly elevated with Poly I:C, increased levels of IL-10 were detected at 24hrs (4.9pg/mg tissue) and continued to increase up to 48hrs (6.5pg/mg tissue) following Poly I:C stimulation. This observation may indicate that in contrast to the LPS response that is TNFα dependent, other cytokines are involved in inducing IL-10 production during viral

replication. Indeed, the response from Poly I:C was observed to be much slower than that of LPS, the latter of which was significant from 6hrs (3pg/mg tissue) and peaked at 24hrs (13pg/mg tissue) before the response declined. Similar results for the induction of IL-10 by Poly I:C have also been documented in BAL samples of healthy patients undergoing low dose stimulus inhalation (Claries et al 2005).

Interestingly, it was observed that there was a great deal of inter-patient variability for cytokine production amongst all patients analysed. This observation was most apparent for the three chemokines analysed. From the results of *figure 3.7*, it can clearly been seen that for most patients, chemokine production increases significantly above control at 24hrs. There are however, patients who do not seem to respond to Poly I:C stimulation and furthermore, there are patients which demonstrate that chemokine production is inhibited following Poly I:C stimulation when compared to control. These vast inter-patient differences explain why the results obtained ENA-78 and other chemokines teetered on statistical significance. Unfortunately, although many patient variables were analysed, no statistical significances were found and therefore requires further investigation. Some possible suggestions may include differing chemokine receptor expression or other patient variables that have not been taken into account such as prescription drugs.

As part of the analysis, relationships were investigated between different cytokine responses. Overall, it was found that there was no significant correlation between any of the cytokines analysed. Interestingly, however, it was found that there was a weak positive relationship between ENA-78 and IL-8 and a weak negative correlation between IL-10 and ENA-78 production (*figure 3.8*). To begin with, it is well known that both ENA-78 and IL-

8 are synthesized from human lung epithelial cells (Chris et al 1998). This pattern would therefore make biological sense. Clearly, more research and greater sampling is required to investigate this observation further. There is also evidence to suggest that IL-10 inhibits the production of CC-chemokines. Healthy subjects intravenously injected with LPS following pre-treatment with recombinant human IL-10, were demonstrated to inhibit the release of MIP-1α and MIP-1β from peripheral blood mononuclear cells (PBMCs) (Olszyna et al 2000). Similar observations have also been documented in alveolar macrophages (Berkman et al 1995). Again, more research is required to pursue this observation further.

Bacterial LPS appeared to demonstrate a different cytokine profile to that of Poly I:C. It was observed that LPS induced pro-inflammatory increases for both TNFα and IL-1β. It is well known that the macrophage is an important source for TNFα production within the lung and that such cells are a plentiful source of TLR4 receptors (Simpson et al 1999). Therefore it is likely that one of the major sources of TNFα *in vivo* following LPS stimulation is the macrophage (Khair et al 1996). However since the majority of macrophages have been leached during washing phases of the tissue protocol, it raises the question as to what cells may be contributing to the response observed in these explants? It is known that small amounts of TNFα are also produced by mast cells and endothelial cells within human lung tissue in response to LPS, which could explain this phenomenon (Simpson et al 1999). Early maximal production of TNFα triggers an acute inflammatory cytokine cascade latter dampened by stimulation of anti-inflammatory cytokines (Barnes 2006). During this cascade, IL-6, IL-8 and IL-10 are all significantly elevated following TNFα production. It was likewise observed in this study that IL-10 was significantly

elevated by LPS and that the maximal response was indeed several hours later than TNFα. IL-6 production was not significantly raised above control in LPS stimulated tissue. Like Poly I:C, the main sources of LPS induced IL-6 *in vivo* are the tissue fibroblasts, few of which are likely to have been cultured in the lung explants. Interestingly, there is evidence to suggest that IL-6 production can also occur from epithelial cells following LPS stimulation (Chang, 2001). This could suggest that there were limited epithelial cells within the tissue parenchyma or that there were inhibitory mechanisms regulating the release of IL-6 from the tissue. Further research is required to understand these observations (*see chapter 5 discussion)*. It has also been observed that for LPS, chemokine expression increased significantly from control, peaking and plateuing from 24 to 48hrs for MIP-1β and IL-8, and continuing to increase up to 48hrs for ENA-78. Like viruses, bacteria also stimulate the production of numerous chemokines and chemoattractants for the influx of various inflammatory cell types including eosinophils, neutrophils, lymphocytes and macrophages and monocytes (Simpson et al 1999). These cell types are clearly essential for the rapid local inflammatory response for the isolation and removal of such foreign pathogens. LPS, like Poly I:C, stimulates the production of ENA-78 and IL-8 from predominantly lung epithelial cells (Chris et al 1998). Interestingly, LPS has been demonstrated to increase the synthesis of MIP-1β, primarily a macrophage derived product. Due to the low numbers of macrophages within the system, it is likely that synthesis is also occurring from elsewhere in the tissue. One recent publication has suggested that macrophage inflammatory proteins can also be induced from pneumocytes by LPS, which may explain these results (Kumar et al 2006).

In this chapter the role of the viral pattern-recognizing receptor TLR3 in human lung tissue has been explored, showing that exposure with Poly I:C resulted in activation of pathways leading to cytokine production. The response of human lung tissue to that of a known bacterial inflammatory stimulus and TLR4 activator, LPS, has also been investigated. A summary of the data presented in this chapter can be found in *table 3.3* below.

Cytokines Investigated (All results expressed as mean pg/mg tissue±SEM)	Kinetic time-points			Dose producing maximal response for Poly I:C (μg/ml)
	6hrs	24hrs	48hrs	
TNFα	3.3±0.3	4.3±0.2	5.0±0.4	
	23.1±10.9*	77.2±12.1*	62.9±17.6*	
	4.3±0.5	4.7±0.6	5.2±0.7	Not significant at any dose
IL-1β	0.9±0.2	1.4±0.4	1.9±0.5	
	2.5±0.9	4.8±1.2*	8.5±1.1*	
	1.5±0.3	2.4±0.8	3.1±1.3	Not significant at any dose
IL-6	721±201	1005±581	2476±1502	
	1059±501	3567±1590	3210±2700	
	850±220	2750±1010	4211±2330	Not significant at any dose
MIP-1β	3.4±2.1	5.9±1.1	6.1±1.4	
	15.7±4.1	57.1±15.1*	57.3±10.9*	
	9.8±1.9	22.6±3.0*	47.8±4.1*	10
IL-8	431±87	601±101	988±211	
	466±305	2866±529*	3005±432	
	150±109	2301±531*	3150±611*	100
ENA-78	20.5±8.9	123.2±41.1	101.5±31.1	
	52.1±12.5	175.4±50.8*	413±41.6*	
	51.8±10.7	172.9±56.1	467±38.7*	Not significant at any dose
IL-5	0.21±0.1	0.42±0.17	0.49±0.19	
	1.01±0.29	2.02±0.12	0.05±0.14	
	0.39±0.11	1.78±0.13	1.34±0.27	Not significant at any dose
IL-13	0.40±0.32	0.99±0.89	1.01±0.92	
	1.4±0.39	2.78±0.22	2.51±0.99	
	0.57±0.02	2.17±0.19	1.15±0.89	Not significant at any dose
IL-10	0.81±0.41	0.89±0.29	0.95±0.37	
	2.45±0.94*	13.2±2.33*	11.6±1.46*	
	0.99±0.38	4.11±0.61*	6.87±0.50*	100

KEY

Control
LPS
Poly I:C

Table 3.3 Summary table of data generated in chapter 3. For each cytokine studied, tissue production expressed in mean pg/mg tissue has been documented for control (in white boxes), LPS (in dark grey boxes) and Poly I:C (in light grey boxes). The summary table shows results over a range of three kinetic time-points; 6, 24 and 48hrs. * Indicates results were significant from control ($P<0.05$). The final column demonstrates the dose of Poly I:C (100, 10 or 1μg/ml) that generated the maximal tissue response for cytokine production. A known submaximal 100ng/ml of LPS was used throughout dose response work to compare to the effects of Poly I:C.

It is interesting to observe from the results of *Table 3.3*, that there are clear inflammatory profile differences between Poly I:C and LPS. LPS rapidly stimulated significant production of both TNFα and IL-1β in under 24hrs, whereas Poly I:C had no significant effects on any of the observed inflammatory cytokines. This already begins to indicate that both Poly I:C and LPS maybe inducing different cytokine profiles. It is also apparent from *Table 3.3* that chemokine expression is equally elevated by both Poly I:C and LPS. Generation of chemokines by both stimuli maybe important in the pathogenesis of COPD exacerbations through recruitment and activation of inflammatory cell types and therefore requires further investiagation. It is interesting to note that MIP-1β appears to be more sensitive to the dose of Poly I:C than IL-8 and therefore raises questions as to its roles in early response to virus in the lung. Both IL-5 and IL-13 were not significantly elevated by Poly I:C or LPS, suggesting the mechanisms of exacerbation are likely to be the result of a Th_1 response. This certainly appears to follow with the concensus of work published to date.

One reason for the general slower response of Poly I:C compared to LPS may be explained by the locational differences between the two receptor subtypes. TLR4 is found in high concentration on the surface membrane of cells where upon it can readily interact with extracellular LPS to rapidly activate the NFκB pathway. The intracellular localization of TLR3 however, is consistent with its role in sensing viral replicative elements. To activate the cells, Poly I:C must first be internalised and then dsRNA delivered to intracellularly located TLR3 present on matured endosomes. This internalisation and presentation of Poly

I:C takes time, and as a result may account for the kinetic differences between the two stimuli.

