Neutrophil Extracellular Trap Formation and Citrullination in Bronchiectasis

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Abstract

Bronchiectasis (BR) patients show risk for developing rheumatoid arthritis (BROS). As citrullination is implicated in rheumatoid arthritis pathogenesis it is possible that neutrophil extracellular trap (NET) formation, which is associated with PAD enzyme activity (i.e. citrullination), may be the mechanism connecting the two diseases. This body of work took 4 separate approaches to study the process of NETosis/PAD activity in the context of BR.

Firstly, healthy peripheral blood neutrophils were assayed for changes in NETosis/PAD following stimulation with BR relevant molecules. Both NETosis and PAD activity increased following incubation, suggesting BR related stimuli promote NETosis and citrullination. Secondly, LPS signalling was assessed to determine how inhibition of the two arms of the signalling pathway (TRIF and MyD88) impacted NETosis/PAD activity. Pre-treatment with either inhibitor downregulated both NETosis rates in vitro, suggesting molecular signalling underpinning NETosis is broader and more complex than predicted.

Thirdly, the impact of Cl-amidine (a PAD inhibitor) on NETosis, PAD activity and neutrophil function was assessed in vitro. Cl-amidine was shown to significantly reduce NETosis and PAD activity in response to BR stimuli. Superoxide production and phagocytosis was also shown to be inhibited by Cl-amidine, suggesting PAD plays some role these aspects of neutrophil function. Finally, NETosis, PAD activity and neutrophil function was assessed in healthy, BR and BROS neutrophils. Several differences in these results were observed between the groups, however limited sample size and lack of age matched healthy comparators complicate results interpretation.

These results imply that the molecular mechanisms underpinning NETosis/PAD activation is likely more complicated than previously suggested, with the results also indicating that PAD activity may play a role in neutrophil function. BR relevant stimuli appear to promote both NETosis and citrullination, with preliminary data suggesting there may be some difference in these processes between healthy, BR and BROS patients.

This thesis is dedicated to my parents David and Denise Cole

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Abbreviations Used

ACPA	Anti-citrullinated protein antibody
ANCA	Anti-neutrophilic cytoplasmic antibodies
ANOVA	Analysis of Variance
AU	Arbitrary Units
BACI	Bronchiectasis aetiology and co-morbidity index
BCOS	Bronchiectasis COPD overlap syndrome
BR	Bronchiectasis
BROS	Bronchiectasis rheumatoid overlap syndrome
BSA	Bovine serum albumin
BSI	Bronchiectasis severity index
CCP	Cyclic citrullinated peptide
CD4	Cluster of differentiation 4
CF	Cystic fibrosis
COPD	Chronic obstructive pulmonary disorder
CRF	Clinical research facility
CRP	C-reactive protein
CT	Computed tomography
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
ExRA	Extra-articular rheumatoid arthritis
FACED	FEV1, Age, Chronic colonisation, Extension, Dyspnoea
FC	Flow cytometry
FEV	Forced expiratory volume
FITC	Fluorescein isothiocyanate
fMLP	N-Formylmethionyl-leucyl-phenylalanine
HBSS	Hanks buffered salt solution
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IF	Immunofluorescence
IFN-γ	Interferon gamma
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MeoSAAPvN	N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide
Mg	Magnesium
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary response 88

Na	Sodium
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAD	Peptidylargininge deiminase
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PI	Propidium iodide
PKC	Protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
SOD	Superoxide dismutase
TNF-α	Tumour necrosis factor alpha
TRIF	TIR-domain-containing adapter-inducing interferon-β
WCL	Whole cell lysate

Chapter 1: Introduction

1.1 Bronchiectasis as an emerging problem

1.1.1 *Pathogenesis underpinning the onset of bronchiectasis*

Bronchiectasis is a chronic pulmonary condition defined by thickening and dilation of the bronchial airways and airflow obstruction which was first identified in the 1800's by Rene Laënnec (Lohani 2011, Rougin 2006). Commonly the disease is viewed to arise from a "vicious cycle" of pulmonary infection and inflammation. Typically, in healthy individuals' pulmonary infections are short lived and eradicated by a specific controlled immune response. However, in some individuals the ability to clear these infections is decreased leading to an exaggerated or imbalanced inflammatory response to resolve infection (Cole 1986).

This exaggerated inflammatory response leads to damaged local airway structures resulting in impaired clearance of mucus by airways, which increases the risk for severe repeat pulmonary infections to occur (Mandal 2013). Over time this cumulative damage from chronic airway inflammation and pulmonary infections leads to destruction of the airway infrastructure, which results in the permanent dilation of the bronchi and airway obstruction defining the disease (Nikolic 2018).

As bronchiectasis results from impaired immune defences within the lung, which results in accumulating damage due to infections/inflammation, there are a variety of diseases that progress to bronchiectasis (Bergin 2013). When examining bronchiectasis patient cohorts, the widespread aetiology is often easily observable with commonly seen causes including: cystic fibrosis (a major cause of paediatric bronchiectasis), severe respiratory infection, inherited immunodeficiency, cilia dysfunction etc. (Dodd et al 2015, Shoemark 2007, McDonnel 2013). However, a majority of bronchiectasis cases are idiopathic, highlighting the gap in knowledge surrounding the pathogenesis of the disease (Milosevic 2013).

1.1.2 *Epidemiology of bronchiectasis and economic/healthcare burden*

In the past decades bronchiectasis was often viewed to be an uncommon pulmonary disease with a low prevalence worldwide (Mandal et al 2013). However, in recent years

this perception has become increasingly challenged. Largely due to the wider implementation of CT-scans in diagnosing the disease in the developed world, coupled with advancements in the technology allowing more accurate diagnosis and reduced cost (Goeminne 2016).

Epidemiological studies have already demonstrated an increasing prevalence over a relatively short timescale of observation. In 2012 Seitz *et al* carried out a study in patients of the Medicare health system in the USA. They found that over 1% of those identified as using Medicare were already diagnosed with bronchiectasis. In addition, they also demonstrated over the course of their study that the prevalence of bronchiectasis seemed to be increasing by almost 95% per year (Seitz et al 2012).

Two European bronchiectasis epidemiological studies have shown similar results. In the UK population it has been observed that the prevalence and incidence of bronchiectasis in the population appeared to be increasing every year (Quint et al 2012). Furthermore, a similar study in Germany indicated that 67 out of 100,000 individuals in the country are diagnosed with bronchiectasis, which is higher than previously estimated (Ringshausen et al. 2015).

Hence there is a consistent increasing trend across developing countries that bronchiectasis is far more frequent than previously believed. With the knowledge that bronchiectasis patients are frequently hospitalised (with longer stays in hospital), need more follow up appointments and need repeated antibiotic treatments during exacerbation it is no surprise that bronchiectasis places a significant burden on public healthcare systems (Poppelwell 2014, Weycker 2005), in addition having severe implications on patient quality of life and mortality (Loebinger et al. 2009, Chalmers et al 2014). For these reasons there is great interest in further research into bronchiectasis to improve patient quality of life and potentially reduce healthcare burden.

1.1.3 *Bronchiectasis as heterogeneous disease*

Heterogeneity in bronchiectasis extends beyond the aetiology underpinning individual bronchiectasis cases. High-resolution CT scans demonstrate that many patients differ in the morphology of the airway dilation. There are 3 established classes: cystic, varicose and cylindrical, with significant variation in the location/distribution of the airway

damage in the pulmonary compartment e.g. single lobar diseases and multi lobar disease (Silva et al. 2010).

The clinical manifestation of the disease also varies between patients. The most common symptoms are productive cough and increased mucus production, however the combination of symptoms that manifest and their severity varies greatly between patients (ten Hacken 2007). Due to this heterogeneity in clinical phenotypes seen bronchiectasis there is surprisingly no single factor that can accurately indicate current disease severity or predict disease trajectory.

1.1.4 Assessing severity of bronchiectasis

The challenge in assessing bronchiectasis severity has resulted in many studies attempting to identify clinical indices that help in defining disease severity. A number of certain clinical factors (e.g. % FEV1, airway colonisation, exacerbations per year) have been shown to be related to the severity of disease. This has allowed the development of severity assessment tools such as the bronchiectasis severity index (BSI), which help guide physicians in diagnosing/monitoring/treating patients (Chalmers et al. 2014). This index has been improved upon in later years by the development of the bronchiectasis aetiology comorbidity index (BACI) and FACED score, which both support the diagnostic capabilities of the BSI (McDonnel et al 2016, Guan et al 2015, Martínez-García 2014).

Given time most patients with bronchiectasis are seen to develop a more severe phenotype of disease (Athanazio 2012). However, the rate at which this occurs varies greatly, creating great problems for physicians in attempting to predict which patients are at most risk from the disease and require more stringent monitoring and/or treatment.

1.1.5 *Bronchiectasis and its overlapping disease states*

Frequently patients with bronchiectasis are diagnosed with the disease after having been previously diagnosed with another pulmonary disease state such as asthma, chronic obstructive pulmonary disorder and cystic fibrosis (CF) (Gao et al 2016). However, CF bronchiectasis patients are often viewed as a distinct group separate from the rest of the BR patient population (i.e. non-cystic fibrosis bronchiectasis) due to differences in the pathogenesis and effective treatment between CF and non-CF

bronchiectasis (McShane et al 2013). These patients can be described as “overlap patients” and are understandably expected to exhibit a more severely affected quality of life and survival rates than patients with bronchiectasis alone.

One established and studied example of these bronchiectasis overlap patients are those diagnosed with bronchiectasis and overlapping COPD (BCOS). As predicted BCOS patients appear to have worse prognosis in terms of airway colonisation and exacerbations (Martinez-Garcia et al. 2015). The mortality rates in BCOS patients has also been seen to be far worse in comparison to all other non-cystic fibrosis bronchiectasis patient subgroups, with 55% of BCOS patients dying over a 5-year period whereas the 5 year mortality rates of the idiopathic and post-infectious BR patient groups was reported as 14% and 16% respectively (Goeminne 2014). Furthermore, in their 2015 paper on BCOS, Hurst et al. argued strongly that there are distinct differences within the BCOS cohort between primary-bronchiectasis patients (bronchiectasis developed first then COPD) and primary-COPD patients (COPD developed first then bronchiectasis) and that increasing our understanding of the mechanistic link between the two is critical to improving patient care.

The co-existence of pulmonary conditions such as COPD and cystic fibrosis with bronchiectasis is largely understandable given the pathogenesis of bronchiectasis (i.e. chronic pulmonary damage), however a sub-group of bronchiectasis patients exist whom are simultaneously diagnosed with rheumatoid arthritis (RA). These patients are referred to as “bronchiectasis rheumatoid overlap syndrome” (BROS) patients.

1.2 Rheumatoid arthritis: intra and extra articular effects

1.2.1 *Rheumatoid arthritis pathogenesis*

RA is a common autoimmune condition which primarily affects joints, typically those in the: fingers, wrists, elbow, ankle and knees (Glynn et al. 1972). Current understanding surrounding the initiation of the disease is limited, with many “triggers” (e.g. autoreactive antibodies) being suggested to play a potential role in paving the way for the inflammatory response seen in the disease. What is well established is that the early stages of the disease feature the synovial membrane becoming extremely inflamed

(with a large number of blood derived immune cells migrating into the tissue). The damage from this inflammation leads to a loss of anatomical integrity within the synovial lining associated with increased elasticity (Choy et al. 2012). Following this inflammation, invading neutrophils and osteoclasts are believed to drive degradation of bones and cartilage within the joint (Sato et al. 2006, Pillinger et al. 1995). Over time this inflammation of the synovial tissue coupled with the destruction of the bone and cartilage leads to the clinical manifestations classically associated with RA: joint pain, deformities and loss of mobility (Choy et al 2012).

1.2.2 *Extra-articular rheumatoid arthritis*

In addition to the debilitating joint destruction seen in RA, patients are also observed to exhibit systemic inflammation which places them at risk of various clinical complications outside of the joint. This manifestation of the disease is commonly referred to as extra-articular rheumatoid arthritis (ExRA). Systemic inflammation is argued to be the source of these extra-articular symptoms on the basis that many patients with ExRA have elevated levels of inflammatory associated molecules such as TNF-α, C-reactive protein (CRP) and rheumatoid factor (Sattar et al 2003, Turesson et al. 2007, Jonsson et al. 1995). Some of the commonly observed extra-articular complications seen in patients with RA include: fatigue, scleritis, stroke, limb nodules, anaemia, pericarditis, interstitial lung disease, cardiovascular disease (Vela 2014, Cimmino et al 2000, Shaw et al 2015).

As expected the implications of ExRA appear to have a largely negative impact on patient prognosis. Of the various observed ExRA phenotypes it has been suggested that those involving impaired cardiovascular function have been shown to have the highest mortality rate (Turesson et al 1999). However, there is some disagreement from other researchers who suggest that ExRA related pulmonary disease is the most common cause of mortality in patients with RA (Bluett et al 2017).

1.2.3 *Frequency of extra-articular rheumatoid arthritis*

The frequency of ExRA in RA patients is a controversial issue with several different international studies reporting different estimates. For example, three individual studies carried out in the USA, Sweden and Saudi Arabia respectively reported ExRA frequencies within the RA patient cohort of 70%, 8% and 41% (Turesson et al. 1999, Al-Ghamdi et al

2009, Turesson et al 2003). Furthermore, a study in 2000 by Cimmino et al showed that in Italy (which has an ExRA prevalence of ~40% in the RA patient cohort) there was a significant difference in the number of ExRA cases in northern Italy compared to the south. This suggests that the RA population may be diverse on a global level with important differences between the patient groups from different nations (or even regions), meaning different approaches to monitoring the healthcare of RA patients may be needed for different regional populations.

1.2.4 *Extra-articular rheumatoid arthritis and pulmonary disease*

As ExRA is argued to be the result of systemic inflammation it is unsurprising that manifestations are commonly seen in the pulmonary compartment which has a high amount of vascular tissue distributed throughout it (Cojocaru et al 2010). This relatively high frequency of pulmonary manifestations within the ExRA cohort poses a significant problem considering that a diagnosis of pulmonary ExRA is often associated with far greater morbidity and mortality rates and therefore a far worse prognosis for the patient (Shaw et al 2015).

A great challenge with regards to treating/managing healthcare for pulmonary ExRA patients relates to the wide diversity in tissues affected and pulmonary diseases presenting. It has been shown that RA is able to impact all manner of pulmonary tissue including: the parenchyma, pleura, bronchial airways and the vascular tissue (Shaw et al. 2015). Each of which has a variety of pulmonary disease states that have been linked to RA (*table 1.1)*.

Table 1.1 Pulmonary diseases in various regions of the respiratory system which are associated with extra-articular rheumatoid arthritis*. Summary of some of the different diseases associated with different compartments of the lung (Adapted from published work by Shaw et al 2015).*

Pulmonary compartment	Diseases associated with extra-articular rheumatoid arthritis
Parenchyma	• Interstitial lung disease
Pleura	• Pleural Effusion • Pleural effusion • Pneumothorax
Airway	• Bronchiectasis • Obliterative bronchiolitis • Follicular bronchiolitis
Vasculature	• Pulmonary hypertension • Rheumatoid vasculitis

1.2.5 *Bronchiectasis as an extra-articular manifestation of rheumatoid arthritis?*

As ExRA may result in a variety of disease states in several pulmonary tissues, including the airway, there is an understandable link between RA and the onset of bronchiectasis. However, published studies of the BROS patient cohort suggest that many developed the bronchiectasis often a decade before RA, with one study suggesting that this was the case for 90% of clinically monitored patients (McMahon et al. 1993, Despaux et al 1997). As BROS patients are shown to have poorer 5 year survival than healthy, bronchiectasis and RA patients (Swinson et al 1997) there is potential clinical benefit in developing a better understanding of how bronchiectasis could potentially lead to the onset of RA.