Overall, although Poly I:C has been very useful in our understanding of the cytokines involved in viral exacerbations of COPD, its relevance to true viral infection in the lung remains controversial. It is well established that there are many other interactions between virus and host cells (prior to viral replication) that contribute to the complete inflammatory response. For example, viral capsulate proteins are capable of inducing an acute inflammatory response, through direct interaction with surface receptors on neutrophils and macrophages (Contoli et al 2007). Therefore, an actual model of inflammation by virus in human lung tissue would help aid our overall understanding of viral-induced exacerbations of COPD.

Chapter 4:

The acute inflammatory response to killed influenza virus.

4.1 Introduction

Respiratory viruses are a major cause of acute exacerbations of COPD. The specific mechanisms through which viruses invoke exacerbations, however, remain unclear. Investigations have recently improved with commercialisation of the viral mimic, Poly I:C, however its relevance to true viral infection within the lung remains to be proven. To date, there is little information on the inflammatory response induced by many different viruses and likewise the kinetics and dose dependency of this response. Therefore, an actual model of inflammation by virus within human lung tissue could help aid our overall understanding of viral-induced exacerbations of COPD.

Growing evidence suggests that direct viral infection of the lower respiratory tract, leads to a robust host inflammatory response, which via as of yet undetermined mechanisms, trigger an exacerbation. It is known that many viruses infect both the upper and lower respiratory tracts, with the principal site of infection being the airway epithelial cell. Viruses cultured in human airway epithelial cells have been shown to result in the production of numerous pro-inflammatory cytokines and chemokines, including IL-6, IL-8, IL-10, IL-15, IL-18, transforming growth factor β (TGF-β), MIP-1β, ENA-78, interferon (IFN)-inducible protein of 10 kDa (IP-10), regulated on activation normal T-cell expressed (RANTES), granulocyte macrophage-colony stimulating factor and eotaxin (Spurrell et al 2005, Chen et al 2006, Korpi-Steiner 2006). This profile of mediators could enhance airway inflammation via the recruitment and retention of a wide range of inflammatory cells that contribute to the pathogenesis of exacerbations (Spurrell et al 2005). It is hoped that a better understanding of the acute inflammatory response by virus will help our overall understanding of viral-induced exacerbations of COPD. In the future this may allow for novel therapeutic targets.

Influenza virus is one of the major causative pathogens of all acute COPD exacerbations. Approximately one third of all viral-induced exacerbations are caused by influenza (Bayder et al, 2005). Influenza is a disease with global impact that causes enormous morbidity and mortality on an annual basis. It causes a broad range of illness ranging from asymptomless infection to fulminant primary viral and secondary bacterial pneumonia. The severity of infection depends on both the virus strain and a number of host factors, primarily age and the presence of comorbid conditions such as cardiopulmonary disease and those with COPD (Kristin et al 1999). Increasing use of vaccination and the development of new antiviral drugs hold out hope that the burden of disease associated with influenza can be reduced. However, the constant emergence of new influenza strains and the current risk of an avian flu pandemic serve as warnings that influenza will remain a serious pathogen for the foreseeable future. This chapter aims to investigate the local inflammatory response of human lung tissue to killed influenza virus. Killed virus was chosen for its safer use in the laboratory and allowed greater control over dose without concern over rates of cell infectivity in the model system.

4.2 Methods

Human lung tissue was removed from mild to moderate COPD patients undergoing resection for cancer. The tissue used was from the non-cancerous margin surrounding the tumour. Data was collected regarding patient age, gender, smoking status and lung function at the time of sample collection (*table 4.1*). Tissue fragments were processed as previously described (*Methods 2.3*) and prepared for primary cell culture.

No. Subjects	n=8
Age	64.3±1.7
Gender	5 Males 4 Females
Lung function (FEV_1/FVC)	0.65±0.12
Smoking Status	Ex =6 Current =1 Unknown=1
Pack years	42.9±1.5

Table 4.1 Patient characteristics of subjects prior to removal of human lung tissue.

4.2.1 Dose response and kinetics preparation

Tissue fragments from 8 patients were cultured in RPMI-1640/PSG and stimulated with viral concentrations of 200, 20, 2, 0.2 and 0.02 $x10^6$ vp/ml. These values corresponded to a multiplicity of infection (MOI) of 10, 1, 0.1, 0.01 and 0.001 viral particles per tissue cell respectively. MOIs were obtained by firstly calculating the total amount of DNA in a 20mg mass of lung tissue. Following this, a factor for the average amount of DNA per cell was used to calculate the approximate number of cells in the explant. Once the number of cells in an average explant was obtained, viral stock concentrations could be diluted accordingly to achieve the desirable MOIs. In addition, appropriate buffer controls and LPS stimulated tissues were also prepared. Tissue fragments were harvested and weighed at 24, 48 and 72hrs and lung supernatants aliquoted and stored at -80°C prior to analysis. A 72hr time point was included based upon the results of Poly I:C kinetics data, which suggested cytokine responses are still increasing after 48hrs.

4.3 Results: Killed virus dose response

4.3.1 Inflammatory cytokines

Three inflammatory cytokines were measured using ELISA (*figure 4.1*). These included TNFα (*left*), IL-1β (*centre*) and IL-6 (*right*). The production of all three pro-inflammatory cytokines showed no significant response for any of the concentrations of killed virus, when compared to appropriate controls at 24hrs. In the cases of TNFα and IL-1β, the levels of cytokine production by the highest dose of killed virus were similar to that produced by 100μg/ml Poly I:C (*see results 3.1*). In comparison, the levels of IL-6 produced by a 10vp/cell viral dose were several orders lower than that produced by 100μg/ml Poly I:C (Mean±SEM = 109.9±31.3pg/mg tissue vs 6700±2500pg/mg tissue respectively).

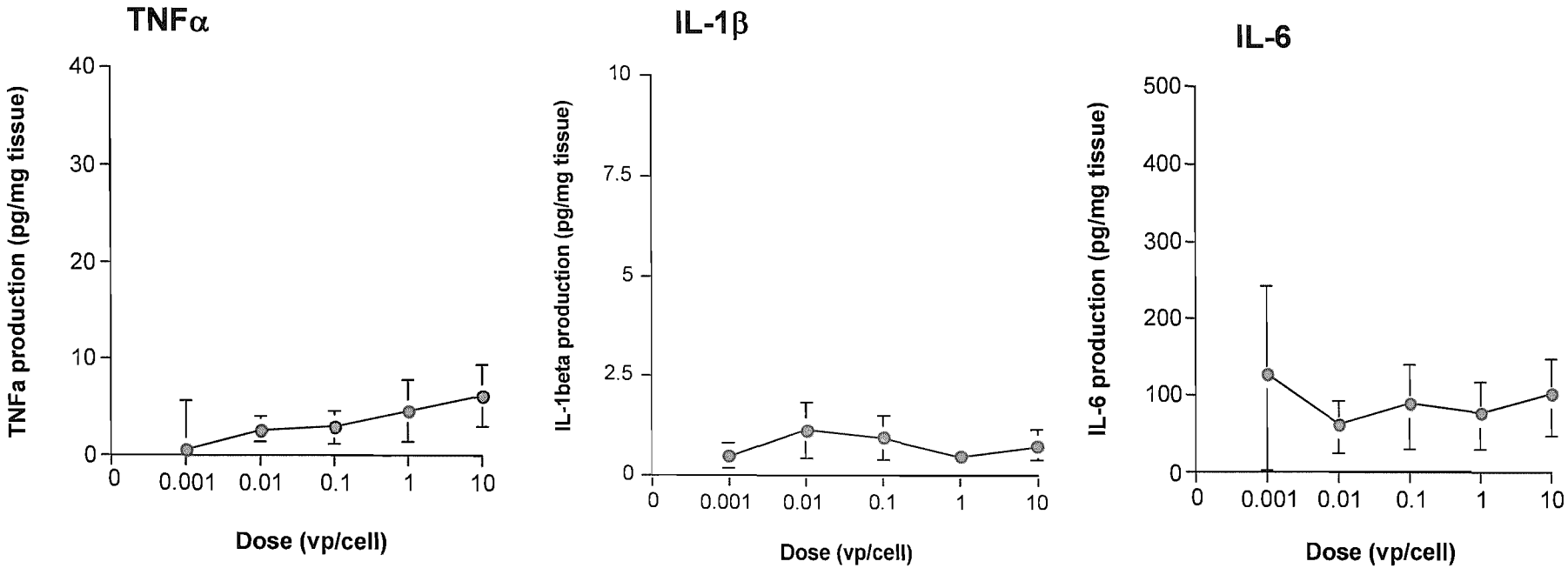

Figure 4.1: Cytokine levels detected in the supernatant for TNFα (left), IL-1β (centre) and IL-6 (right), following human lung tissue stimulation with varying doses of killed influenza virus at 24hrs. Release of cytokines was measured using ELISA. Values shown are the mean ± SEM and are expressed as pg/mg tissue. Groups were compared to controls using Wilcoxon signed rank; * indicates a P value <0.05.

4.3.2 Chemokines

A group of three chemokines were investigated as shown in *figure 4.2*. In comparison to the pro-inflammatory cytokines, all three chemokines demonstrate significant production with stimulation by killed influenza. MIP-1β production (*left*) appeared significant from control at 48hrs with tissue stimulation of 1 vp/cell (5.26±0.69pg/mg tissue vs 3.13±0.56pg/mg tissue in control) and 10 vp/cell respectively (5.62±0.58pg/mg tissue vs 3.13±0.56pg/mg tissue in control). No significant difference in MIP-1β production was found between these two doses of killed virus. Similar levels of MIP-1β were observed with killed influenza at a dose of 10 and 1 vp/cell, when compared to results of 100μg/ml Poly I:C stimulated tissue (*results section 3.3.2 and 3.4.2*).

Levels of IL-8 (*centre*) detected in the supernatant were once again found to be much greater in concentration than any other chemokines and cytokines analysed. IL-8 was found to be significantly elevated in tissue stimulated with 10 vp/cell killed virus when compared to control (7300±2690pg/mg tissue vs 2500±870pg/mg tissue). Interestingly, this latter response was much smaller than that observed by 100μg/ml Poly I:C at 48hrs.

ENA-78 (*right*) production was found to be similar in dose response characteristics to that of IL-8. ENA-78 production was significantly elevated in tissue stimulated with 10vp/cell of killed influenza at 48hrs (202±100.5pg/mg tissue vs 38.5±20.1pg/mg tissue in control). Production of ENA-78 with the highest dose of viral stimulation generated more cytokine production than that with 100μg/ml Poly I:C at 48hrs.

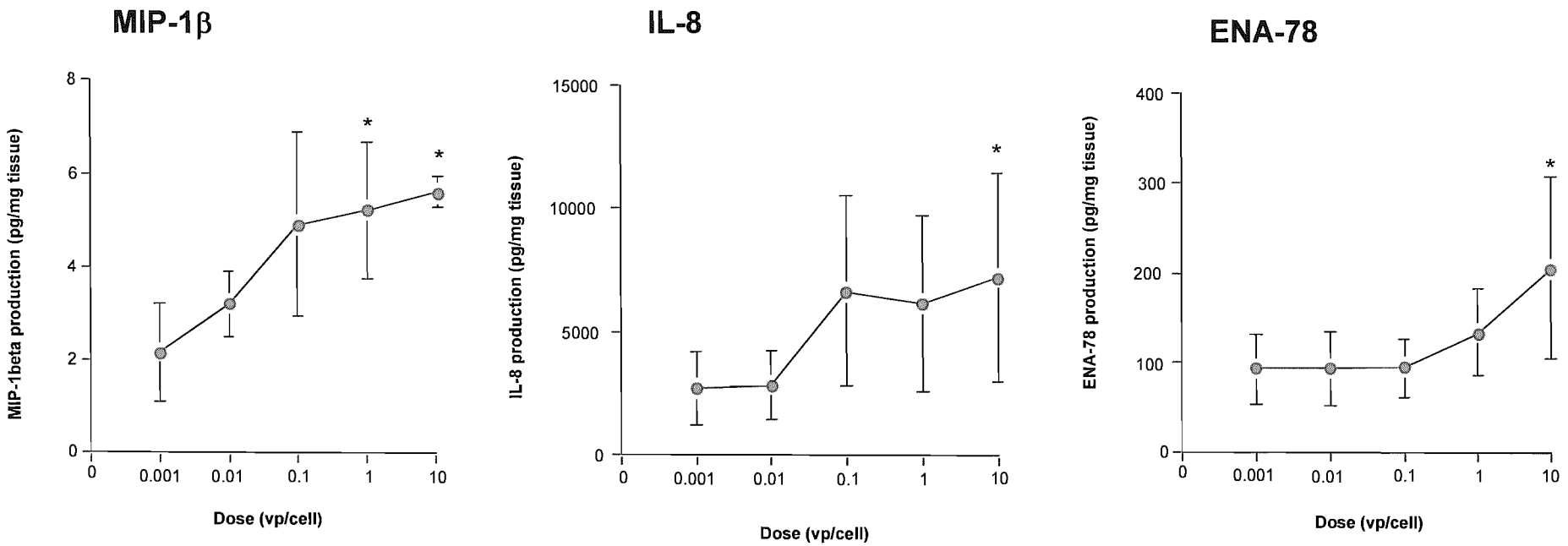

Figure 4.2: Cytokine levels detected in the supernatant for MIP-1β (left), IL-8 (centre) and ENA-78 (right), following human lung tissue stimulation with varying doses of killed influenza virus at 48hrs. Release of cytokines was measured using ELISA. Values shown are the mean ± SEM and are expressed as pg/mg tissue. Groups were compared to controls using Wilcoxon signed rank; * indicates a P value <0.05.

4.3.3 Th_2 cytokines

Interleukins 5, 10 and 13 are cytokines associated with a Th_2 response (*figure 4.3*). Similarly to observations with Poly I:C, there were no significant differences in the release of IL-5 (*left*) or IL-13 (*centre*) with any concentration of killed virus at 48hrs. In both cases, cytokine production was just above the limit of detection. In contrast to IL-5 and IL-13, *figure 4.3 right* shows that IL-10 production was significantly elevated by increasing doses of killed virus. At 48hrs, it was apparent that IL-10 production had reached significance with a dose of only 0.1vp/cell (0.6±0.05pg/mg tissue vs 0.2±0.01pg/mg tissue in control). This response was elevated with a higher dose of 1vp/cell (0.75±0.02pg/mg tissue vs 0.2±0.01pg/mg tissue in control) and even further by 10vp/cell (0.92±0.015pg/mg tissue vs 0.2±0.01pg/mg tissue in control).

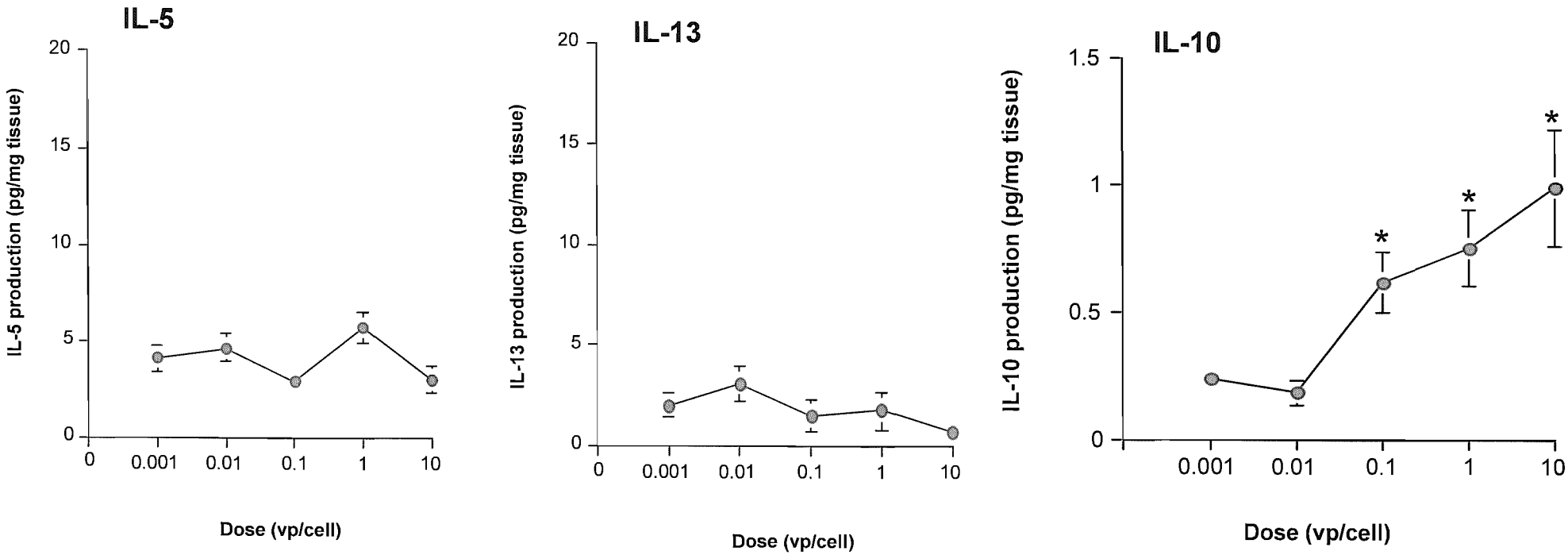

Figure 4.3: Cytokine levels detected in the supernatant for IL-5 (left), IL-13 (centre) and IL-10 (right), following human lung tissue stimulation with varying doses of killed influenza virus at 48hrs. Release of cytokines was measured using ELISA. Values shown are the mean ± SEM and are expressed as pg/mg tissue. Groups were compared to controls using Wilcoxon signed rank; * indicates a P value <0.05. No statistical differences from control were observed for both IL-5 and IL-13.