1.2.6 *Bronchiectasis lung as an origin of rheumatoid arthritis?*

The observation that a large number of BROS patients developed bronchiectasis long before RA has evoked several debates within the literature with regards to an explanation for this phenomenon. One emerging explanation over the years being that the accumulating infections commonly seen in bronchiectasis may result in the generation of an autoimmune response and the onset of RA by an at first unknown mechanism (Al-Shirawi et al 2006). This is supported by several studies showing that

clinical samples from bronchiectasis patients (without RA) are often seen to possess significantly higher quantities of biological markers associated with the onset of RA including rheumatoid factor and anti-CCP, which suggests that the bronchiectasis lung may potentially act as a trigger for the onset of RA (Hilton et al. 1978, Perry et al. 2014, Chatzidionisyou et al 2016).

Some opposed the suggestion that the bronchiectasis lung can initiate RA, with an alternative explanation for these studies results being that the RA related autoimmune molecules (i.e. autoantibody production) in clinical samples are not originating from the BR lung, but are instead the result of the underlying RA which has not yet been diagnosed and/or progressed to presenting joint disease. Therefore, implying the presence of these molecules is simply a marker of extra-articular inflammation that may predict RA but is not a cause of the disease.

However, there is growing support for the concept that RA imunopathogenesis and the loss of tolerance may in several cases be initiated outside the primarily affected joints, which suggests the development of auto-antibodies and RA-related inflammatory molecules begins in other tissues. One of the best-defined arguments for this being periodontitis. Periodontitis is a gum disease heavily linked to the onset of RA (Lappin et al 2013). Initially the disease was believed (much like bronchiectasis) to be the result of underlying RA inflammatory activity. Research has however demonstrated that *P. gingivalis* (the main pathogen seen in periodontitis) expresses an enzyme homologous to a protein modifying enzyme expressed in humans known as peptidylarginine deiminase (PAD), which could potentially modify host proteins in the infected gum leading to the formation neoantigens with citrullinated peptide motifs. In a genetically susceptible individual this could elicit the production of anti-citrullinated peptide antibodies (ACPA) that may play a role in the onset of RA (Maresz et al 2013).

This phenomenon of the diseased gums serving as an origin point of the onset of RA has given greater consideration for the concept that the lung may also act as a site for the development of autoimmune responses that promote RA. To determine whether this is the case we must examine the cells implicated in each disease and the potential biological mechanisms which may play a role in the onset of autoimmune responses relevant to RA.

1.3 Cellular components in bronchiectasis and rheumatoid arthritis

1.3.1 *Inflammatory cells seen in the bronchiectasis lung and airway*

With the two key features in bronchiectasis being inflammation and infection it is no surprise that immune cells are heavily implicated in the disease. Bronchial mucosa biopsies taken from adult bronchiectasis patients are known to contain a diverse range of infiltrating immune cells including macrophages, CD4+ lymphocytes and a large number of polymorphonuclear neutrophils (Gaga et al 1998). The importance of infiltrating CD4+ lymphocytes in bronchiectasis is still relatively unexplored. A prior study by Emad et al suggested that CD4+ lymphocytes may have a role in initiating bronchiectasis based on their observation that the number of CD4+ lymphocytes in bronchoalveolar lavage (BAL) appears to directly correlate with patient diagnostic HRCT scores (Emad et al. 2007). However, as this paper specifically focused on war veterans with bronchiectasis resulting from exposure to mustard gas it is unclear if these results extend to the broader bronchiectasis population.

A further study by Zheng et al (2001) supported the findings of Gaga et al (1998), by demonstrating that the lamina propria of bronchiectasis airways possessed significantly higher amounts of macrophages and neutrophils than healthy comparators. Although macrophages are present in higher number in bronchiectasis patients with more frequent bouts of sputum production there appears to be no direct correlation between macrophage number and markers for bronchiectasis severity/onset. This raises doubts that macrophages play a major role in the progression of the disease. However, pulmonary macrophages may still contribute to bronchiectasis pathogenesis indirectly, by promoting neutrophil migration to the lung via TNF-α secretion (Zheng et al 2001).

1.3.2 *The role of neutrophils in bronchiectasis disease progression*

Neutrophils are classically known for their abundance in circulation and their role of migrating into infected/damage tissue where they phagocytose any potential pathogens in addition to releasing a host of protective anti-microbial and pro-inflammatory molecules to protect from any threat to the host tissue (Amulic et al. 2012). Given their function of migrating to inflamed/infected tissue it is no surprise that neutrophils have

been shown to be present in high amounts within the bronchiectasis lung (Gaga 1998, Zheng et al 2001).

Given the importance of neutrophils in protecting tissues from infection it was in the past believed that neutrophils served simply a protective function in BR, which they no doubt do. However, as our understanding of neutrophil biology and bronchiectasis pathophysiology has improved we are now becoming aware that in BR neutrophils do not only serve a protective role. Rather they can carry out a destructive role via an exaggerated and prolonged response in the bronchiectasis lung (in response to the repeated cycle of inflammation and infection) that results in the progression to a more severe disease phenotype for the patient.

The link between pulmonary neutrophils and bronchiectasis disease severity progression is understandable given their function of releasing pro-inflammatory cytokines and broad targeting destructive proteinases such as proteinase 3, neutrophil elastase and cathepsin G following migration to inflamed/infected tissue (Wiedow et al. 2005). The release of these molecules in large amounts would plausibly lead to the destruction/degradation of airway structural tissue critical for the progression seen in bronchiectasis (Schaaf et al. 2000). This hypothesised mechanism of neutrophils driving airway destruction is furthermore supported by recent work from Chalmers et al that identified neutrophil elastase in sputum as a biomarker for predicting decline in lung function and increased risk of exacerbation in bronchiectasis (Chalmers et al 2017).

Therefore, although bronchiectasis lungs are infiltrated by a variety of immune cells from circulation, neutrophils are the most frequent migrator into the disease tissue and their dysfunction likely play a key role in the progression of the disease state.

1.3.3 *Cells implicated in rheumatoid arthritis*

As already established, the progression of RA within the joint is known to classically feature inflammation of the synovial membrane (i.e. synovitis) and the degradation of the bone/cartilage structure. This progressive destruction is enabled by a large influx of a variety of leukocytes (*figure* 1.1) into the RA afflicted joints due to the increased local angiogenesis resulting from increased expression of pro-angiogenic molecules such as VEGF and Hif-1α (Szekanecz et al. 2009).

Typically, synovitis is believed to occur first in joints affected by RA and has been linked to the presence of various cell types within the synovial membrane including: dendritic cells, B cells, plasma cells, T cells, macrophages and mast cells (Choy 2012). Our current understanding of RA pathology indicates that the synovial inflammation underpinning the disease largely depends upon the interaction between the antigen presenting cells in the joint (e.g. macrophages and B cells) and the large number of CD4+ T cells present within the joint (Choy 2012). These activated T cells then secrete several pro-inflammatory cytokines (IFN-γ, IL-17, IL-2), which activate resident macrophages and B cells leading to the production of auto-antibodies (e.g. rheumatoid factor) and more cytokines (e.g. IL-1, IL-6, TNF-α) that results in an extreme pro-inflammatory environment in the joint causing damage to the synovial lining structure (Smolen et al. 2007).

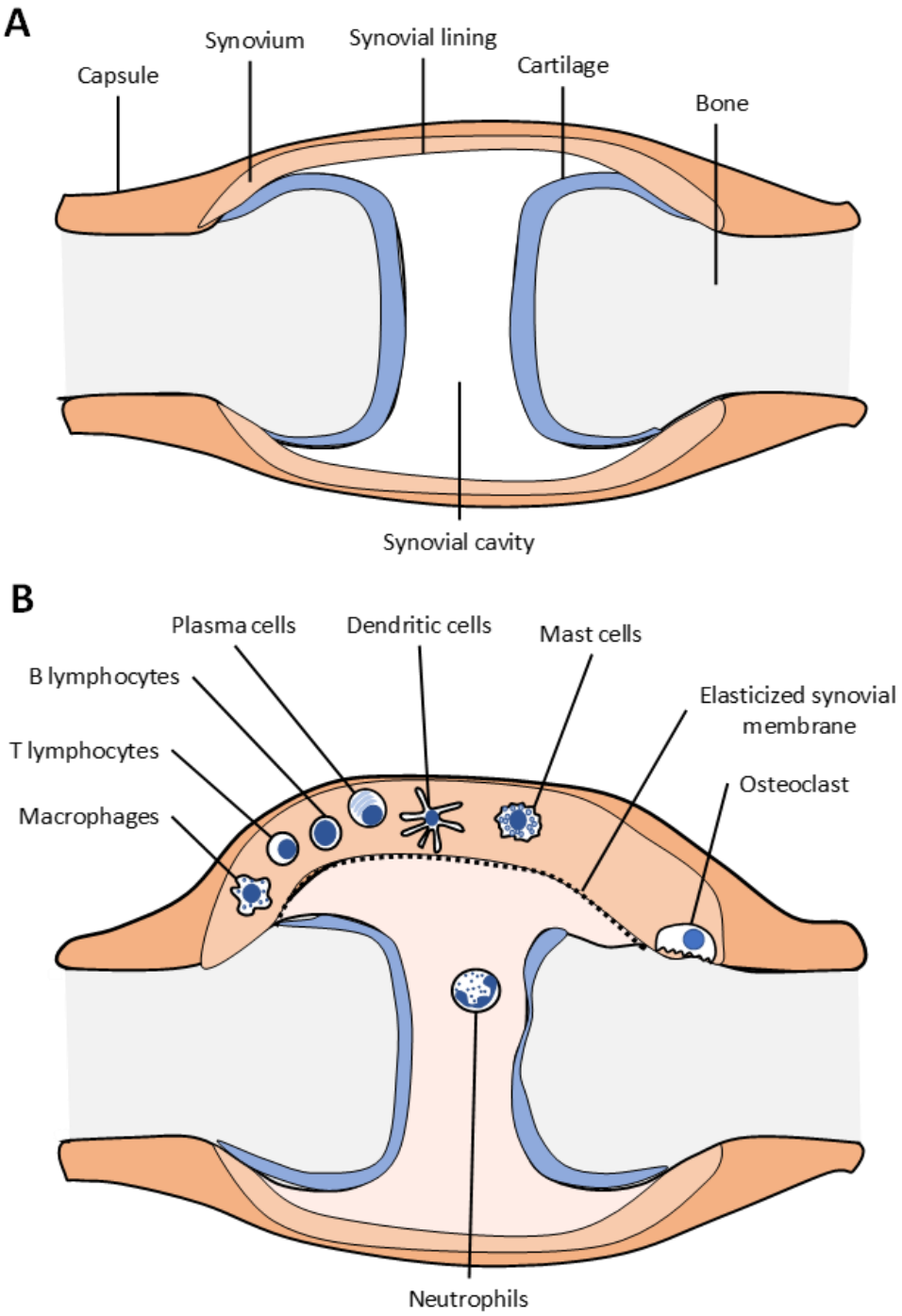

Figure 1.1 Comparison of cells within healthy and rheumatoid arthritis afflicted joints (A) Healthy joints are typically seen to have minimal swelling and minimal presence of infiltrating cells within the synovium. (B) In rheumatoid arthritis joints become severely damaged due to the influx of fluid and a variety of immune cells into the synovium, which promote synovitis (leading to hyper elasticity and loss of controlled architecture) and the destruction of the cartilage and bones, driven by neutrophils in the synovial cavity and osteoclasts in the synovium (figure adapted from Choy et al 2012)

1.3.4 *Activation of bone/cartilage destroying cells in rheumatoid arthritis*

The presence of these pro-inflammatory molecules leads to both the activation of osteoclasts, which degrade the bone tissue within the joint, as well as the recruitment and activation of neutrophils to the site of inflammation. Once recruited and activated neutrophils contribute to RA by secreting various proteases into the synovial fluid which destroy the cartilage structures contributing to the infrastructure damage widely seen in joints of affected RA patients (Kudo et al. 2003, Lally et al. 2005). In addition to their ability to destroy cartilage tissues, once activated neutrophils will also secrete CXCL8 (that recruits more neutrophils to the inflamed tissue) and reactive oxygen species (ROS) which cause tissue damage (Hitchon et al 2004). There is also some evidence suggesting neutrophils may alter the structure of local antibodies leading to the production of immune complexes such as rheumatoid factor which can also activate other neutrophils present (Rasheed 2008).

With our increased understanding of the key players in RA pathology more therapeutic targets have become identified. In previous decades many of the developed therapies for RA have targeted elements of the adaptive immune system (e.g. anti-B cell and T cell treatments) or cytokines (e.g. anti-TNF-α treatments) known to be central in RA. Perhaps due to the relative success of these treatments research has largely focused on understanding the roles of the targets of these therapies in RA. Meaning neutrophils in the disease has arguably been under investigated in terms of importance to the progress of the disease. However, there are some studies that suggest the observed success of some of these therapies (particularly those targeting TNF-α) may be effective at limiting RA severity partly due to their impact on neutrophil recruitment and activity in the synovium (Dominical et al 2011, Capsoni et al 2005). This implies a more important role for neutrophils in RA pathogenesis than initially thought and potential for neutrophils to be a target of interest in RA treatment.

1.3.5 *The common ground between rheumatoid arthritis and bronchiectasis*

From these studies it is clear that the pathogenesis of RA is an extremely complex system of interactions between several types of cells within the joint and the action of various cytokines. The pathogenesis of bronchiectasis has been comparatively understudied; however, it is suggested that neutrophils drive the progression of the disease by destroying the tissue structure within the airway (Russel et al 2016) similar to the action of neutrophils on the cartilage in the RA afflicted joints.

As this thesis aims to study the potential role the bronchiectasis lung plays in leading to the onset of RA it stands to reason that, as the drivers of bronchiectasis, neutrophils likely play some role within the bronchiectasis lung which contributes to the development of this autoimmune disease. The question this creates is how could the activity of neutrophils in the lung lead to the onset of RA?

1.4 NETosis, PAD enzymes and citrullination

1.4.1 *Neutrophil extracellular traps*

The primary roles of neutrophils has classically been identified as phagocytic activity and degranulation of protective molecules such as superoxide, elastase, cathepsin G and myeloperoxidase to defend against pathogen invasion and proliferation. However in 2004 Brinkmann et al described a newly discovered method of protection against pathogens by neutrophils in which neutrophils release their intracellular contents to the external environment to trap potential pathogens. This structure release by neutrophils for this purpose is now defined as a neutrophil extracellular trap (NET).

NETs are the result of a unique method of cell death now called NETosis. Following specific stimuli (e.g. activation of protein kinase C pathway with Phorbol 12-myristate 13-acetate) the chromatin structures with the neutrophils decondense after which the membrane of the nucleus disintegrates allowing the nuclear material and the cytoplasm/granular contents to mix. This results in proteins such as myeloperoxidase (MPO) and neutrophil elastase (NE) binding to the chromatin structure forming a large macromolecular complex. The cell membrane then ruptures releasing the complex to the extracellular environment (Kobayashi 2015) (*fig 1.2*).

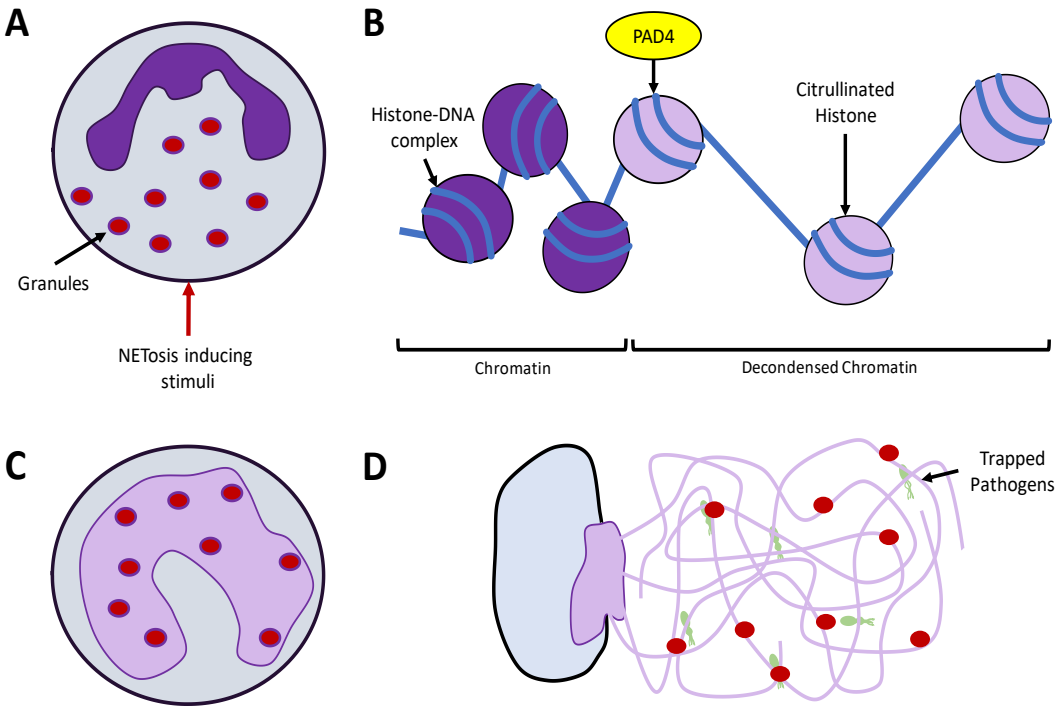

Figure 1.2 Cellular processes underpinning the release of NETs. (A) Neutrophils encounter NETosis inducing stimuli that promotes activation of NADPH oxidase activity and PAD4. (B) PAD4 is translocated to the nucleus and targets histones within the chromatin structure. Histone citrullination leads to the chromatin structure becoming decondensed. (C) Cytoplasmic and granular contents in neutrophils (e.g. neutrophil elastase and myeloperoxidase) mixes with the contents of the nucleus. (D) In the final stage of NETosis the neutrophil undergoes lysis, releasing the intracellular content to the external environment as a neutrophil extracellular trap which traps pathogens within the vicinity of the neutrophil (Adapted from figure produced by Abdallah et al 2012).

1.4.2 *NETs as a protection mechanism against infection*

The release of NETs is largely believed to be of importance in infection where the structures act as a physical barrier to pathogens to prevent dissemination. It is possible that molecules such as MPO and elastase contained within NETs may also directly kill trapped pathogens (Brinkmann et al 2004, papayannopolous et al. 2009). However, this has yet to be directly demonstrated. One study by Menegazzi et al (2012) has claimed to demonstrate the opposite, i.e. that microbes (*S. aureus* and *C. albicans*) trapped by NETs were not killed, however given the pathogens in the study were only exposed to NETs for 20 minutes before NET degradation by DNase it is possible that NETs may kill bacteria with longer exposure time. Therefore, the precise role of NETs in killing

microbial pathogens remains unclear.

Some have suggested that NETosis is defensive mechanism targeted primarily against fungi by showing a preferential formation of NETs in response to large pathogens (e.g. *C albicans* hyphae structures) over small single cell bacteria and yeast (Branzk et al. 2014, Urban et al 2009). Whilst this may potentially be true this does not negate the existing evidence that NETs may offer some protection against non-fungal pathogens. Already there is ample evidence that NETs form in response to the presence of a variety of bacteria (e.g. *S. aureus* and *E. coli*) as well as protozoa such as *T. gondii,* suggesting that NETosis is a broad protection mechanism against infection (Pieterse et al. 2016, Abdallah et al 2012).

1.4.3 *NETs are implicated in a variety of inflammatory/infectious disease*

Due to their apparent involvement in immune function and restricting pathogens it is no surprise that several studies have suggested that NETs may be implicated in several diseases. Potential markers for the presence of NETs (e.g. DNA-elastase complexes, high elastase concentrations, microbes entangled in DNA) have been detected in a range of clinical samples (i.e. sputum and endobronchial biopsies) from patients with pulmonary diseases such as: cystic fibrosis (Manzenreiter et al 2012, Dwyer et al 2014), asthma (Dworski et al 2011) and COPD (Obermayer et al 2014). Recently a paper by Dicker et al (2018) reported a strong correlation between the presence of NETs and severity of COPD, with the conclusion drawn by the author being that NETs in serum/sputum could act as a biomarker for disease severity

Several studies have also been published suggesting a relationship between NETosis and the onset of autoimmunity. Serum samples from systemic lupus erythematosus (SLE) patients have been reported to contain self-DNA containing immune complexes believed to be derived from neutrophils undergoing NETosis (Lande et al 2011), in addition mature SLE peripheral blood neutrophils have been observed to be undergo NETosis at a higher rate than healthy controls (Garcia-Romo et al 2011). Similar results have been seen in patients with anti-neutrophilic cytoplasmic antibodies (ANCAs) associated vasculitis, whom have higher levels of NETs detected in circulation than healthy controls (Söderberg et al 2015), with additional animal studies showing that myeloid dendritic

cells stimulated with NETs promote the production of ANCAs and disease relevant renal damage when injected into mice (Sangaletti et al 2012).

1.4.4 *Molecular processes underpinning NETosis*

Since they were first reported in 2004 a great deal of research has gone into determining the molecular mechanisms involved in the onset of NETosis. Whilst a variety of stimuli have been suggested to induce NETosis including those from an infectious origin (e.g. LPS) and an endogenous inflammatory origin (e.g. CXCL8) (Fuchs et al 2007), only three intracellular processes have been identified as being linked to NETosis. Firstly, it has been shown that transport of calcium stores following activation is essential for a neutrophil to successfully undergo NETosis, but the precise explanation as to why this is necessary remains elusive (Gupta et al 2014).

Secondly, it has been observed that NADPH oxidase deficient mice neutrophils (both *in vivo* and *ex vivo*) are unable to form NETs in response to *Aspergillus fumigatus* (Röhm et al 2014). Therefore activation of NADPH oxidase (by protein kinase C and/or MAPK activation) has been identified as essential for NETosis (Branzk et al 2013). There have been several explanations put forward to explain the importance of NADPH oxidase in NET formation. One explanation suggests that ROS, resulting from NADPH oxidase, causes the release of NE and MPO from their granules which are then transported to the nucleus to assist in decondensation of chromatin structures (Papayannopoulos et al 2010). Another is that ROS production is needed in order to inhibit pro-apoptotic caspases and trigger autophagy which results in breakdown of cellular membranes and mixing of cytoplasmic/nuclear compartments that is essential to NETosis (Remijsen et al 2011, Kaplan et al 2012). Despite the various explanations suggested the precise role of NADPH oxidase activity in NETosis remains unclear.

The final NETosis linked intracellular process involves the citrullination (i.e. a post-translational modification) of neutrophil histones within the nucleus. This process may initiate the decondensation of the chromatin within the nucleus that forms the core structure of the NET prior to release (Wang et al. 2009).

1.4.5 *Citrullination and PAD enzymes in human biology*

Citrullination describes a post-translational modification involving the conversion of an arginine residue within a peptide sequence to a non-coded citrulline residue (*figure 1.3*), typically catalysed by peptidylarginine deiminase (PAD) enzymes. The modification has been implicated in several key cellular processes including cell differentiation, apoptosis and epigenetic regulation (Slade et al 2014, Asaga et al 1998, Osamar et al. 2016).

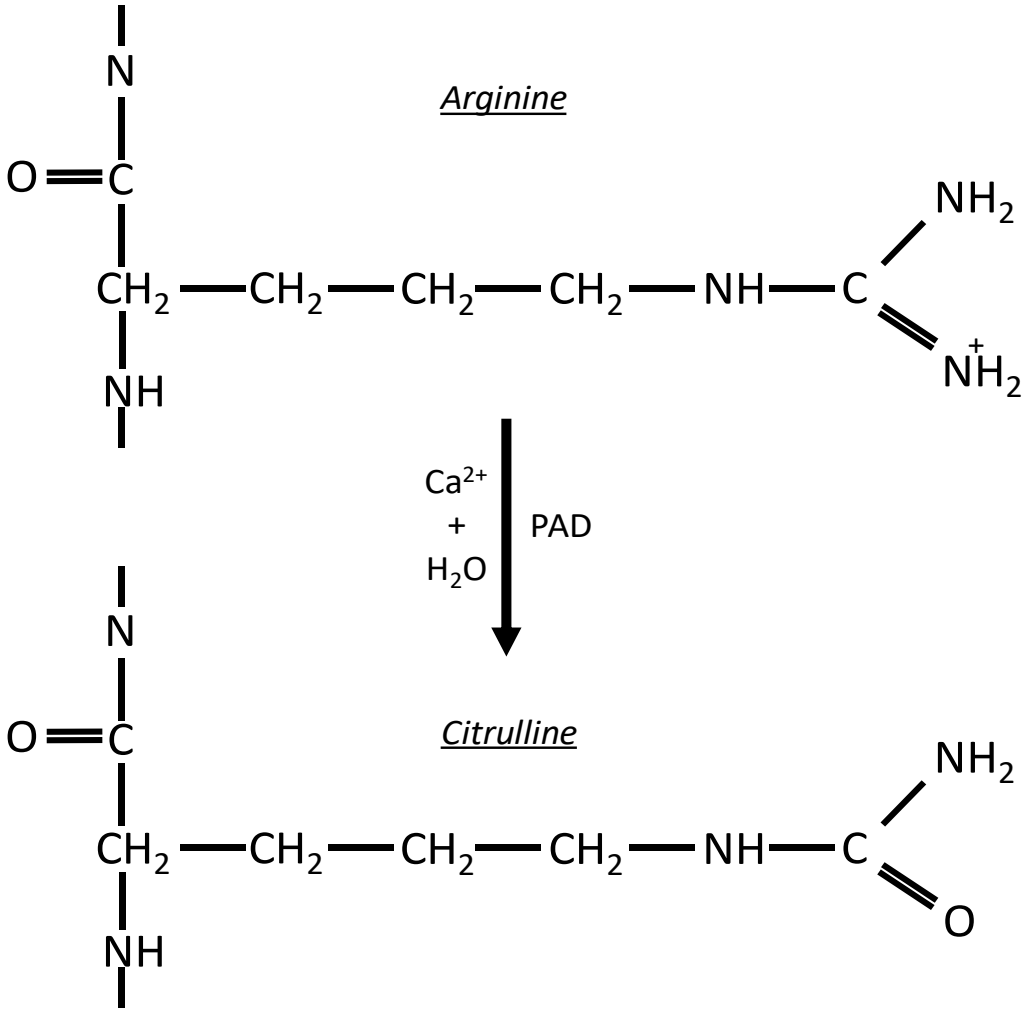

Figure 1.3 Conversion of an arginine residue to citrulline by a PAD enzyme. Positively charged citrulline residues within a peptide sequence are converted to citrulline via a PAD enzyme in the presence of calcium ions and water molecules. This results in the loss of a positive charge from the residue which can have a major impact on the protein structure and function (Adapted from Van Venrooji et al 2000).

In humans there have been 5 PAD enzymes identified to date: PAD1-4 and PAD6. Each has been shown to differ in terms of tissue expressing the enzymes and their individual roles (summarised in *Table 1.2*). PAD1,2, 3 and 6 are currently identified as being cytoplasmic proteins. PAD4 differs in this regard with structural studies showing the protein also localises to the nucleus due to a nuclear localisation signal within the peptidyl sequence (Bicker et al 2013, Nakashima K et al 2002).

Table 1.2 Summary of the differences between human PAD enzymes. The enzymes are expressed in a variety of tissue types and have been shown to target a broad range of protein targets which have various physiological effects (adapted from Mohanan et al 2012).

PAD enzyme	Tissue expressed in	Targets/Roles
PAD1	• Uterus • Epidermal epithelial cells	• Citrullinates keratin. Maintains epithelial barrier.
PAD2	• Ubiquitously expressed • Ovaries, muscle, stem cells, blood cells, neurones	• Citrullinates histones, myelin basic protein and vimentin • Epigenetic modification, maintaining neurophysiological function, apoptosis
PAD3	• Epithelium • Follicular cells	• Citrullinates filaggrin • Maintains epidermal barrier
PAD4	• Variety of leukocytes including: neutrophils, macrophages and monocytes • Also expressed in several carcinomas	• Variety of substrates can be citrullinated by PAD4: Vimentin, α-enolase, antithrombin, histones • Potential functions regulated by PAD4 include: cytoskeletal remodelling, angiogenesis epigenetic modification and NETosis
PAD6	• Embryos and oocytes	• Appears vital for development of embryo to multi-cellular state

Due to PAD4 being primarily expressed in neutrophils (as well as its ability to localise towards the nucleus), PAD4 is the member of the PAD family most explicitly linked to NETosis. Furthermore, animal studies have shown that PAD4$^{-/-}$ mice are both unable to form NETs in response to NET inducing stimuli (e.g. LPS and live bacteria) and have greater susceptibility to bacterial infection, but not to viral infections as can be expected given no evidence has been published demonstrating beneficial anti-viral defence from the ability to undergo NETosis (Li et al 2010, Kaplan et al 2012).

It is worth noting, with regards to PAD enzymes and NETosis, that several papers have reported elevated levels of histone citrullination correlating with an increase in PAD2 transcription/activity in the absence of any increase in PAD4 concentration (McNee et al 2016, Zhang et al 2012). However, given that to date there is no publications detailing how PAD2 localises to the nucleus, and that it has been demonstrated that PAD4 activity can increase up to 10,000-fold under given conditions (e.g. high intracellular calcium) without any change in concentration (Knuckley et al. 2011), there is some debate whether PAD2 does truly play a large role in histone modification and NETosis.

1.4.6 *Citrullination in disease*

Due to the variety of targets of the PAD enzymatic family, and the broad range of physiologically functions they are implicated in, the process of citrullination is believed to be linked to several clinically important diseases including: cancer, multiple sclerosis and Alzheimer's disease (György et al 2006). As citrullination is key to NETosis and given that NETosis and citrullination are observed in a large range of diseases there is strong interest in examining these phenomena in diseases with neutrophilic component, such as RA and BR, in relation to these characteristics.