4.3.4 Th_1 cytokines

Since Poly I:C was found to be a poor stimulus for pro-inflammatory cytokine production, two further Th_1 cytokines were investigated using killed virus. These were interferons (IFN) alpha and beta respectively (*figure 4.4*). IFNα/β share a common multicomponent, cell surface receptor and elicit a wide range of biological responses, including antiviral, antiproliferative, and immunomodulatory activities in response to viral infection in the lung. Interestingly, it was observed that production of both interferons peaked at 24hrs with a low dose of 0.01vp/cell, before the response hereafter declined with increasing dosages of killed virus. In the case of IFNα, it was observed that there was a sharp decline in the response following 0.01vp/cell (3.75±1.01pg/mg tissue vs 0.02±0.01pg/mg tissue in control), suggesting that its production is dependent upon low concentrations of viral particles. The observed patterns for the two interferons were not statistically significant, which maybe due in part to a low sample size. Further experiments would therefore be beneficial.

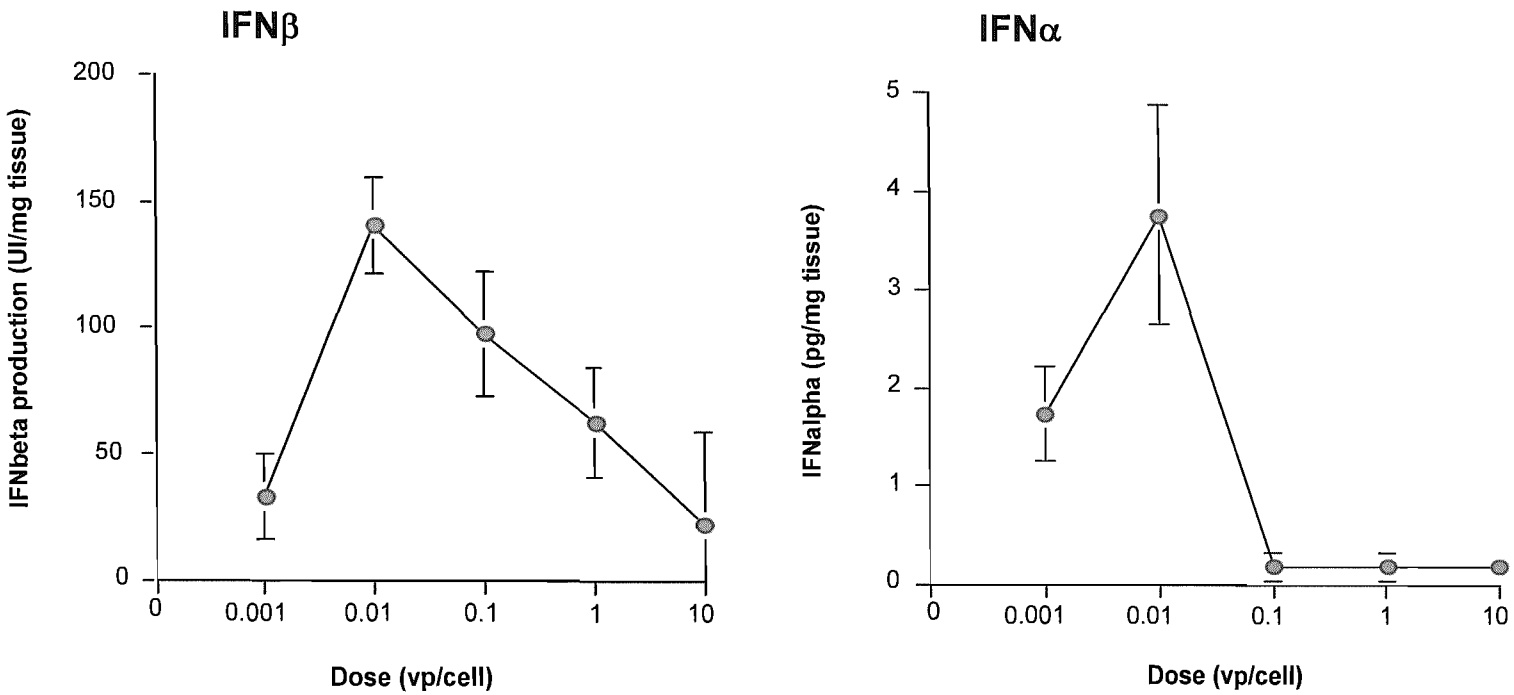

Figure 4.4: Cytokine levels detected in the supernatant for IFNβ (left) and IFNα (right), following human lung tissue stimulation with varying doses of killed influenza virus at 24hrs. Release of cytokines was measured using ELISA. Values shown are the mean ± SEM and are expressed as pg/mg tissue for IFNα and units/mg tissue for IFNβ. Groups were compared to controls using Wilcoxon signed rank; * indicates a P value <0.05.

4.4 Results: Killed virus kinetics

4.4.1 Inflammatory cytokines

Kinetic profiles for the release of the three inflammatory cytokines TNFα, IL-1β and IL-6 were observed over 24, 48 and 72hrs (not shown). The release of inflammatory cytokines followed the results that had been observed with both Poly I:C kinetics and killed virus dose response data. In all, neither TNFα, IL-1β or IL-6 were significantly elevated from control over the 72hrs respectively.

4.4.2 Chemokines

Killed influenza virus was found to generate significant release of chemokines from human lung tissue that increased with time. *Figure 4.5* shows the results for production of MIP-1β (*left*), IL-8 (*centre*) and ENA-78 (*right*). Interestingly, all three chemokines appear to share similar kinetic response characteristics and closely resemble data observed with Poly I:C kinetics over 48hrs. We can see from the results of *Figure 4.5*, that MIP-1β production increases rapidly from 24hrs, becoming significant at 48hrs (10.1±2.6pg/mg tissue vs 2.1±0.99pg/mg tissue in control) and increasing further up to 72hrs (12.3±4.8pg/mg tissue vs 2.1±0.99pg/mg in control tissue). The release of chemokine IL-8 (*centre*) followed a similar pattern to that of MIP-1β production, with a maximum response at 72hrs (6.2±0.51ng/mg tissue vs 1.1±0.33ng/mg tissue in control). The production of ENA-78 was almost identical to the MIP-1β response, with production increasing from 24hrs, becoming significant at 48hrs (130±25.5pg/mg tissue vs 42±21.3pg/mg tissue in control) and increasing further to 72hrs (150±27.1pg/mg tissue vs 42±21.3pg/mg tissue in control).

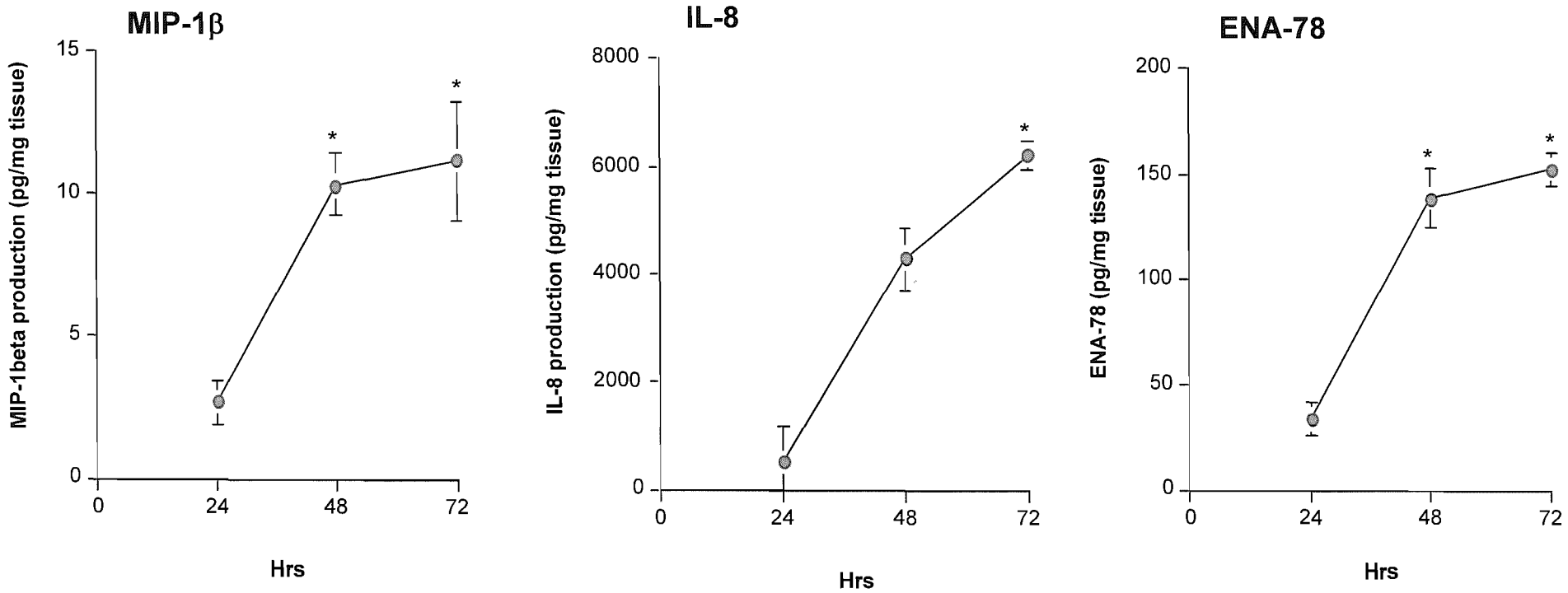

Figure 4.5: Cytokine levels detected in the supernatant for MIP-1β (left), IL-8 (centre) and ENA-78 (right), following human lung tissue stimulation over 72hrs with killed influenza virus. Release of cytokines was measured using ELISA. Values shown are the mean ± SEM and are expressed as pg/mg tissue. Groups were compared to controls using Wilcoxon signed rank; * indicates a P value <0.05.