1.4.7 *Citrullination and NETs in rheumatoid arthritis*

Since the 1960s several autoantibodies have been discovered within serum and synovial fluid from RA patients (e.g. rheumatoid factor, anti-perinuclear factor), however In the late 1990s a new RA-associated autoantibody was discovered which was directed solely against citrullinated peptides (Puszczewic et al. 2010, Girbal-Neuhause et al. 1999). These anti-citrullinated peptide antibodies (ACPAs) have since been recognised as a commonly observed phenotype within a majority of the RA patient population, to such an extent that the antibodies are now used as a marker for confirming diagnosis

(Aletaha et al. 2010). There is also growing evidence suggesting the ACPAs may also be implicated in the early pathogenesis of RA. Patients testing positive for ACPAs are often seen to have a more severe phenotype of disease and display differences in response to conventional RA therapies (e.g. corticosteroids) than APCA negative patients (Seegobin et al 2014).

Comparatively less is known in relation to NETs in RA. *In vitro* studies of peripheral blood neutrophils from RA patients have shown elevated markers for NET formation including production of ROS, secretion of MPO and elastase, with elevated histone citrullination also being detected in comparison to controls (Chowdhury et al. 2014). However these factors are arguably broad markers of neutrophil activity and could simply indicate neutrophil activation (which would be expected in RA). This highlights one of the greatest problems with studying NETosis in a biological setting, largely that many methods for detecting the structures focus on a molecule commonly associated with other physiological processes (e.g. elastase and degranulation, extracellular DNA and cell death).

A separate study by Khandpar et al (2013) also assessed NETosis in RA using two commonly observed approachs: immunofluorescent staining of NETs and the detection of extracellular DNA. From their comprehensive work they drew several conclusions relevant to NETosis in RA. Firstly that RA neutrophils in circulation and in the synovial fluid had higher levels of NETosis than controls. Secondly, NETosis rates appeared to correlate with ACPAs and levels of pro-inflammatory cytokines. Thirdly, their proteomic analysis suggested that both vimentin and α-enolase (two common targets of clinical ACPAs) were present in NET structures formed from healthy and RA neutrophils. Finally, exposure of NETs to synovial fibroblasts appeared to alter immunomodulatory function with greater expression of molecules such as IL-6 and CXCL8 observed, with similar results shown by Papadaki et al (2016) in regard to dendritic cell activity and maturation differing following exposure to NETs.

Overall this evidence suggests that NETosis is linked to the formation of ACPAs in RA, and that NETs may alter the expression of other immune cells within affected RA joints contributing to the pathogenesis of the disease.

1.4.8 *Citrullination and NETs in bronchiectasis*

Relatively little has been published regarding NETosis in bronchiectasis, with most studies into pulmonary-relevant NETs instead focusing on detecting the presence of NET structures in cystic fibrosis patients pulmonary tissue (Law et al 2017). However, research by Pumphrey et al presented in abstract form at the American thoracic society 2017 conference suggests that NETs can be detected in both serum and sputum samples from BR patients and could serve as a biomarker for a variety of disease outcomes (e.g. exacerbation risk, mortality).

Citrullination is gaining interest as a phenomenon in bronchiectasis, particularly in relation to the BROS patient cohort. Work by Quirke et al (2015) showed that although RA-relevant autoantibodies (e.g. anti-vimentin) were observed in some patients with BR, only autoantibodies obtained from BROS patients were specific to citrullinated versions of these target peptides. From this the authors concluded that a switch to a "citrulline specific" autoimmune response occurs in BROS following gradual breakdown of immune tolerance in patients with bronchiectasis. Furthermore, Clarke et al (2017) have reported similar results by showing that the levels of antibodies to citrullinated calreticulin (a RA-related pro-inflammatory molecules) are higher in BROS patient serum than BR and RA patient groups (Clarke et al. 2017).

1.5 Neutrophil citrullination as a generator of autoimmunity in bronchiectasis?

The overlap between bronchiectasis and RA has created great interest in the two fields of research. As there is documented evidence of RA pathogenesis being initiated at extra-articular sites (e.g. periodontitis) it stands to reason that the pulmonary environment in bronchiectasis may be the origin site for the onset of RA in BROS patients. The literature supports that neutrophils play a role in the pathogenesis of both diseases (more so in bronchiectasis), furthermore it appears that NETs and citrullination (which is associated with NETosis) appears to be implicated in early pathogenesis/progression of RA.

Information regarding NETosis and citrullination in bronchiectasis is limited however available data suggests some difference in the citrullination responses in BROS patients in comparison to those whom are diagnosed with bronchiectasis alone. Despite this

however there is little available data examining the possibility of increased NETosis and/or citrullination in bronchiectasis and BROS, which would be useful for gaining a better understanding of the link between the two disease states and guiding future developments in therapeutics and diagnosis for prevention/treatment of BROS.

In this thesis I aim to summarise my work examining the various aspects of neutrophil biology in relation to NETosis and citrullination with a focus on how this applies to bronchiectasis as a disease state.

Chapter 2: Aims and Hypotheses

The following aims and hypotheses were established for the four chapters of experimental work presented in this thesis.

2.1 Chapter 4: Impact of Bronchiectasis relevant molecules on healthy volunteer neutrophils citrullination and NETosis

Chapter four aimed to assess whether bronchiectasis relevant stimuli induce NETosis in healthy peripheral blood neutrophils and promote a pro-citrullination extracellular environment *in vitro*. The following hypotheses was established for experimental work:

- Neutrophils stimulated *in vitro* with bronchiectasis disease relevant molecules and bacterial cell lysates will show signs of elevated NETosis
- Neutrophil mediated citrullination will increase following stimulation with bronchiectasis relevant stimuli in comparison to unstimulated neutrophils.

2.2 Chapter 5: Assessing the LPS signalling pathway and its role in neutrophil citrullination and NETosis

Chapter five aimed to investigate the roles of the two arms of the LPS signalling pathway (MyD88 dependent and TRIF dependent) in neutrophil mediated citrullination and NETosis. As prior studies imply the MyD88 pathway (via MAPK-ERK signalling) is directly related to NETosis and/or PAD activity it is expected that inhibition of MyD88 will have inhibitory effects on citrullination/NETosis whereas TRIF inhibition will have no impact.

The following hypothesis determined this body of work:

- Inhibition of MyD88 function in neutrophils will significantly reduce NETosis rates and/or PAD activity, whereas TRIF inhibition will have no impact on either feature.

2.3 Chapter 6: Examining the effect of Cl-amidine on NETosis, citrullination and neutrophil function

Chapter 6 presents work involving the incubation of healthy peripheral blood neutrophils with a PAD enzyme inhibitor known as Cl-amidine. This work aimed to establish if Cl-amidine successfully inhibited neutrophil PAD activity and NETosis, whilst also assessing if Cl-amidine had any effect on other aspects of neutrophil function.

Two hypotheses were tested in this chapter:

- Pre-treatment of healthy volunteer derived peripheral blood neutrophils *in vitro* with the PAD inhibitor Cl-amidine will significantly decrease PAD activity and NETosis rates.
- Cl-amidine will significantly alter various aspects of neutrophil function (i.e. phagocytosis, superoxide production, lifespan and CXCL8 secretion).

2.4 Chapter 7: Differences in citrullination, NETosis and neutrophil function in the bronchiectasis patient cohort

Chapter 7 details the results of a feasibility study which aimed to produce preliminary data on NETosis rates, PAD activity and neutrophil function in peripheral blood neutrophils obtained from healthy volunteers, bronchiectasis patients, rheumatoid arthritis patients and Bronchiectasis-rheumatoid overlap patients.

As BROS patient neutrophils are theorised to have elevated rates of NETosis and citrullination, we tested this hypothesis in this chapter:

- Peripheral blood neutrophils from BROS patients will exhibit higher levels of NETosis and citrullination than neutrophils from BR patients without comorbid rheumatoid arthritis.

Chapter 3: Methodology

3.1 Equipment and materials

3.1.1 *Reagents Used*

The following reagents used throughout the thesis were obtained from Thermo fisher Scientific (Illinois, USA): Quant-iT# PicoGreen® dsDNA Assay Kit (P11496), SYBR® Green I Nucleic Acid Gel Stain (S7563), DNase I (EN0525), SYTOX green nucleic acid stain (S7020). Sigma-Aldrich (Missouri, USA) provided the following reagents: Mowiol 4-88 (81381), Glycerol (G5516), Paraformaldehyde (P6148), Bisbenzimide Hoechst 33342 trihydrochloride (B2261), Human Neutrophil Elastase lyophilized powder – from human leucocytes (E8140-1UN), N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (M4765), Sodium chloride (746398), Sodium hydroxide (221465), 36.5-38% hydrocholoric acid (H1758), Sodium acetate (S2889), HEPES (H3375), Propidium Iodide (81845), Phorbol 12-myristate 13-acetate (P8139), N-Formyl-Met-Leu-Phe (F3506), Lipopolysaccharide from *Pseudomonas aeruginosa* (L8643), Cytochrome C (Sigma: C6749), SOD (Sigma: S9697), Zymosan from *Saccharomyces cerevisiae* (Sigma: Z4250).

Rabbit Anti-Histone H3 (citrulline R2 + R8 + R17) antibody (ab5103), Goat anti-rabbit IgG FITC conjugated 1 : 1000 dilution (ab6717), Goat anti-mouse IgG antibody Alexa Fluor® 647 conjugated 1 : 200 dilution (ab15011), Human PMN ELISA kit (ab119552), mouse monoclonal antibody to histone H1 (ab71594), rabbit polyclonal antibody to neutrophil elastase (ab21595), goat polyclonal antibody to rabbit IgG – HRP labelled (ab6721) were all provided by Abcam (Cambridge, UK). Recombinant human TNF-α and a human IL-8/CXCL8 DuoSet ELISA kit was purchased from R&D (Minneapolis, USA) systems (210-TA and DY208 respectively). Biolegend (California, USA) provided APC Annexin V (640919) and Annexin V binding buffer (422201). Cayman chemicals (Michigan, USA) supplied a PAD4 human ELISA kit (501460) and Cl-amidine (10599). The PAD activity assay kit (MQ17.101) used throughout the thesis was provided by Modiquest Research (Oss, Netherlands). The following signalling pathway inhibitors were provided by Invivogen (Massachusetts, USA): TRIF inhibitory peptide (tlrl-pitrif) and MyD88 inhibitory peptide (tlrl-pimyd). Finally, anti-neutrophil elastase antibody (SC-53388) was purchased from Santa Cruz Biotechnology (California, USA).

3.1.2 Equipment and software

Filtropor S 0.2µM filters (83.1826.001) and disposable 50ml syringes (94.607.137) for filtration of prepared solutions were purchased from Sarstedt (Nümbrecht, Germany). pH of prepared solutions was confirmed using a Mettler Toledo FiveEasy pH monitor (Greifensee, Switzerland). The Sorvall (Hanau, Germany) Primo R centrifuge, Shandon Cytospin 3 and microscope slides (11562203) were from Thermo Fisher scientific (Illinois, USA). Sigma Aldrich (Missouri, USA) provided the 24 well plates and 96 well plates used in cell culture (CLS3527 and CLS3358) whilst the Prism microcentrifuge used to pellet cultured cells and CO_2 incubator used to incubate neutrophils was from Labnet (New Jersey, USA) and Sanyo (Osaka, Japan) respectively. A FACSCanto II cytometer from Beckton Dickinson Biosciences (New Jersey, USA) was used for all flow cytometric work.

Absorbance and fluorescence in 96 well plates was measured using a FLUOstar Omega Multiplex microplate reader with Omega data analysis for analysis of results, both purchased from BMG labtech (Ortenberg, Germany). Glass circular 13mm coverslips used for immunofluorescence were provided by Agar scientific (London, UK)). Isolated neutrophil counts, purity assessments and phagocytosis counts were carried out using a Laborlux II brightfield microscope from Leitz (Wetzlar, Germany). Fluorescence microscopy was carried out using a Nikon (Amstelveen, Netherlands) A1R confocal microscope initially, with a Zeiss (Oberkochen, Germany) axiomager widefield microscope (with mounted Zeiss AxioCam MRC) being used for counts and image capture of fixed samples, Zen Pro software v2.3 was used for image processing. A Boeco (Hamburg, Germany) S-20 spectrophotometer was used for monitoring optical density (OD) of bacterial cultures.

Microsoft (New Mexico, USA) excel and Minitab (Pennsylvania, USA) 17 software was used for storage of collected data, creation of figures and all statistical analysis.

3.2 Bacterial whole cell lysate preparation

3.2.1 *Clinical bacterial isolate growth*

Aliquots of several clinical strains from two bacterial species implicated in bronchiectasis (*Pseudomonas aeruginosa* and *Haemophilus influenzae*) were thawed and plated out on blood agar plates and incubated at 37°C (See table 3.1 for details of clinical isolates). Once the plate surface showed adequate growth of the bacterial strains, a sterile loop was used to harvest the bacteria present and transfer the bacteria to 1900µl of sterile PBS.

Table 3.1 Details on clinical isolates collected for experimental work. Information on the origins of the *P. aeruginosa* and *H. influenzae* strains used in this work. Details on *P. aeruginosa* clinical isolates are described De Soyza et al (2013), information on *H. influenzae* isolates were provided by staff at the Freeman hospital (Newcastle UK) where the isolates were initially provided from.

Source ID	Bacterial species	Origin
PA01	*Pseudomonas aeruginosa*	Genome sequenced lab strain from Melbourne, Australia
968333S	*Pseudomonas aeruginosa*	Non-cystic fibrosis (CF) bronchiectasis patient from UK
57P31PA	*Pseudomonas aeruginosa*	COPD patient, phenotypically well characterised. USA origin
DK2	*Pseudomonas aeruginosa*	CF patient from Denmark
2386	*Haemophilus influenzae*	Bronchiectasis patient, UK (Amoxicillin and Co-trimoxazole resistant)

3.2.2 *Killing and degradation of bacteria*

Bacterial strain suspensions were then split into five 380µl aliquots. Each aliquot was then placed on ice and sonicated eight times at 15 kHz (30 seconds on and 30 seconds off). Following sonication, DNAse I was added (final concentration of 200µg/ml) for 1 hour at 37°C. Following DNAse treatment proteinase K (final concentration of 1mg/ml) was added to the suspension and incubated for 2 hours at 60°C. To inactivate the proteinase K bacterial lysate suspensions were then incubated at 100°C for 20 minutes. Samples were stored at -80°C for use in future work. Prior work has demonstrated these contain no viable bacteria but are rich in LPS (De Soyza et al 2004).

3.2.3 *Confirmation of bacterial death*

Prior to storing the samples at -80°C, a sterile loop was used to spread each of the bacterial strains on a sterile blood agar plate. Plates were then sealed and incubated at 37°C for 72 hours. At 12, 24 and 72 hours the plates were checked for the presence of any bacterial colonies forming on the plate surface to confirm the protocol had successfully killed the clinical strains. *Figure 3.1* shows images of the plate at 12, 24 and 72 hours with no bacterial colonies visible.

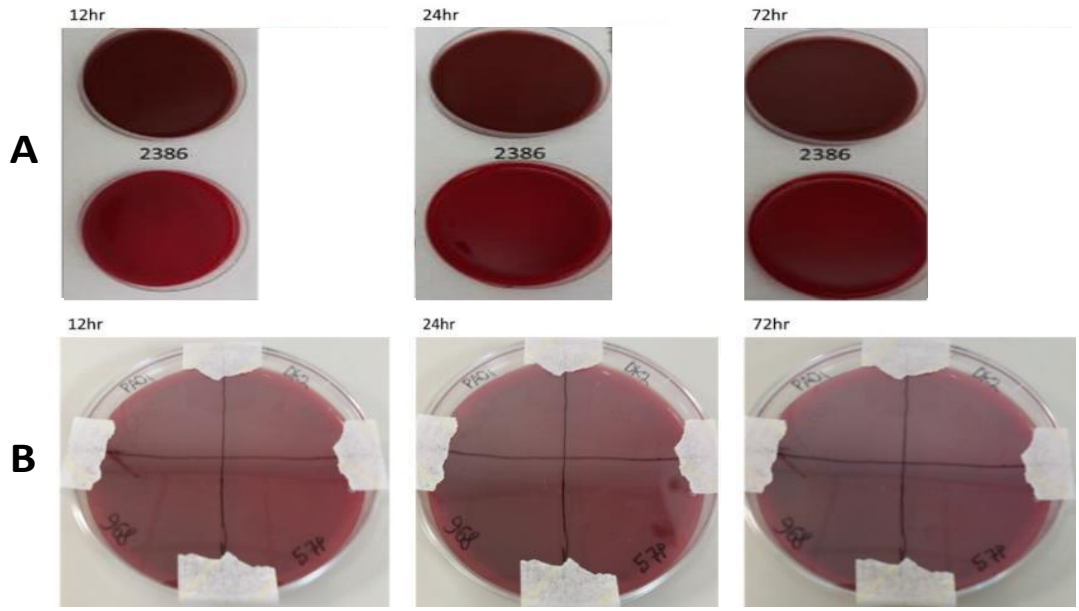

Figure 3.1 Images of growth plates streaked with bacterial whole cell lysates of Pseudomonas aeruginosa and Haemophilus influenzae. Row A shows Haemophilus WCLs influenzae (strain 2386) incubated on blood agar and chocolate agar plates for 12, 24 and 72 hours with no growth being visible. Row B shows 4 strains of Pseudomonas aeruginosa incubated on a single blood agar plate for 12, 24 and 72 hours with no growth being visible after 72 hours, confirming successful killing of the clinical strains.

3.3 Peripheral blood neutrophil preparation

3.3.1 *Obtaining healthy volunteer blood samples*

Ethical approval for obtaining blood from healthy volunteers for use in the research was provided by the County Durham and Tees Valley regional ethics committee (12/NE/0121). Healthy volunteers attended morning appointments at the clinical research facility (CRF) in the Royal Victoria Hospital (Newcastle, UK). A trained clinician obtained written consent and collected between 50-150ml of blood into falcon tubes containing 1ml of citrate (4%) per 10ml of blood (*Fig 3.2A*). Whole blood was then transported from the CRF to the lab (<3-minute transit time) for the cell separation protocol to be carried out.

3.3.2 Dextran sedimentation and Percoll separation

Whole blood was centrifuged at 300xg for 20 minutes at room temperature resulting in separation of plasma from the erythrocyte and leukocytes of the blood (*Fig 3.2B*). During this centrifugation step which 6% dextran (in 0.9% NaCl) and saline (0.9% NaCl) solutions were warmed to 37°C in a water bath. Once centrifugation of the blood was complete the platelet rich plasma upper layer was carefully transferred to a glass tube (*Fig 3.2C*). 220µl of 1M $CaCl_2$ was then added to the plasma (per 10ml of plasma) before incubating the solution 37°C, by 60 minutes a clot was formed within the former plasma solution, due to the activation of clotting factors by $CaCl_2$, which resulted in a clear serum to be used in later experiments (*Fig 3.2D*).

To the remaining cell pellet warmed 6% dextran solution was added (2.5ml of dextran per 10ml of cell pellet), after which warm saline was added to make the cell suspension up to the original volume of the whole blood. The tube was then mixed by gentle inversion and left to sediment at room temperature. After 30 minutes a leukocyte rich translucent upper layer should form within the solution (*Fig 3.2E*). This upper layer was then carefully extracted (taking care to not disturb the erythrocyte rich cell pellet) and transferred to a new 50ml falcon tube, with warm saline added to reconstitute to the original volume of whole blood before centrifuging at 200xg for 5 mins at room temperature (*Fig 3.2F*).

During this spin the Percoll concentrations needed for the upcoming separation step were set up. Percoll plus was diluted to 90% v/v in 10x PBS without Ca^{2+} and Mg^{2+} (the absence of Ca^{2+} and Mg^{2+} is required to avoid priming the neutrophils). This 90% Percoll solution was then mixed with 1x PBS without Ca^{2+} and Mg^{2+} in 15 ml falcon tubes (See table 3.2 for volumes of reagents used) to give 55%, 70% and 81% 2.5ml Percoll solutions. Once these solutions were created the 70% Percoll solution was then gently overlaid on top of the 81% Percoll solution to avoid mixing or disturbing the two layers.

Once the leukocyte suspension had finished centrifugation the supernatant is discarded and the remaining cell pellet is gently resuspended in 55% Percoll solution. The cell/Percoll solution was then gently overlaid on top of the 70%/81% Percoll solution gradient previously set up (*Fig 3.2G*). Once complete the Percoll gradient was then centrifuged at 720xg for 20 minutes at room temperature.

Table 3.2 Volumes of 90% Percoll solution and PBS needed to make up 55%, 70% and 81% Percoll solutions for neutrophil isolation. Three concentrations of percoll were created to separate cells based on size and density during the neutrophil isolation procedure

Percoll Percentage	90% Percoll solution volume (ml)	1x PBS without Ca^{2+} and Mg^{2+} volume (ml)	Total volume (ml)
55%	1.375	1.125	2.5
70%	1.75	0.75	2.5
81%	2.025	0.475	2.5

Following centrifugation two bands of cells are visible. One at the 55%/70% Percoll solution border containing peripheral blood monocytes (PBMCs) and a larger band at the 70%/81% Percoll solution border containing polymorphonuclear cells (PMNs) (*Fig 3.2H*). Each layer was then carefully extracted and placed into two separate falcon tubes prior to being suspended in HBSS (without Ca^{2+} / Mg^{2+}) and centrifuged (200xg for 5 minutes). After this wash step the supernatant was discarded and the remaining cell pellets were suspended in a suitable volume of HBSS (without Ca^{2+} / Mg^{2+}) after which cell counts and purity assessments are carried out.

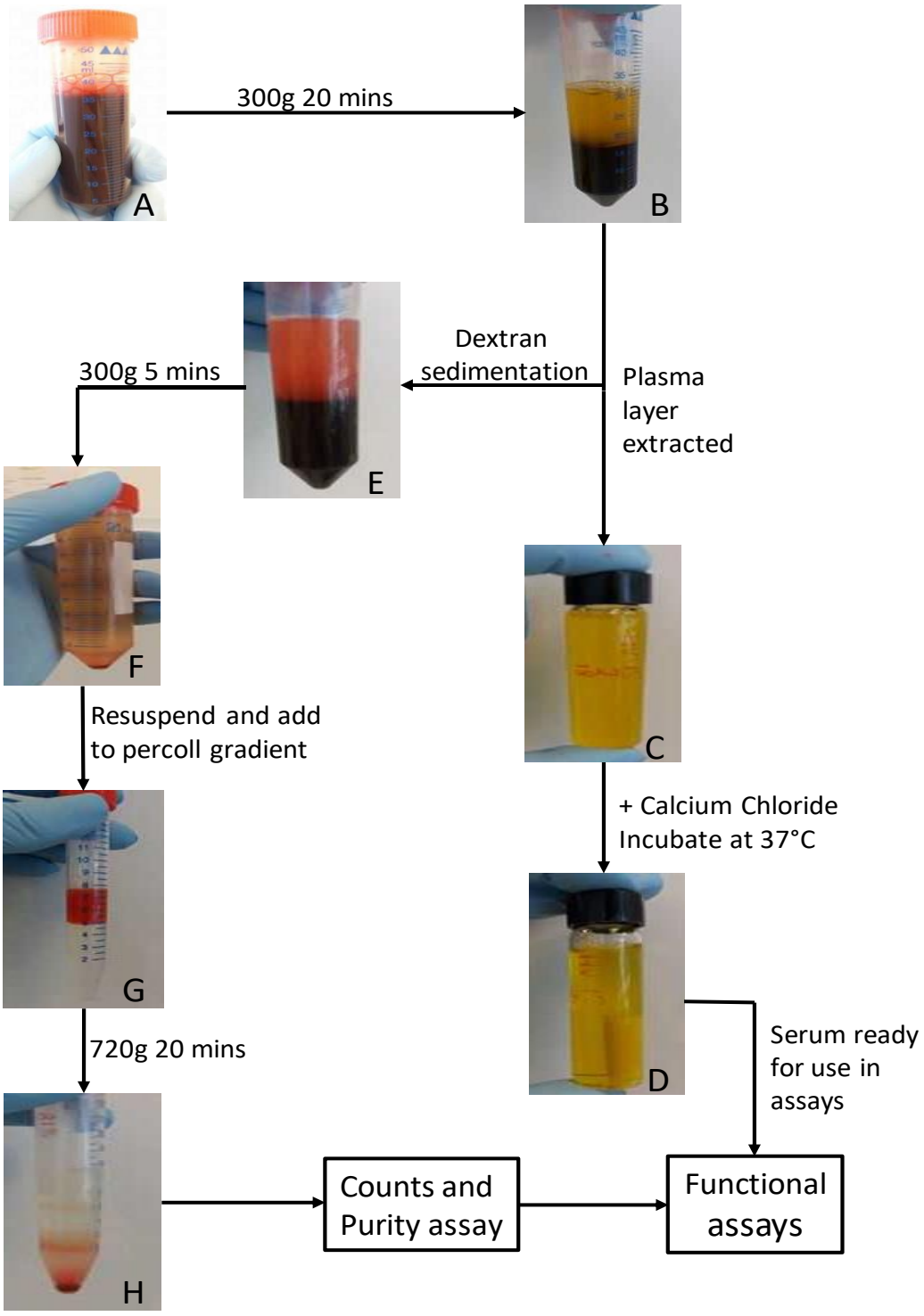

Figure 3.2 Summary of the neutrophil isolation procedure. (A) Whole blood is taken from mixed with citrate. (B) Centrifugation results in the formation of a plasma upper layer, (C) which is extracted and placed in a glass vial. (D) Activation of plasma clotting factors results in the formation of plasma for later use. (E) The remaining cell pellet from previous is suspended in dextran and saline resulting in a leukocyte rich upper layer, (F) which is then removed and centrifuged. (G) After discarding the supernatant, the cell pellet is resuspended in 55% Percoll and added to a 70%/81% Percoll gradient. (H) Centrifugation allows separation of monocytes and PMNs.

3.3.3 *Cell counts and purity assessments*

For cell counts a 100µl aliquot of the PMN cell suspension was taken. 5µl of 0.4% Trypan blue solution was added to the 100µl cell aliquot and gently mixed with the pipette. 100µl of this suspension was then applied directly to the grid of a haemocytometer (Hausser Scientific: cat. no. 1483) and sealed with a coverslip. Brightfield microscopy (x40 magnification) was used to examine the haemocytometer and record the number of cells within the 25 squares of the grid. The number of observed cells was then used to determine the number of cells per ml for the original PMN stock, which allows aliquoting of accurate numbers of cells for individual experiments.

It was also essential to record the purity of the isolated PMN cell suspension to be consistent with the quality of the work conducted and determine if any unusual results obtained are due to a large contamination of individual samples by other leukocytes. To determine purity a 150µl aliquot of the PMN suspension was loaded into a cytospin chamber attached to a microscope slide. Chambers are centrifuged at 300xg for 3 mins after which the chambers are dismantled. The microscope slides were then fixed in acetone for 10 minutes before being removed and allowed to dry. A 1:10 dilution of giemsa (diluted in PBS with 0.05% Tween) was then prepared and pipetted onto the surface of the slides. After 10 minutes the slides were gently rinsed with water, leaving the fixed and now stained PMNs on the surface. As mature neutrophils possess a characteristic multi-lobed shape it was possible to determine the purity of neutrophil isolations by nuclear morphology. Using brightfield microscopy, 300 cells were examined and assessed according to their nuclear morphology. The number of recorded "suspected neutrophils" within the sample are then divided by the total number of counted cells (i.e. 300) giving the proportion of neutrophils, which acts as an indicator of purity of the PMN stock. To maintain a set standard throughout the work only results from cell preps with a purity of 85% or higher would be used to obtain results for the thesis.

3.4 Neutrophil stimulation and inhibition assay

3.4.1 *Incubation of neutrophils with stimulatory molecules*

Following isolation of peripheral blood neutrophils and cell counts, the volume of PMN stock needed to provide adequate cell number for all conditions of the stimulation assay was aliquoted. This aliquot was then centrifuged at 200xg for 5 minutes, after which the supernatant was discarded with the remaining the cell pellet being gently resuspended in IMDM media at a concentration of 8 million cells/ml (phenol red free).

Neutrophil stimulation was carried out in a 24 well plate at 37°C, with 7 stimulatory conditions used in the experiments: untreated, 50nM PMA, 1µg/ml LPS, 100nM fMLP, 10ng/ml TNF-α, 2.5% WCL *Pseudomonas aeruginosa* and *2.5% Haemophilus influenzae*. To each well of the plate; IMDM media, autologous serum (final volume 1% v/v) and 250µl of cell-media suspension was added giving 2 million neutrophils per condition (see table 3.3 for example of volumes). After 30 minutes incubation at 37°C, set volumes of stimuli were added to relevant wells to bring the final volume in each well to 500µl and each stimuli to set final concentration. Cells were then incubated for 4 hours at 37°C.

Table 3.3 Example of volumes of cell suspension, serum, stimuli and media used in neutrophils stimulatory experiments

Experimental conditions	Volume Cell suspension (8million/ml)	Volume autologous serum	Volume stimuli	Volume IMDM media	Final volume
Untreated	250µl	5µl	-	245µL	500µl
50nM PMA	250µl	5µl	2.5µL	242.5µL	500µl
1µg/ml LPS	250µl	5µl	5µL	240µL	500µl
100nM fMLP	250µl	5µl	0.5µL	244.5µL	500µl
10ng/ml TNF-α	250µl	5µl	2.5µL	242.5µL	500µl
2.5% WCL *P. aeruginosa*	250µl	5µl	12.5µL	232.5µL	500µl
2.5% WCL *H. influenzae*	250µl	5µl	12.5µL	232.5µL	500µl

3.4.2 *Isolation of cell supernatant and storage*

After 4 hours incubation, the cell solutions in each of the wells of the plate were collected and transferred to a corresponding labelled 1.5ml Eppendorf tube. Samples were then centrifuged at 7200RCF for 5 minutes separating the supernatant and cell fraction. Following centrifugation, the supernatant was carefully aspirated and transferred to another labelled 1.5ml Eppendorf tube. Supernatants were then stored at -80°C for use in future experiments.

3.4.3 *Confirmation of neutrophil activation by CXCL8 ELISA*

A "Human IL-8/CXCL8 Duoset ELISA" kit from R&D systems (D8000C) was used to measure CXCL8 secretion by stimulated neutrophils to determine if the PAMPs and cytokines used in the stimulatory assay were biologically activating the neutrophils. Supernatants collected from stimulated neutrophils were removed from -80°C storage and left to thaw at room temperature before being diluted 1:5 in assay specific reagent diluent and ran in duplicate with concentrations determined from a standard curve of recombinant CXCL8. The assay was run to manufacturers specifications at all points of the protocol.