4.4.3 Th_2 cytokines

The time course for the production of Th2 cytokines in response to killed influenza virus was studied. As previously observed for IL-5 and IL-13 (*not shown*), there were no significant changes in the time course for cytokine production by killed virus over 48hrs. Levels of IL-5 and IL-13 in all conditions were just above the detectable limit.

In contrast, the results obtained for the IL-10 (*figure 4.6*) demonstrate that cytokine production was significant from 24hrs (0.5±0.01pg/mg tissue) and peaking at 48hrs (1±0.04pg/mg tissue), before the response declined to 72hrs (0.5±0.02pg/mg tissue). At all time points investigated, IL-10 production was significantly elevated compared to control.

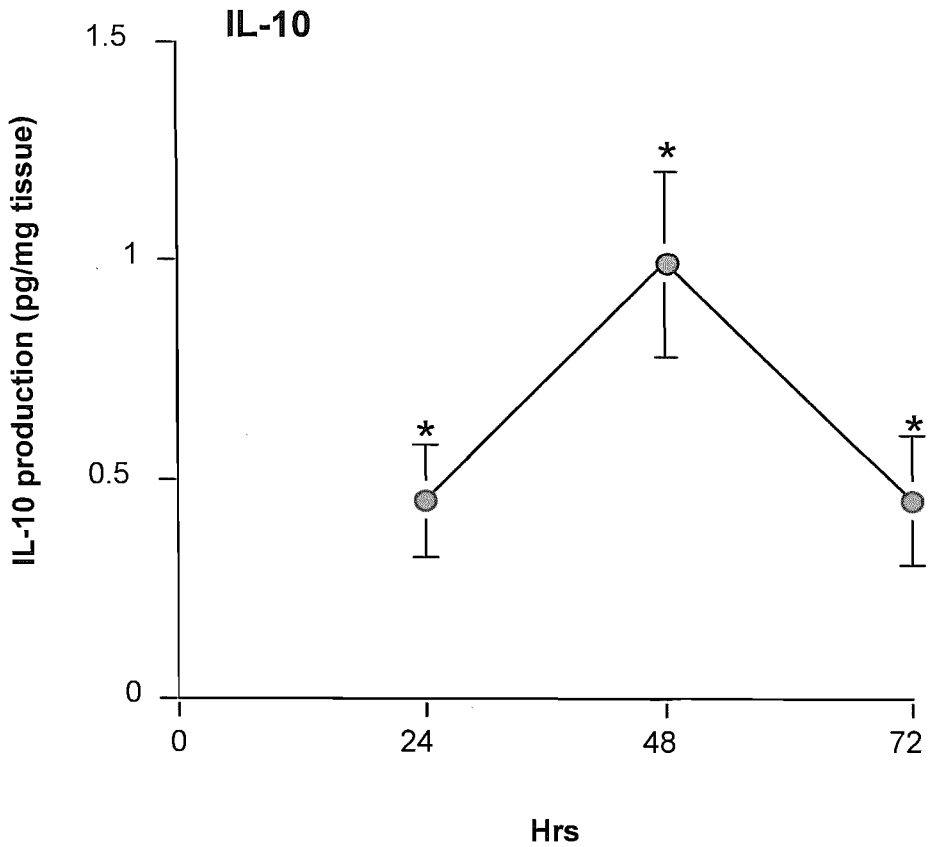

Figure 4.6: Cytokine levels detected in the supernatant for IL-10 following human lung tissue stimulation over 72hrs with killed influenza virus. Release of IL-10 was measured using ELISA. Values shown are the mean ± SEM and are expressed as pg/mg tissue. Groups were compared to controls using Wilcoxon signed rank; * indicates a P value <0.05.

4.4.4 Th_1 cytokines

The time course for the release of two interferons alpha (*left*) and beta (*right*) were next investigated (*figure 4.7*). In both cases, cytokine production followed a very similar pattern. IFNα/β reached maximal production at 24hrs (3.75±1.01pg/mg for IFNα and 140±12.5UI/mg tissue for IFNβ) before the response rapidly declined at 48hrs and plateaued at 72hrs respectively. Clearly, earlier time points are required to observe the initial kinetics of the two interferons prior to 24hrs. The observed patterns for the interferons are not statistically significant, which maybe due in part to a low sample size. Further experiments would therefore be beneficial particularly with the current interest surrounding interferons and viral infection (*see chapter 5 discussion*).

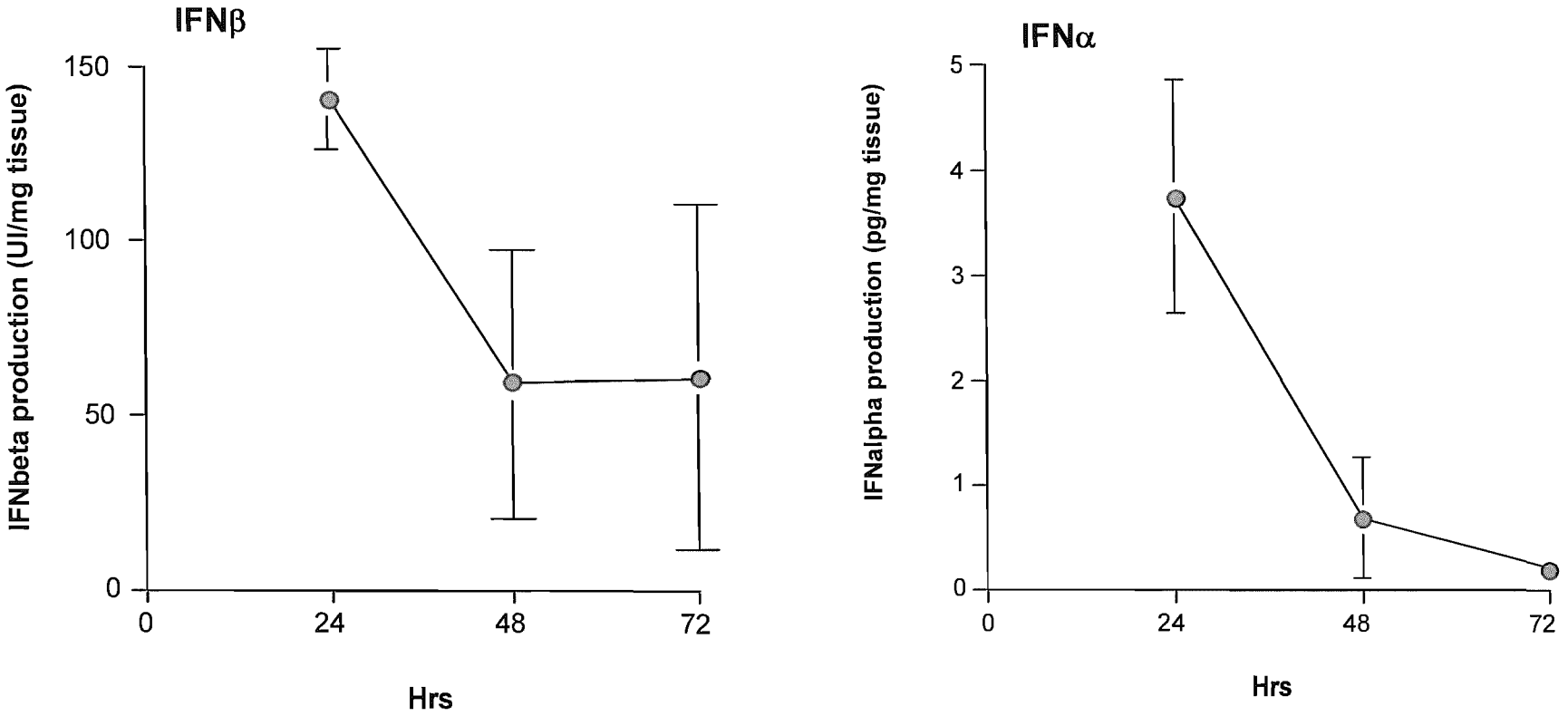

Figure 4.7: Cytokine levels detected in the supernatant for IFNβ (left) and IFNα (right), following human lung tissue stimulation over 72hrs with killed influenza virus. Release of cytokines was measured using ELISA. Values shown are the mean ± SEM and are expressed as pg/mg tissue for IFNα and units/mg tissue for IFNβ. Groups were compared to controls using Wilcoxon signed rank; * indicates a P value <0.05.

4.5 Discussion

The mechanisms of viral-induced exacerbations of COPD are very poorly understood. Investigations have recently improved with commercialisation of a viral mimic, Poly I:C, however its relevance to viral infection within the lung remains to be proven. More studies using intact viral infection in the lung are required to understand the full implications of host and viral mechanisms of inflammation. Using a model system, it has been demonstrated that an assortment of chemokines, Th_1 and anti-inflammatory cytokines are elevated in response to a killed influenza stimulus in human lung tissue. A summary of the information generated from this chapter can be found in *table 4.2* overleaf. For a detailed summary comparison of the effects of killed virus compared to Poly I:C and LPS please refer to *table 4.3*.