3.5 Assessing PAD activity and PAD4 concentration

3.5.1 *PAD activity assay*

A "PAD enzyme assay kit" (Modiquest research) was used to assess the levels of PAD activity in neutrophil supernatant samples. Stored supernatant samples were thawed at room temperature then diluted 1:10 in the kits provided reagent diluent. Manufacturer's instructions were followed for the whole protocol.

3.5.2 PAD4 ELISA

Neutrophil supernatant samples stored at -80°C were thawed at room temperature before being diluted 1:10 in reagent diluent. Diluted samples were analysed for PAD4 concentration using a "PAD4 ELISA kit" (Cayman chemicals), following manufacturer's protocol.

3.6 Measuring Neutrophil extracellular traps

3.6.1 *Selection of methods to measure NET formation*

Due to NETs being a relatively new phenomena discovered in neutrophil biology, (coupled with the mechanism involving death and lysis of the neutrophil) there is great difficulty in accurately assessing NETosis rates *in vitro.* Consultation with Prof James Chalmers and his team at Dundee university revealed that although several methods of measuring NETosis have been proposed, each has flaws in their design that result in questionable accuracy/validity.

For this reason several approaches were used to measure *in vitro* NETosis in order to provide a clearer picture of whether NETosis rates were increasing/decreasing across the experiments. These methods include semi-quantitative measures of free-NETs in supernatant (with methodology provided by Prof Chalmers), measures of extracellular DNA and counts of immunofluorescent stained adherent NETs which according to Prof Chalmers and published research examining NET formation (Ginley et al 2017, Chan et al 2017) is currently the gold standard for measuring NETosis *in vitro.* The protocol for each of these methods will now be discussed in greater detail with their strengths and limitations discussed further in the thesis.

3.6.2 *Early attempt at immunofluorescent NET staining*

Initial attempts at establishing an immunofluorescent staining protocol followed the same process outline later in section 3.6.4, with three distinct differences. Firstly, NETs were only stained for the presence of DNA and neutrophil elastase. Secondly neutrophils were incubated in 24 well plastic bottomed plates. Thirdly as the neutrophils adhere to a plastic surface (rather than thin glass coverslips) confocal microscopy was needed to visualise neutrophils/NETs. This protocol was ultimately abandoned due to the low number of neutrophils observed in each well (believed to be due to loss of neutrophils during the harsh wash steps) and the poor-quality staining seen for neutrophils incubated and imaged in this manner (*Fig 3.3*). To correct these issues, a new staining protocol was established which instead incorporated three stains (DNA, elastase and citrullinated histones) to confirm the presence of NETs (improving the validity of the assay) and had glass coverslips placed at the bottom of each well for neutrophils to

adhere to, which allowed more delicate washing of neutrophils (reducing undesirable loss of cells) and the use of widefield microscopy to count NETs.

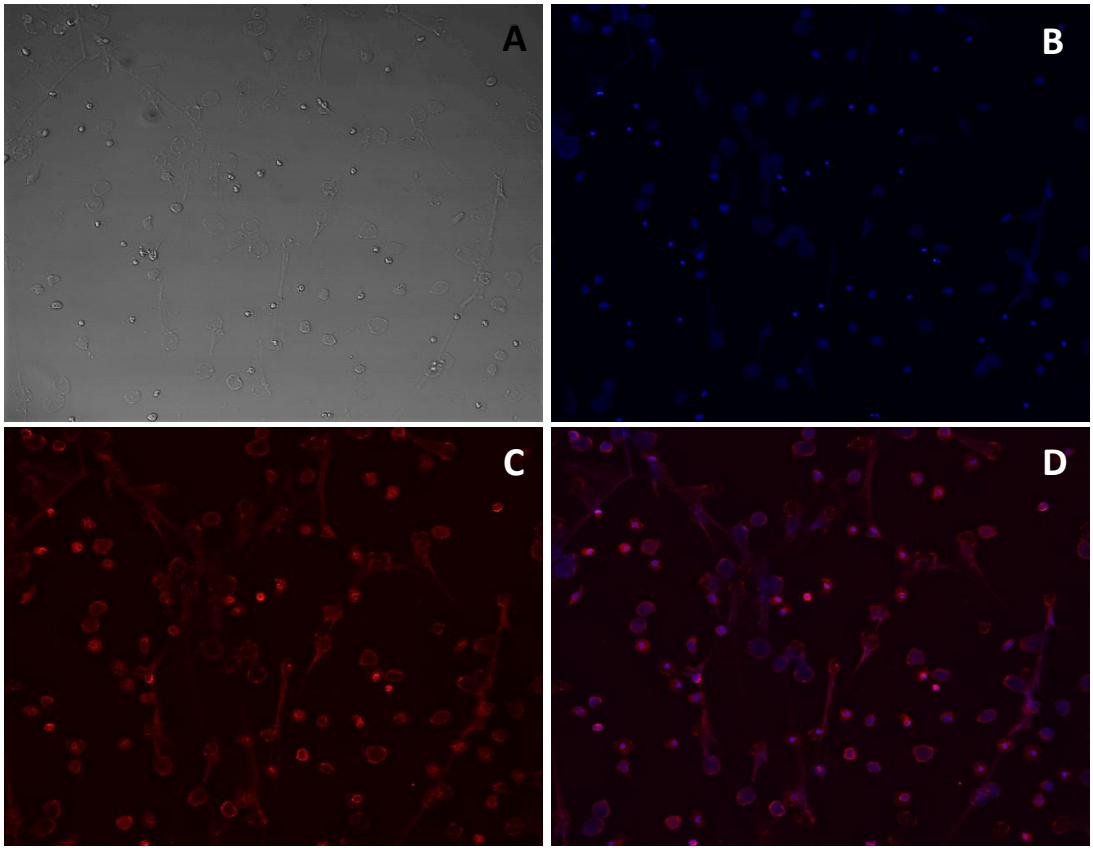

Figure 3.3 Image of NET staining using initial immunofluorescent staining protocol. Images were captured of 50nM PMA treated neutrophils adherent to the plastic base of a 24 well plate. High numbers of neutrophils (500,000) were added to each condition/well to compensate for loss of cells during washing/staining. Staining was seen to be problematic with clear issues detecting fluorescence in neutrophil elastase staining (image C) where excessive exposure was needed to detect any possible fluorescence (A = brightfield, B = DNA, C = Elastase, D =merge).

3.6.3 *Coverslip preparation for immunofluorescent staining*

Coverslips were sterilised by autoclaving then washing in 100% ethanol for 15 minutes, followed by two more 15-minute washes in 70% ethanol. Once dried, the sterile coverslips were placed on the bottom surface of a 24 well plate using sterile tweezers. 300µl of autologous serum was then added to each well with the plate to coat the coverslips. The plate was then incubated at 37°C for 20 minutes before being washed 5 times with ultrapure culture grade water and allowed to dry prior to addition of cells.

3.6.4 *Paraformaldehyde and Mowiol solution preparation for immunofluorescent staining*

Preparation of 8% paraformaldehyde (PFA) solution for use in cell fixation was carried out using all appropriate personal protective equipment and safety guidelines. To make the solution, 2g of Paraformaldehyde was first added to 50ml falcon tube containing 40ml of distilled water. This solution was then heated to 60°C and incubated with mixing for 2 hours. After 2 hours 1M NaOH was added to the PFA (dropwise) until the solution became clear in appearance. Following this the volume was made up to 50ml final volume using distilled water and mixed by vortex. The solution was then divided into twenty-five 2ml aliquots and stored at -20°C for future use.

Mowiol solution needed to mount and preserve fixed samples for long term analysis was prepared by following the protocol (Technical data sheet 777) described by *Polysciences Inc*. (Pennsylvania, USA). 4.8g of Mowiol and 12g of glycerol were added to a 50ml falcon tube containing 12ml of distilled water. The tube was then left on a shaking incubator at room temperature for 3 hours, after which sodium azide (final concentration 0.02%) and 24ml of 0.2M Tris HCl was added to the solution. After brief mixing to solution by vortexing the solution was then divided into 36 x 1ml aliquots and incubated at 60°C for 30 minutes until the solution became clear. Aliquots were then centrifuged at 5000 RCF for 15 minutes, after which 500µl was aspirated from the supernatant and transferred into a 2ml Eppendorf. These aliquots were then stored at -20°C until further use. Once opened the aliquots were stored for a maximum of one month at 4°C

3.6.5 *NET immunofluorescent staining protocol*

A protocol published by Brinkmann et al (2010) was used to assess *in vitro* NET formation rates. Once neutrophils were isolated and cell counts determined, cells (suspended in IMDM media with 1% v/v autologous serum) were added to each well and

left incubating at 37°C for 30 minutes, giving neutrophils time to adhere to the serum coated glass coverslips.

After 30 minutes stimulatory PAMPs and cytokines were added to the wells to give the desired final concentration. The plate was then incubated for 4 hours at 37°C, after which PFA was carefully added to each well at a final concentration of 4% (v/v) and incubated at room temperature for 40 minutes to fix the adherent cells. Following fixation, the PFA-media solution was aspirated with the remaining coverslips being removed with tweezers and placed inverted (cells facing down) on a 350µl drop of sterile PBS (on sterile lab parafilm) for 5 minutes to wash the coverslip. This washing procedure was repeated a further 3 times.

Slips were then transferred to a 250µl drop of 0.5% triton-X-100 for 5 minutes to permeabilise the fixed cells, after which the slips were then washed 4 more times in sterile PBS. Coverslips were then blocked by placing the slips on a 300µl drop of 5% BSA solutions (BSA diluted in PBS) for 30 minutes at 37°C. After blocking, slips were placed on a 250µl droplet of primary antibody solution (Mouse anti-neutrophil elastase antibody 1:50 dilution, Rabbit anti-Histone H3 (citrulline R2 + R8 + R17) antibody 1 : 200 dilution) and incubated at 4°C overnight.

The following day coverslips were again washed in a droplet of sterile PBS 4 times (5 minutes per wash) before being incubated with 250µl of secondary antibody solution (Goat anti-rabbit IgG FITC conjugated 1:1000 dilution, Goat anti-mouse IgG antibody Alexa Fluor® 647 conjugated 1:200 dilution) for 2 hours at 37°C. Coverslips were then washed 5 times in sterile PBS and incubated at room temperature for 10 minutes in 1µg/ml Hoechst 33342 DNA staining solution (protected from light exposure).

The coverslips were then washed 4 times (5 minutes per wash) in ultrapure culture grade water. During these wash steps 7µl of mowiol solution was placed onto a labelled glass slides, with the coverslips being placed onto the mowiol (cells facing the mowiol). The slides were then left in the dark at room temperature for 1 hour, giving the mowiol time to solidify and seal the coverslip. Slides were then stored at 4°C for microscopic analysis within three weeks.

3.6.6 *Establishing number of neutrophils needed for immunofluorescent staining*

Once the final staining protocol using coverslips was established images were taken of staining procedures carried out on wells with varying numbers of neutrophils. *Fig 3.4* shows an image of neutrophils/NETs taken from LPS stimulated neutrophils with 100,000, 75,000, and 50,000 neutrophils per well respectively. Based on the number of neutrophils/NETs per field of vision, to avoid overcrowding which would increase difficulty of counting individual NETs, 50,000 cells/condition was selected as an optimal number of cells for all future immunofluorescent staining.

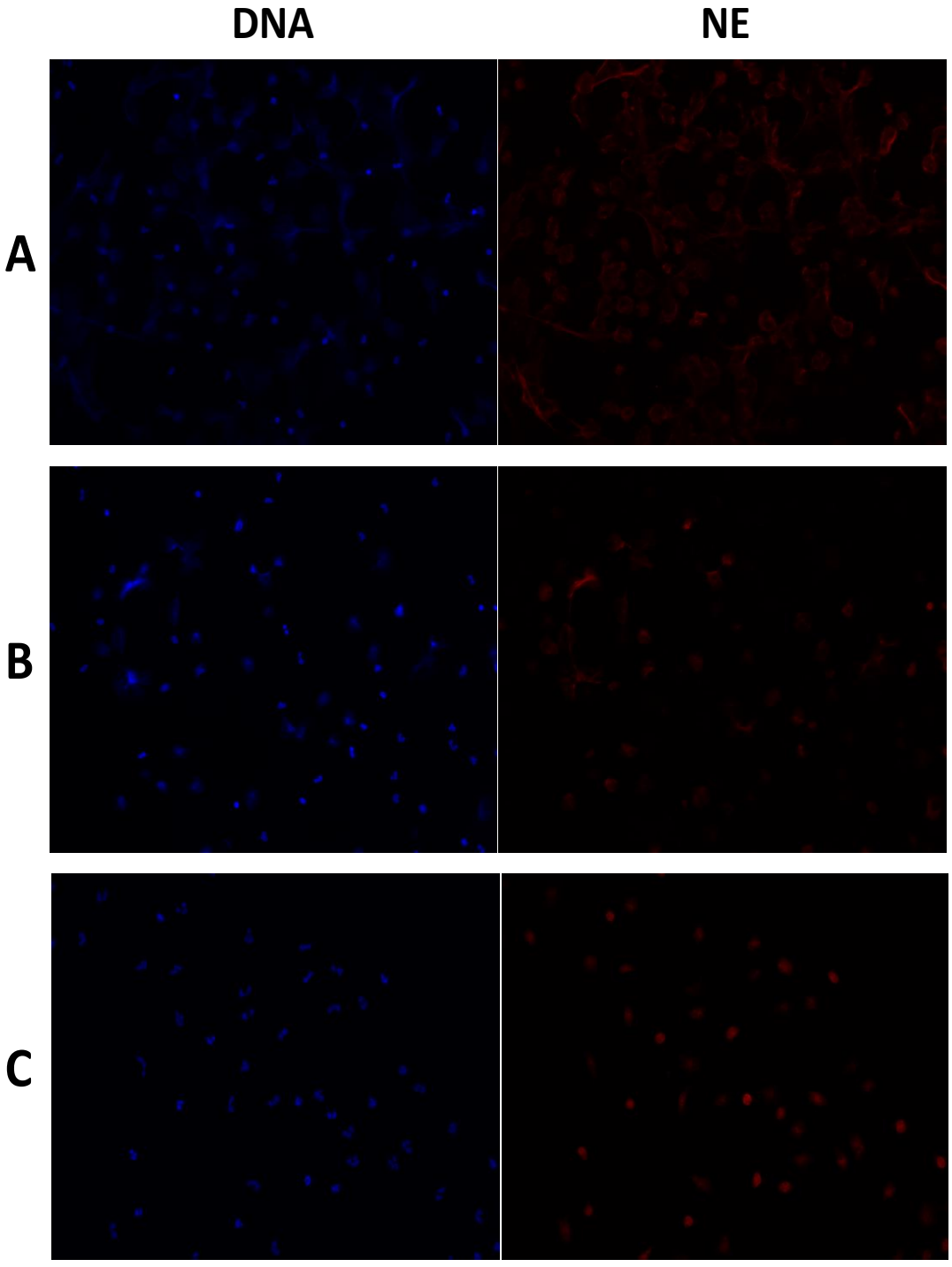

Figure 3.4 Immunofluorescent staining of DNA and neutrophil elastase for differing numbers of LPS treated neutrophils. Row A shows immunofluorescent staining of 100,000 cells, Both DNA and elastase staining show heavy overcrowding which would make accurate individual cell/NET counts impossible. Row B (75,000 cells) shows a decrease in the number of cells within the field of vision compared to row A. Row C (50,000 cells) resulted in a field of vision without excessive crowding (similar to as seen in row B). For this reason, 50,000 cells/ condition was used for all future immunostaining experiments.