Cytokines Investigated All results are expressed as mean pg/mg tissue ± SEM unless stated	Kinetic time-points			Dose producing maximal response for Killed Virus (viral particles per cell)
	24hrs	48hrs	72hrs	
TNFα	0.99±0.19	2.1±0.49	2.9±0.33	
	1.3±0.32	3.1±0.71	3.1±0.11	Not significant at any dose
IL-1β	0.75±0.19	1.0±0.11	1.2±0.47	
	0.99±0.13	1.1±0.72	3.2±1.13	Not significant at any dose
IL-6	323±121	1211±912	1865±1444	
	420±190	1992±781	1999±1390	Not significant at any dose
MIP-1β	2.11±0.79	2.1±0.31	2.7±1.02	
	2.61±0.98	10.1±2.6*	12.3±4.8*	10
IL-8	661±176	4029±113	4031±101	
	675±198	4200±219	6200±510*	10
ENA-78	30.5±3.9	35.4±5.5	37.4±6.7	
	39.1±5.2	130±25.5*	150±27.1*	10
IL-5	0.20±0.05	0.47±0.19	0.55±0.31	
	0.22±0.01	0.92±0.26	1.11±0.69	Not significant at any dose
IL-13	0.41±0.29	0.91±0.77	1.18±0.82	
	0.44±0.10	1.45±0.42	1.49±0.43	Not significant at any dose
IL-10	0.01±0.01	0.39±0.09	0.11±0.01	
	0.5±0.01*	1±0.04*	0.5±0.02*	10
IFNα	0.29±0.22	0.41±0.29	0.19±0.07	
	3.75±1.01	0.75±0.55	0.32±0.23	0.01
IFNβ (mean UI/mg tissue +SEM)	26±9.2	23±8.7	32.9±13.9	
	140±12.5	56.5±33.1	63.5±50.1	0.01

KEY

Control

Killed virus

Table 4.2: Summary table for data illustrated in chapter 4. Tissue production of cytokines were measured over 24, 48 and 72hrs following stimulation with killed influenza virus (Dark grey boxes) compared with control (white boxes). All cytokine production is expressed as mean pg/mg tissue ±SEM with the exception of IFNβ, which is expressed as UI/mg tissue. * Indicates results were significant from control ($P<0.05$). The final column lists the MOI of killed virus (10, 1, 0.1, 0.01 or 0.001 viral partials per tissue cell) that generated the maximal tissue response for cytokine production. In the case of pro-inflammatory and Th_2 cytokines, no responses were significant.

A whole range of cytokines and chemokines are known to be induced during influenza infection in many different lung cell types, including bronchial epithelial cells (Flory et al 2000), alveolar macrophages (Julkunen et al 2001) and dendritic cells (Papadopoulos et al et al 1999). Collectively, these include IL-1, IL-2, IL-5, IL-8, IL-10, IL-15, IL-18, transforming growth factor beta (TGFβ), TNFα, IFNα/β, IFNγ, GM-CSF, MIP-1α/β, and RANTES (Flory et al 2002, Donninger et al 2003, Papadopoulos et al 1999). It can be observed from the results of *table 4.2* that killed virus was not an effective stimulus for the production of pro-inflammatory cytokines TNFα, IL-1β and IL-6. This response

is vastly different to LPS in which the majority of early up-regulated cytokines are pro-inflammatories. On the other hand, this response was identical to that observed with Poly I:C. Concordance for limited IL-1β production by virus has been documented in other studies of influenza-induced exacerbations of COPD (Van Reeth et al 2000). In contrast, there is evidence to suggest that TNFα production is significantly elevated in isolated alveolar macrophages when cultured with live influenza (Wilkinson et al 2004). This response however, is dependent upon the stain of the virus, its ability to replicate and the multiplicity of infection. More studies are required to investigate these findings. It was also observed that like Poly I:C, there was also no increase in Th_2 cytokines, IL-5 and IL-13, which can once again be related to the significance of COPD as primarily a Th_1 response (Hogg et al 2001).

It has been shown that chemokine expression increased significantly up to 72hrs upon stimulation with killed virus. Interestingly, for all three chemokines studied, the highest dose of killed virus was required to produce the largest tissue response. These results followed previous observations seen with the highest dose of 100μg/ml Poly I:C up to 48 hours (*Results 3.4.2*). Increased chemokine expression has also been detected in airway secretions during viral infections (Message et al 2004) and in sputum samples of mild/moderate COPD patients during a viral exacerbation (Chen et al, 2006). Induction of chemokines IL-8 and ENA-78 have been shown to occur within the first 24hrs following influenza infection and binding to ICAM-1 on human lung epithelial cells (Flory et al 2002). Furthermore, it is apparent that this process is not dependent upon viral replication, which supports our observations with *killed* virus (Contoli et al 2007). Despite the potential for chemokines to recruit multiple cell types to the airways, evidence of sputum from viral exacerbations of COPD (Saetta et al 2002) and

experimental infections of mice with rhinovirus (Mallia et al 2005) and influenza (Donaldson et al 2007), have suggested there is selective recruitment of neutrophils and lymphocytes. This implies that mechanisms must exist to limit the cell types recruited, however these mechanisms are not well understood.

In contrast to the rapid generation of chemokines by many viruses, other responses to viral infection, such as the generation of anti-inflammatory cytokines TGFβ and IL-10, do not occur until 24hrs post viral exposure (Flory et al 2002). This late phase response is believed to be dependent upon viral replication; however from results of this study it has been shown that *killed* influenza virus is an effective stimulus for the generation of IL-10 in human lung tissue. It was observed that IL-10 production was significant from 24hrs and peaked at 48hrs before the response tailed off. A very similar result was also documented with the use of Poly I:C, of which the response peaked at 48 hours. IL-10 is a multifunctional cytokine with diverse effects on most hemopoietic cell types. It inhibits the secretion of TNFα and IL-8 from macrophages and decreases the expression of matrix matalloproteinases (MMPs), while increasing the expression of endogenous tissue inhibitors of MMPs (TIMPs) (Cassatella et al 1993). Interestingly, evidence suggests that IL-10 concentrations are reduced in sputum from patients with COPD, thus highlighting a possible mechanism for increased lung inflammation (Barnes et al 2004). The development of a selective agonist for IL-10 receptors or unique signal transduction pathway activators could therefore be of potential benefit for patients with COPD (Takanashi et al 1994).

Table 4.2 illustrates a summary of the interferon kinetics and maximal dose response data. Interferons are a group of specialized cytokines produced in response to many

viruses, bacteria, and their products including viral glycoproteins, viral RNA, bacterial endotoxin, bacterial flagella and CpG DNA. In this model system, we observed interesting patterns with IFNα and IFNβ. Both IFNs were elevated with low doses of killed virus (0.01vp/cell) peaking early at 24hrs, before the responses hereafter declined at 48 and 72hrs respectively. Although the kinetics of the response appear to suggest that interferons are up regulated early during viral stimulation, more repeats and further investigations are required to see if these patterns are significant and furthermore if IFNs trigger cytokine cascades. It is clear that interferons play an important role in innate immunity; interferon deficient mice fail to mount a sufficient inflammatory response to both viral infection and stimulation with LPS or Poly I:C, leading to death (Mogensen et al 2001). Interferons are particularly important in inhibiting viral replication, in particular during stages of dsRNA synthesis. It is well known that both IFNα and IFNβ are secreted in large amounts in response to dsRNA, which fits with the observations using virus (Chen et al 2006). Viral dsRNA or the synthetic mimic, Poly I:C, activate TLR3 leading to phosphorylation of the transcription factor IRF3 and a late phase NFκB response (Mallia et al 2006). The genes that encode for interferons are switched on in an infected cell, and synthesized interferon is secreted to surrounding cells, thus preparing them for potential viral infection (Laza-Stanca et al 2006). IFNα and IFNβ reduce the cells ability to translate, therefore preventing viral replication and inhibiting normal cell ribosome function, killing both the virus and host cell if the response is active for a sufficient amount of time (Guerrero-Plata et al 2005). Due to its relative importance for inhibiting viral replication, it is not surprising that we should therefore observe high interferon production with low doses of killed virus.

Finally, *Table 4.3* overleaf provides an overall summary of the kinetic differences between Poly I:C and killed virus compared to LPS. These differences will be discussed in detail in Chapter 5.

Cytokines Investigated (Results expressed as Mean pg/mg tissue ± SEM)	Kinetic time points		
	24hrs	48hrs	72hrs
TNFα	4.3±0.2	5.0±0.4	-
	77.2 ±12.1*	62.9±17.6*	-
	4.7 ± 0.6	5.2±0.7	-
	0.99±0.19	2.1±0.49	2.9±0.33
	1.9 ± 0.21	2.7±0.61	3.1±0.11
IL-1β	1.4±0.4	1.9±0.5	-
	4.8±1.2*	8.5±1.1*	-
	2.4±0.8	3.1±1.3	-
	0.75±0.19	1.0±0.11	1.2±0.47
	1.5±0.28	1.5±0.67	3.2±1.13
IL-6	1005±581	2476±1502	-
	3567±1590	3210 ±2700	-
	2750±1010	4211±2330	-
	323±121	1211±912	1865±1444
	1782±883	3301±1764	1999±1390
MIP-1β	5.9±1.1	6.1±1.4	-
	57.1±15.1*	57.3±10.9*	-
	22.6±3.0*	47.8±4.1*	-
	2.11±0.79	2.1±0.31	2.7±1.02
	2.61±0.98	10.1±2.6*	12.3±4.8*
IL-8	601±101	988±211	-
	2866±529*	3005±432	-
	2301.3±531*	3150±611*	-
	661±176	4029±113	4031±101
	675±198	4200±219	6200±510*
ENA-78	123.2±41.1	101.5±31.1	-
	175.4±50.8*	413±41.6*	-
	172.9±56.1	467±38.7*	-
	30.5±3.9	35.4±5.5	37.4±6.7
	39.1±5.2	130±25.5*	150±27.1*
IL-5	0.42±0.17	0.49±0.19	-
	2.02±0.12	0.05±0.14	-
	1.78±0.13	1.34±0.27	-
	0.20±0.05	0.47±0.19	0.55±0.31
	1.79±0.33	1.69±0.21	1.11±0.69
IL-13	0.99±0.89	1.01±0.92	-
	2.78±0.22	2.51±0.99	-
	2.17±0.19	1.15±0.89	-
	0.41±0.29	0.91±0.77	1.18±0.82
	2.19±0.76	2.12±0.54	1.49±0.43
IL-10	0.89±0.29	0.95±0.37	-
	13.2±2.33*	11.6±1.46*	-
	4.11±0.61*	6.87±0.50*	-
	0.01±0.01	0.39±0.09	0.11±0.01
	0.5±0.01*	1±0.04*	0.5±0.02*

KEY

Controls
LPS
Poly I:C
Killed Virus

Table 4.3: Summary table illustrating kinetic data for three stimuli; 100ng/ml LPS (dark grey boxes), 100 μg/ml Poly I:C (light grey boxes) and 10vp/cell killed Influenza virus (black boxes) at 24 and 48 hours respectively. Only 72 hours time points were conducted for killed virus but have been included for clarity of results. For each cytokine, lung tissue production has been expressed in mean pg/mg tissue. * Indicates results were significant from appropriate controls ($P<0.05$). Two controls are shown (white boxes). For each cytokine, the first control shown refers to LPS and Poly I:C experiments, the second control refers to killed virus experiments.

To summarise, this model system has been used to show that killed influenza virus can induce a whole range of cytokines and chemokines that demonstrate markedly similar profiles and kinetic characteristics to that of Poly I:C. Chemokines are most noticeably increased in response to influenza virus and continue to increase significantly up to 72hrs compared to controls. It has also been demonstrated that anti-viral interferons, rather than pro-inflammatory cytokines, are rapidly generated early on during viral stimulation. The ability of IFNα and IFNβ to increase rapidly in response to low doses of viral MOI suggest it may play an important role in the initial stages of anti-viral inflammation, however, more research is required to investigate these observations.

Chapter 5:

Discussion

5: Discussion

To better understand the innate immune response of human lung tissue to viral infections, this study investigated the role of TLR3 in response to the TLR3 agonist Poly I:C and killed influenza virus. In addition, a bacterial agonist for TLR4, lipopolysaccharide, was also studied for comparison. Using this model system, it has been shown that ligands for TLR3 and TLR4 can initiate responses in human lung tissue of mild to moderate COPD patients. It is already apparent from the summaries of chapters 3 and 4 however, that the responses between the ligands for these receptor subtypes differ markedly based upon the cytokines produced and their kinetic profiles.

In general, it can be clearly observed from the results of *Table 4.3* that both killed influenza virus and Poly I:C demonstrate very similar kinetic responses to one another (see Chapter 4 discussion). Both these stimuli do not generate significant production of pro-inflammatory cytokines TNFα, IL-1β and IL-6, from human lung explants over 48 hours. Comparing these two stimuli now to that of LPS, it is clear to see that the kinetic profile is vastly different. LPS induced TLR4 activation resulted in significant production of both TNFα and IL-1β, but not however IL-6. Please refer to the discussion of chapter 3 for probable causes of limited IL-6 production. TNFα is one of the key cytokines initially involved in triggering a cytokine cascade in LPS induced TLR4 activation from primarily macrophages/monocytes but also pulmonary epithelial cells and T cells. Indeed studies neutralising the biological activity of TNFα are known to block the production of IL-6, IL-8, ENA-78, MIP-1β and IL-10 (Khair et al 1999). It is therefore likely that the significant production of the three chemokines and anti-inflammatory IL-10, also induced by LPS, were due to the initial production of TNFα. This appears to fit with evidence that TNFα synthesis from the tissue by LPS was

significant from as early as 6hrs post stimulation, while all other cytokines only became significant from 24 hours. In converse, there is little information describing the induction of cytokine cascades and kinetics of the inflammatory response during a viral exacerbation. Since TNFα production was not observed during TLR3 stimulation in these studies, it could be speculated that different cytokines are at work for the initial induction of the viral inflammatory cascade. Many recent studies to date have implicated interferons as a possible candidate (Contoli et al 2007, Doyle et al 2002). These interferons include IFNα, IFNβ and IFNγ respectively. It is known that both IFNα and IFNγ in particular induce the production of many chemokines including IL-8, ENA-78 and interferon inducible protein 10 or IP-10 in bronchioepithelial cells *in vivo* (Medoff et al 2002). In addition, IFNα is capable of inducing numerous pro-inflammatory cytokines including IL-1β and IL-4, which it could be proposed may lead to a further inflammatory cytokine cascade (Mogensen et al 2001). The work in this thesis has highlighted some interesting observations for anti-viral specific interferons. Both IFNα and IFNβ demonstrated patterns of early release with low doses of killed virus. This data is consistent with other published literature to date (Van Reeth 2000). More research focusing upon the kinetics and stimulatory factors inducing interferon production during viral exacerbations of COPD may be interesting for future development.

COPD is a debilitating disease characterized by recurrent episodes of leukocyte infiltration in the lung parenchyma, causing progressive pulmonary tissue damage and loss of function (Panina et al 2006). Recruitment of neutrophils, macrophages and $CD8^+$ T cells are linked to disease progression and is under control of chemotactic mediators produced in the inflamed lung. In this thesis it has been observed that three

of these chemotactic mediators, IL-8, MIP-1β and ENA-78 were all increased in response to the three inflammatory stimuli. To begin with, it can be seen from summary *table 5.1* that LPS and Poly I:C generated significant production of both IL-8 and MIP-1β from control at 24 hours respectively. In contrast, killed virus was a much slower stimulus for the production of both these cytokines and only became significant from 48 to 72 hours. One possible explanation for the kinetic differences between Poly I:C and killed virus is that Poly I:C can be more rapidly presented to TLRs within the cell endosomes, not requiring enzymatic breakdown and removal of the viral coating prior to dsRNA release by killed virus. In this fashion, Poly I:C could induce TLR3 activation and generation of inflammatory cytokines more rapidly than internalised virus.

It is also apparent from the results of *table 4.3* that LPS and Poly I:C demonstrate markedly similar kinetics and response patterns for ENA-78 production. In both cases, results are of a similar magnitude and appear significant from 24 hours increasing up to 48 hours respectively. Compare this to the response with killed virus however, and it can clearly be seen that the scale of ENA-78 production is a lot smaller than both LPS and Poly I:C and only becomes significant from 48 hours increasing up to 72 hours. Again this phenomenon could be explained by the time differences taken between internalisation and presentation of viral dsRNA and Poly I:C to TLR3.

IL-8, MIP-1β and ENA-78 all stimulate the chemotaxis of predominantly neutrophils and are potent angiogenic factors (Korpi-Steiner et al 2006). IL-8 and ENA-78 are synthesised and released from primarily human lung epithelial and endothelial cells *in vivo* (Contoli et al 2007). Expression of ENA-78 has also been observed in eosinophils (Julkunen et al 2001) and in type II pneumocytes (Korpi-Steiner et al 2006) and is

inhibited by the interferon IFN-γ (Contoli et al 2007). IL-8 can also be synthesised by a variety of inflammatory cell types expressing TLRs, with particular reference to neutrophils, which thus provides a positive feedback mechanism for inflammatory cell influx into infected tissues. Interestingly, while being as potent as IL-8 in inducing neutrophil responses, ENA-78 tissue expression often does not usually coincide with the expression of IL-8 for many viruses (Rieder et al 2001). It may therefore be of interest to any further research with influenza virus, that these two chemokines were simultaneously and significantly increased above control. One suggestion for this phenomenon could be that the tissue was overwhelmed by the concentration of killed virus, thus releasing both chemokines. This is unlikely however since even the highest dose of viral MOI was well within the recommended user range for tissue culture techniques and furthermore the virus was clearly not replicating. MIP-1β on the other hand belongs to the family of chemotactic Macrophage Inflammatory Proteins (or MIPs). In humans, there are two major forms, MIP-1α and MIP-1β that are now officially named CCL3 and CCL4 respectively. Both are major factors produced by macrophages after they are stimulated with bacterial endotoxins. MIP-1α and MIP-1β activate human granulocytes (neutrophils, eosinophils and basophils), which like ENA-78 and IL-8 lead to the production of acute neutrophilic inflammation. They induce the synthesis and release of pro-inflammatory cytokines such as IL-1, IL-6 and TNFα from fibroblasts and macrophages. It has been suggested that increases in chemokine or chemokine receptor expression may result in the phenotypic characteristics that are associated with COPD exacerbations (Chen et al 2002). Unfortunately more work is required to substantiate these ideas. Nevertheless, there is a lot of supporting research and publications that demonstrate increases in neutrophils and other types of

inflammatory cells within sputum and BAL fluids of patients with COPD, linked to increases in chemokine gene over-expression (Olszyna et al 2000).