3.6.7 *Adherent cell counts and NET counts using microscopy*

Coverslips which had been fixed, stained and mounted onto microscope slides were examined by fluorescent microscopy for the presence of FITC (Citrullinated histones), Cy5 (neutrophil elastase) and Hoechst (DNA) positive staining. For determination of NETosis rates, 300 neutrophils per condition were examined for staining and morphology. Structures which appeared positive for all described stains and possessed the characteristic NET shape (i.e. a large dispersed web like) were recorded as being a NET+ve (*Fig 3.5*), those that did not stain positive for all three stains and/or showed a classical neutrophil nucleus morphology (e.g. multi-lobed, band cell) were recorded as being NET-ve (*Fig 3.6* And *3.7*).

To compare neutrophil adhesion to the coverslips between different conditions (which could feasibly impact NETosis estimates), five fields of view (20x magnification) were selected at random for each coverslip and the number of cells (determined Hoechst positive staining and nuclear morphology shape) in each field was recorded in an unblinded manner (i.e. with the observer aware of each condition being assessed). This was used to calculate the mean number of neutrophils per field of vision for the conditions and determine if there were any differences in the number of neutrophils remaining adherent at the tested conditions.

For the staining protocols a number of staining controls were included with each run to address potential concerns of factors including autofluorescence, antibody cross-reactivity and non-specific binding. The details of the various staining controls are summarised in *Table 3.*4.

Table 3.4 The staining controls used for immunofluorescent staining of NETs in vitro. A variety of controls were included with each immunofluorescent staining procedure in order to control variables such as autofluorescence, antibody cross-reactiviy and antibody non-specific binding

Control	Description
No stain	Samples incubated with no primary or secondary staining antibodies.
Single stain	Samples stained with one set of primary and secondary antibodies (e.g. “Rabbit anti-Histone H3 primary” and “Goat anti-rabbit IgG FITC conjugated).
Primary stain	Samples incubated with primary staining antibodies (i.e. “Rabbit anti-Histone H3” and “mouse anti-neutrophil elastase”) without secondary antibodies.
Secondary stain	Samples incubated with secondary staining antibodies (i.e. “goat anti-rabbit IgG FITC conjugated” and “goat anti-mouse IgG Alexa Fluor® 647 conjugated) without primary antibodies.
Mixed stain	Samples incubated with non-matching antibody pairs (e.g. “rabbit anti-Histone H3” primary and “goat anti-mouse IgG Alexa Fluor® 647 conjugated” secondary)

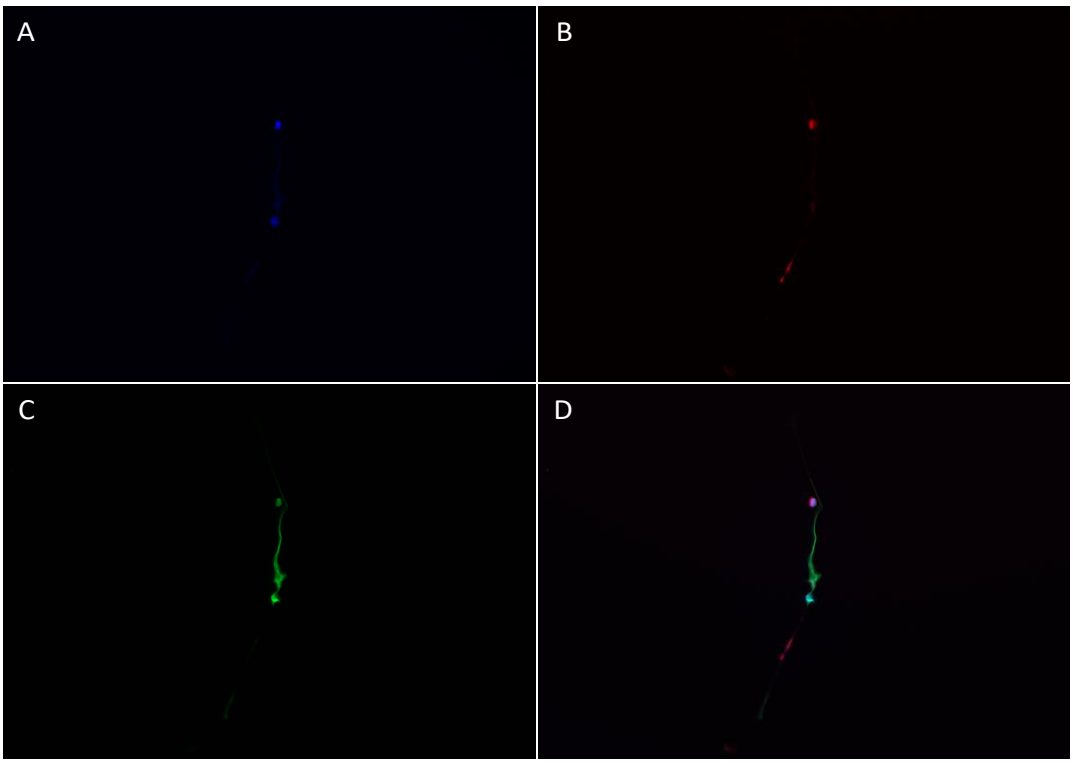

Figure 3.5 Neutrophil recorded as positive for having undergone NETosis. The cell has stained positive for the 3 markers of NETs: DNA (A), neutrophil elastase (B) and citrullinated histones (C) (D representing a merged of the three stains). There is also a clear dispersed nuclear morphology characteristic of NETs.

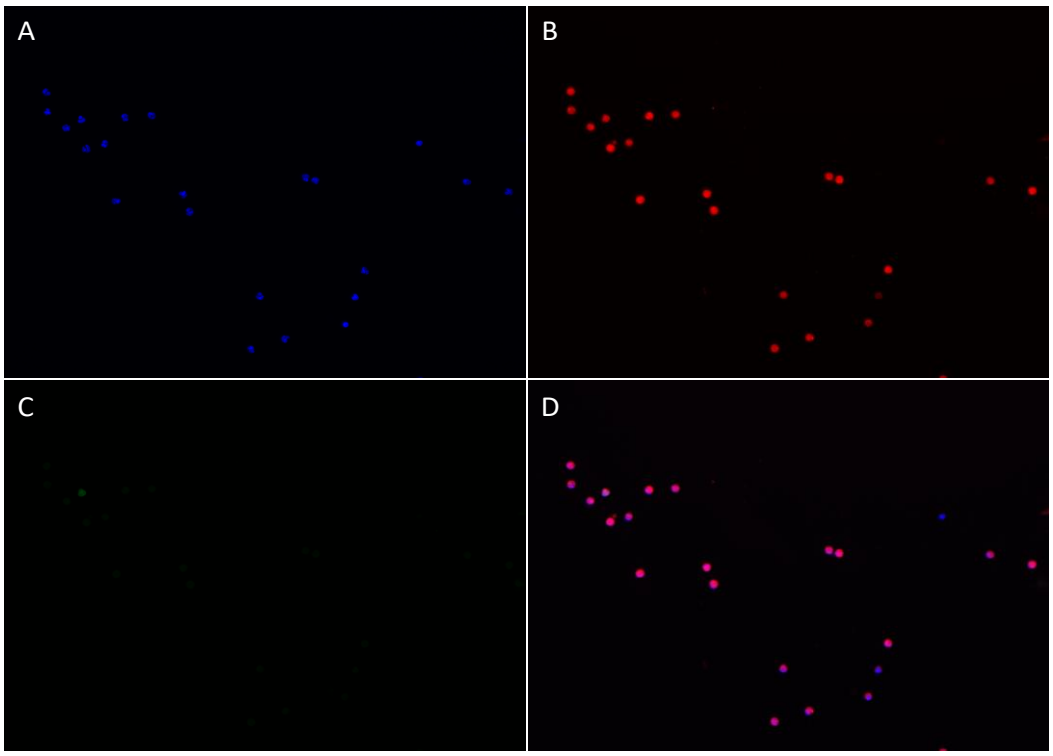

Figure 3.6 Neutrophils recorded as negative for having undergone NETosis due to incomplete staining. The cells in this image have stained positive for DNA and neutrophil elastase (A and B respectively), however no staining was observed in these cells for citrullinated histones (C). This would result in the cells being recorded as negative for NETosis. (Image D = merge).

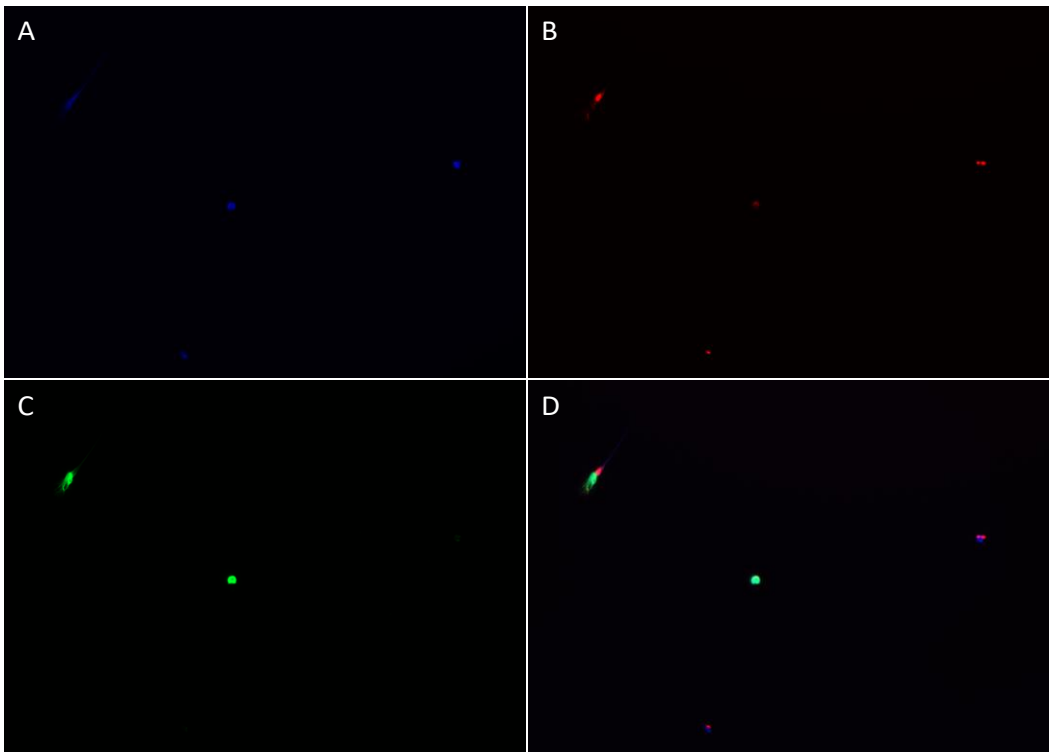

Figure 3.7 Neutrophil recorded as negative for having undergone NETosis due to nuclear morphology. The cell in the centre of each image has stained positive for DNA, neutrophil elastase and citrullinated histone (A, B and C respectively, D = merge). However, as the nucleus does not have a dispersed morphology this neutrophil would be recorded as negative for NETosis. Particular biases that may impact accurate NETosis predictions using this methodology include: unblinded assessment of samples, samples only being assessed by one observer and the lack of spatial validation for NET formation (e.g. the use of a measuring tool to identidy the width of positively stained structures to confirm the presence of a NET). All of which are points for consideration in future work.

3.6.8 *Assessing elastase concentration and activity*

To determine the concentration of neutrophil elastase in supernatant, samples were removed from -80°C and assessed using a "human elastase ELISA" kit (abcam). Samples were diluted by 1:5 in the reagent diluent provided by the manufacturer. All instructions provided with the kit were followed with the standard curve being used to determine individual sample elastase concentration.

For assessing elastase activity in peripheral blood neutrophils, the neutrophils were stimulated as previously described (chapter 3.5.1), however the neutrophils were incubated in the absence of autologous serum. The rationale for this decision is that autologous serum contains protease inhibitors that would block the elastase function *in vitro*. After stimulation neutrophil supernatants were collected as previously described by centrifugation and stored at -80°C.

By adapting a protocol kindly provided by Prof. James Chalmers (Dundee university) the levels of elastase activity in several samples was also determined. Firstly, 1UN of biologically active human neutrophil elastase (purchased from Sigma) was suspended in suspended in 50mM sodium acetate, 200mM NaCl and diH_2O. On the day of each assay serum free supernatant samples were thawed to room temperature, during which a standard curve of neutrophil elastase was prepared (diluted with a 50mM HEPES, 150mM NaCl. pH8 assay buffer).

Supernatant samples were then added to a 96 well plate (undiluted) along with the prepared elastase standard curve and blank (elastase free assay buffer). Following this N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (MeoSAAPvN), a substrate of elastase which impacts absorbance at 405nm when cleaved, was added to each well to a final concentration of 100µg/ml. Using a plate reader warmed to 37°C the absorbance (405nm) in each well was read 24 hours after addition of MeoSAAPvN. The elastase enzyme activity based standard curve was then used to determine the relative levels of elastase activity in each of the assayed samples.

3.6.9 *Initial attempts at determining extracellular DNA to measure NETosis*

An established methodology for studying the occurrence of NETs involves the use of extracellular DNA binding dyes to detect extracellular NET structures (Hair et al 2018, Patel et al 2018). Initial attempts at developing an assay such as this involved the use of PicoGreen, a fluorescent DNA dye used in a similar protocol in a 2010 study by Hakkim et al, and setting up a DNA standard curve (provided with the PicoGreen commercial kit) to quantify the concentration of extracellular DNA in each condition/sample (allowing easier comparison of separate samples). Despite several attempts there was no clear correlation between the recorded PicoGreen induced fluorescence and DNA standard concentration (see *figure 3.8*). Use of an additional DNA binding fluorescence dye (SYBR green) produced similar results. Due to the inability of this method to produce a consistent and accurate change in fluorescence relevant to a DNA standard, attempts at quantifying DNA using set concentrations of DNA were abandoned for future experiments.