Recent progress into of the molecular mechanisms that regulate the migration of inflammatory cells into the COPD lung have revealed interesting novel targets for therapeutic intervention. Chemokine receptors CXCR1 and CXCR2 expressed on neutrophils and CXCR3 expressed on CD8+ T cells have been identified as potential therapeutic targets to prevent recruitment of pathogenic cells into the inflamed lung (Chen et al 2002). Although promising, other recent observations have suggested that these chemokine receptors are also functionally expressed on various types of resident lung cell including epithelial and smooth muscle cells (Van Reeth et al 2006). This has raised new questions into the role played by chemokine receptors in disease pathology. One suggestion has been put forward that chemokine receptor signalling could contribute to the adaptive response of lung tissue to the micro-environmental changes induced by inflammation (Berkman et al 2005). Clearly, investigation into the roles of chemokines and their receptors in the development of COPD remains a fertile area of research. Hopefully the work provided in this thesis will help contribute to this research.

From the results of *table 4.3*, it can clearly see that all three stimuli generate production of the anti-inflammatory molecule IL-10. In all cases, cytokine production increases significantly up to 48 hours. It makes physiological sense for the synthesis of IL-10 to increase following an inflammatory stimulus. IL-10 is essential for dampening down the inflammatory response of the tissues, which otherwise unchecked can cause structural tissue damage and hence inflammatory lung disease. It has been previously reported that human mast cells have the ability to synthesise and release IL-10 spontaneously (Royer et al 2001). In addition the same group also demonstrated that

inhibition of IL-10 using a neutralising IL-10 antibody removed the inhibitory activity of IL-10 on the release of IL-5, IL-8 and TNFα. Many studies into the molecular mechanisms behind exacerbations of COPD have suggested that there is poor signalling from anti-inflammatory cytokines, in particular IL-10, which may contribute to the severity of inflammation observed in the lungs of these patients (Corsini et al 2005). In concordance with these suggestions, this thesis has illustrated IL-10 correlates with inhibitory effects upon ENA-78 expression, providing a possible mechanism for increased inflammatory cell burden in the lungs of patients during an exacerbation. It could therefore be proposed that low IL-10 levels would permit less regulated TNFα and ENA-78 activity, thus promoting neutrophil influx and a stronger pro-inflammatory response. To see if there were any differences in IL-10 production between disease severities within our patient cohort, subjects were split up according to their GOLD status and analysed for average cytokine production. It was found however that there were no significant differences in IL-10 production between these groups. Similar analyses were also conducted for many other patient characteristics including age, gender, smoking status and recent respiratory infection, however again there were no significant differences in IL-10 production between groups. The same technique was adopted for all other cytokines described within this thesis, however no patterns were found to be of significance. More kinetic investigations with human lung explants into the roles of other anti-inflammatory cytokines, for example TGFβ, should be conducted to identify their inhibitory effects upon other pro-inflammatory cytokine and chemokine production.

It should be considered when reading this thesis that the volume of work has been based upon bacterial and viral products to mimic exacerbations of COPD. To some extent,

even killed influenza virus may not induce the same inflammatory response as live and replicative viral infection. There are many other bacterial products besides LPS that can stimulate an immune response within human lung tissue. For example, unmethylated CpG sites of bacterial DNA are known to activate TLR-9, present on monocytes/macrophages, plasmacytoid dendritic cells and B lymphocytes (MacNee 2005). In addition, bacterial flagellin, long protein tail-like structures essential for locomotion, are known to activate TLR-5 on epithelial cells, stimulating predominantly TNFα production (Lorenz et al 2005). Furthermore peptidoglycans, sugar and amino acid polymers that form a mesh-like layer outside the plasma membrane of bacteria, stimulate TLR-2 activation and synthesis of TNFα, IL-8 and MIP-1β (Lorenz et al 2005). It is likely that these other bacterial products in addition to LPS contribute to exacerbations of COPD, however much more research is required to substantiate these ideas. It remains uncertain as to how all the components of bacteria generate an overall coordinated immune response and how this process may be controlled. It has been suggested that the controlling mechanisms for the inflammatory response may be deficient in exacerbations of COPD however this requires further investigation (Krutzik et al 2001). In this study, LPS was chosen for it known immunogenic properties and TLR4 activation. To further this research, explant studies using killed or preferably live bacteria maybe beneficial to begin to understand the overall immune response to bacteria as a whole organism. In the same way as bacteria, there are also many other *viral* products that are capable of generating an immune response rather than just double stranded RNA. T cell inflammatory responses to viruses can occur against any viral protein, as long as the protein can be processed by the cells and presented at the cell surface in conjunction with MHC molecules (Mallia et al 2006). Furthermore, antigenic viral capsule glycoproteins such as neuraminadase and haemagglutinin, essential for

binding to and entering host cells *in vivo*, can activate TLR7 and TLR9 of human host cells (Wilkinson et al 2004). This could have also explained some of the kinetic and cytokine profile differences observed between Poly I:C and killed virus. Both TLR-7 and TLR9 are also capable but to a lesser degree than TLR3 of detecting ribonucleoside analogues and therefore contribute to the inflammatory response of virus (Nichol et al 1999). Indeed, investigations in mice have demonstrated that production of interferons by TLR7/9 and TLR3 are different but yet critical for protective immunity to various experimental viral infections (Valenca et al 2006). It could be suggested therefore that TLRs need to be able to make different adaptations to the inflammatory response in order to deal with different types of viral stain effectively.

Overall, studying cytokine responses during exacerbations of COPD are hindered by the fact that clinical samples such as BAL are rarely taken during these episodes due to the severe deterioration of lung function in patients. Therefore it is not possible to study the mediators which are released during the inflammatory response *in vivo*. In addition, by the time patients present with exacerbations at hospital the initiation of the inflammatory cascade has already occurred. Work presented in this thesis utilised a model of acute inflammation involving primary culture of human lung tissue from smokers and ex-smokers with a range of lung functions. The advantage of using this model system is that it allows the analysis of the temporal release of cytokines during acute inflammation, which would be unethical to determine using current bronchial alveolar lavage techniques in COPD patients. Hopefully future work using this model system will give us a better insight into the molecular mechanisms and kinetics of acute inflammation during COPD.

5.1: Future work

This thesis has suggested a number of different research areas that require further investigation. To begin with, it is important to identify the cells within the tissue explant that maybe responsible for contributing to the synthesis or release of cytokines. It has already been discussed that some cell types are known to synthesise and generate cytokines, however, the research in this area is not complete and in general does not focus upon lung parenchyma. This work is central for continuing with the results discussed in this thesis. There are a whole host of cell types present within the lung parenchyma including, epithelial cells, endothelial cells, mast cells, Type I and II pneumocytes, as well as a variety of resident tissue macrophages, neutrophil and eosinophils. Understanding the cell types that may be involved in the inflammatory pathogenesis of exacerbations of COPD may help develop novel therapeutic targets in the future. Experiments that may help to identify the cellular source of such cytokines include co-localisation immunohistochemistry in which antibodies targeted for specific cytokines are used in conjunction with staining markers for common cell types. Taking this one step further, pre-treatment of the tissue with cyclohexamide, a protein synthesis inhibitor, prior to tissue stimulation with LPS, poly I:C or killed virus could help identify the sources of *de novo* cytokine synthesis when later performing immunohistochemistry on such prepared sections.

Another important aspect to research to focus upon is in determining whether synthesised cytokines and chemokines are active at the detected concentrations. One problem with using an ELISA based detection method is that it is not possible to tell whether the protein is active or in a functional or complexed state. To solve the latter problem, western blots can be performed using supernatant samples. To investigate

whether cytokines are active, a simple shape change assay could be exploited. With this set up, isolated human neutrophils (or other inflammatory cell types) can be exposed to the chemokine cocktail present within harvested lung supernatants and observed to see if they become active, hence changing shape. Furthermore, the application of specific neutralising chemokine antibodies (e.g. nIL-8 for neutrophils) and other chemotactic inhibitors could be applied to the supernatants to elucidate the key chemokines involved in cell activation. This approach can be adopted for many other chemokines and inflammatory cell types for example MIP-1β and macrophages/monocytes. These experiments are important in determining which cytokines are key to the inflammatory pathogenesis of COPD. This would have particular reference to the interferons, since it has been proposed they are likely to be one of the first elevated cytokines in the viral inflammatory response.

In general, more repeat studies are required to improve upon the accuracy of this data, with reference to the killed virus and interferon work. It may be interesting to do earlier time-point kinetics for the interferons, owing to the fact that they demonstrated an early peak at 24hrs. This may give some stronger indication as to whether interferons are indeed one of the primary cytokines elevated by viral inflammation. In addition to the use of influenza virus in this thesis, other viruses such as rhinovirus or respiratory syncytial virus (RSV) are also commonly implicated in the pathogenesis of COPD exacerbations. This versatile model system could be used to add to data on viral-induced inflammation, by studying the kinetics and dose response of cytokines initiated by different types of virus, particularly since it has been discussed that many viruses do not stimulate the same type of inflammatory response. Defining the key signalling events and cascade initiators of viruses in this manor could help to develop generalised

future therapeutics. A similar approach can also be adopted using bacteria instead of a virus, which would allow us to further examine the kinetics of true bacterial-induced exacerbations of COPD, rather than LPS alone.

Chapter 6:

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