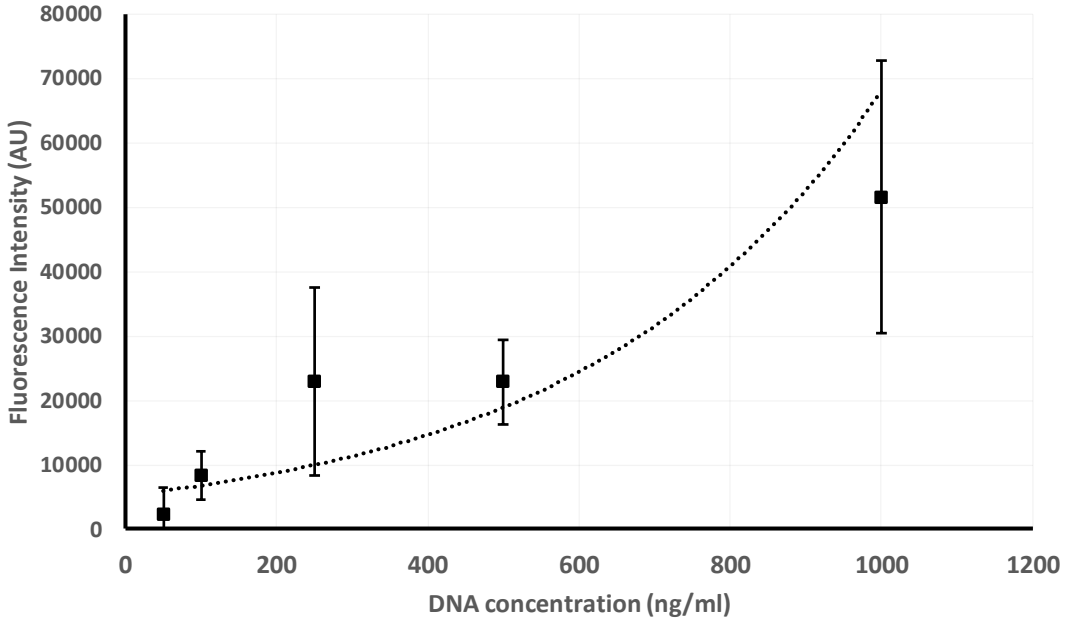

Figure 3.8 Standard curve of DNA concentration against blank subtracted picogreen dependent fluorescence intensity. As the data shows although an increase in fluorescence was observed when comparing the lowest concentrations of DNA (50 and 100ng/ml) to the highest (1000ng/ml), the increase in fluorescence between the concentrations of DNA was often inconsistent (in addition to several overlapping 95% confidence intervals) and not consistently statistically significant (n=4 +/-95% CI).

3.6.10 *Validation and use of SYTOX staining to measure extracellular DNA*

Following a visit to the Chalmers lab at the university of Dundee, a new protocol using a fluorescent DNA binding dye (SYTOX) to measure extracellular DNA within an individual set of samples was obtained and successfully replicated here in Newcastle. To confirm the DNA binding dye was functioning as expected two sets of experiments were performed; the first involved incubating various numbers of cells (ranging from 10,000 to 250,000) in a 96-well plate for 4 hours at 37°C, after which SYTOX was added at a final concentration of 500nM with the fluorescence in each well measured to confirm a correlation between cell number and fluorescence.

The second experiment aimed to confirm the fluorescence detected when mixing SYTOX with cells was due to the presence of DNA. To assess this, cells were incubated for 4 hours either with or without LPS in duplicate with one experimental arm being pre-treated with DNAse I for 30 minutes before the addition of SYTOX after which fluorescence in the wells of each experimental arm was read using a plate reader.

To measure the extracellular DNA release by stimulated/unstimulated peripheral blood neutrophils, the cells were incubated in a 96-well plate in HBSS+ (in the absence of autologous serum) with/without stimuli for 4 hours (50,000 cells per well, ran in quadruplicate). After 4 hours SYTOX was added to each well (final concentration of 500nM) with the fluorescence in each well being measured (480nm excitation, 520nm emission). For each individual volunteer/patient sample the level of extracellular DNA in each stimulatory condition was expressed as a percentage relative to the unstimulated condition (which was set as 100%).

3.6.11 *Histone-elastase complex ELISA*

A H1-elastase complex ELISA protocol (provided by Prof. James Chalmers) was used to assess the relative amount of NETs in cell free supernatants (*figure 3.9*). This assay assumes that the use of two antibodies directed against separate molecules present within NETs (i.e. histones and elastase) will allow quantification of the number of NETs within a given sample. Anti-H1 antibody was diluted 1:4000 in PBS to a final volume of 10ml. 100µl of this antibody solution was then added to each well of a 96-well ELISA plate and incubated for 1 hour at room temperature. Each well of the plate was then washed three times with wash buffer (0.05% Tween 20 in PBS). 100µL of 1% BSA (pH

7.2-7.4) was then added to each well for 1 hour to block the wells. During the blocking step samples are diluted 1:10 in blocking buffer for the assay whilst also setting up a 2 fold serial dilution of a biological standard (sputum sample possessing a high number of H1-elastase complexes, provided by Prof. Chalmers) to be used as a standard curve.

After 1 hour the blocking buffer is aspirated from the wells of the plate and each well washed 3 times with wash buffer. Following this 100µl of sample/standard/blank is added to the relevant well of the plate in triplicate. The plate is then incubated at room temperature for two hours before repeating the previous wash step. 100µl of rabbit anti-neutrophil elastase antibody (1:4000 dilution in block buffer) is added to each well and incubated at room temperature for two hours. After repeating the wash step, 100µl of HRP conjugated goat anti-rabbit IgG (1:40000 dilution in block buffer) is added to each well and incubated for 1 hour at room temperature.

The plate is then washed three more times before 100µl of TMB substrate solution is added to each well. Colour change is monitored and after 10-20 minutes H_2SO_4 is added to stop the reaction. Within 30 minutes the absorbance in each well was measured, with the absorbance values of the standard curve being used to assign each sample on the plate relative arbitrary units (allowing comparisons to samples ran on other plates alongside the same biological standard).

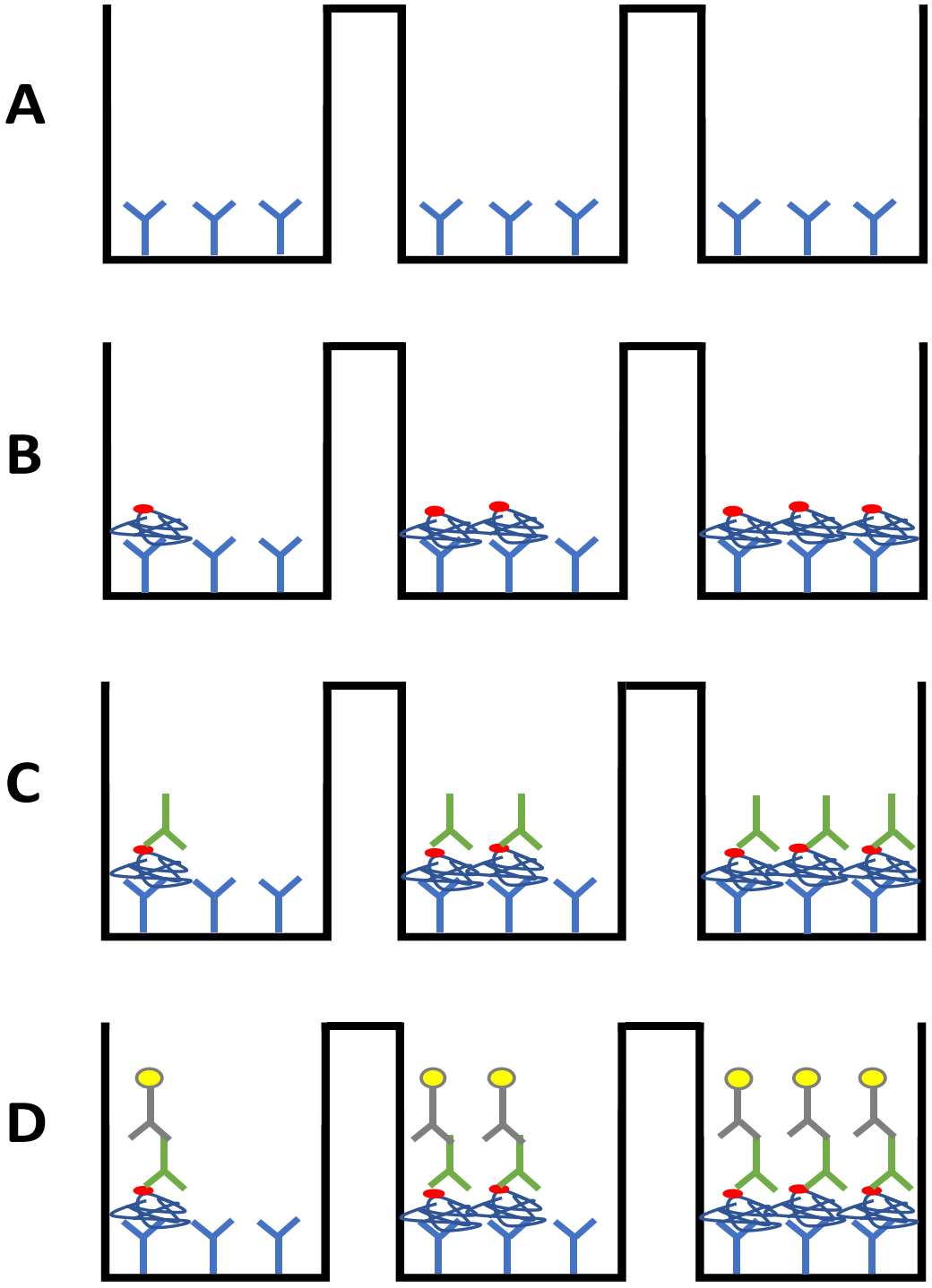

Figure 3.9 Neutrophil extracellular trap ELISA protocol (A) Well plates are coated with a mouse anti-H1 antibody and washed. (B) Samples and standards containing NETs (Dark blue = DNA/Histones, Red = Elastase) are added to each individual well and incubated. (C) After washing the wells, rabbit anti-neutrophil elastase antibody is added to each well, the plate is then incubated and washed to remove unbound antibody. (D) Goat anti-rabbit IgG (conjugated to HRP) is added to each well incubated before the plate is washed a final time and TMB solution is added. Once colour change is observed the reaction is stopped and fluorescence for each well recorded.

3.7 Assessing neutrophil function

3.7.1 *Detecting superoxide production by cytochrome C reduction*

Isolated neutrophils were resuspended in HBSS+ (HBSS media with Ca_{2+} and Mg_{2+}) at a cell concentration of 2 million cells per ml. 250µl of this cell suspension was then added to several marked 1.5ml Eppendorf tubes in duplicate. Using HBSS+ and 1% (final volume) autologous serum each well was made up to 500µl. After being incubated for 30 minutes (37°C), neutrophils were primed by adding platelet activating factor (PAF) at a final concentration of 1µmol/L then incubated at 37°C for 10 minutes. Respiratory burst was then induced by the addition of stimulatory molecules (50nM PMA, 1µg/ml LPS and 100nM fMLP) and an additional 1 hour incubation period at 37°C.

Following incubation 62.5µl of supernatant was carefully removed from each tube. To one set of duplicate tubes 25µl superoxide dismutase (SOD) was added (giving a final enzymatic activity of 200U/ml), to the other set of duplicate tubes an equal volume of HBSS+ was added. 37.5µl of cytochrome c (end concentration 1mg/ml) was then added to each Eppendorf. As oxidation of cytochrome c results in a change of the molecules absorbance at 550 nm, the underpinning theory is that by measuring the absorbance difference between SOD+ and SOD- samples we can determine the change in absorbance due to superoxide (as SOD metabolises superoxide).

All Eppendorf's were briefly and gently mixed, then incubated at 37°C for 1 hour, after which the contents of each Eppendorf was split into five 100µl aliquots and transferred to a respective lane of a 96 well plate. The absorbance in each well at 550nm was then measured and recorded. The average absorbance values for the SOD+ve wells were then subtracted from their counterpart SOD-ve wells in order to determine the change in absorbance due to the presence of superoxide. Using Beer-Lambert law with these absorbance values allows the concentration of superoxide to be determined.

3.7.2 *Zymosan based phagocytosis assay*

Prior to completion of neutrophil isolation from whole blood, 4µl of zymosan stock (0.2mg/ml) was added to a 0.5ml Eppendorf containing 96µl of IMDM media and 100µl of autologous serum. The solution was then incubated in a heated water bath at 37°C for 60 minutes then centrifuged at 10,000xg for 2 minutes. The supernatant was

carefully aspirated, with the remaining zymosan pellet being resuspended in 200µl of IMDM media. This wash step was repeated an additional time with the pellet being resuspended in 200µl of IMDM media for use in the assay.

Once neutrophil isolations and counts were performed neutrophils were resuspended in IMDM media at a cell concentration of 2 million cells per ml. 500,000 neutrophils were added to 4 marked wells of a 24 well plate, with IMDM media being added to make each well up to a final volume of 500µl. After 30 minutes incubation at 37°C, 50µl of supernatant was carefully aspirated from each of the 4 wells. To two wells 50µl of zymosan-media solution was added, to the remaining two wells 50µl of IMDM media was added (giving a duplicate of zymosan +ve and -ve wells). After 1 hour incubation 37°C, all media from each well was carefully removed. Sterile PBS was then carefully used to wash each well a total of 5 times (extreme care being taken to avoid detachment of neutrophils from the plate).

After the final wash, the wells were left to dry before 500µl of absolute ethanol was added to each well for 15 minutes in order to fix the cells. The ethanol was then aspirated from each well with 5 more PBS wash steps being carried out. 500µl of giemsa solution (1:10 dilution of giemsa in 0.05% PBS Tween) was then added to each well and left to stain for 15 minutes. Finally, distilled H_2O was then used to wash each well of the plate a total of 7 times (or until all visible presence of giemsa was removed).

Brightfield microscopy was then used to examine the fixed/stained cells in each well. By examining the cells in the zymosan-positive wells it is possible to see zymosan particles that have been phagocytosed by the neutrophils. For each well 300 cells were examined in total with the number of neutrophils which had phagocytosed 2 or more zymosan particles being recorded and used as a marker of phagocytic ability.

To confirm counts were accurately assessing neutrophil phagocytosis of zymosan, an additional two duplicate wells were included in which neutrophils were incubated with Salbutamol, which has previously been shown by our lab to inhibit phagocytosis was used at a concentration of 10µM (Scott et al 2016).

3.7.3 *Neutrophil viability assay*

Flow cytometric analysis of Annexin V and propidium iodide (PI) binding was used to assess neutrophil viability rates in several experiments. 500,000 neutrophils (suspended in phenol-red free media and 1% autologous serum) were added to 1.5ml Eppendorf's at a final volume of 500µl. After a specified period of incubation relevant Eppendorf's were gently centrifuged at 300xg for 5 minutes. 275µl of supernatant was then carefully extracted and discarded from the centrifuged tubes.

275µl of Annexin V binding buffer was then added to the remaining volume/pellet in each tube before centrifuging the samples again (300xg for 5 minutes). Following this wash step 275µl of supernatant was again extracted and discarded. 225µl of annexin V binding buffer was then added to resuspend the cell pellet. 100µl of the resuspended cell suspension was then added to a corresponding flow cytometry tube either left empty or containing propidium iodide (0.5mg/ml stock) and/or APC conjugated annexin V stock (see table 3.6).

Table 3.5 Volumes of cells, propidium iodide and Annexin V placed in control and sample tubes for viability assay.

Condition	Cell solution (µl)	Propidium iodide (µl)	Annexin V (µl)	Annexin V binding buffer (µl)
Unstained	100	-	-	5
PI single stain	100	1	-	4
Annexin V single stain	100	-	2	3
Experimental sample	100	1	2	2

After 15 minutes incubation (protected from light exposure) 200µl of annexin V binding buffer was added to each tube and gently mixed. Samples were then analysed for Annexin V and PI staining of cells within the sample by flow cytometry. Samples ran through the flow cytometer were gated to isolate neutrophils (excluding cells with low size and granularity) and remove double cells from the assessed population. Using unstained, PI single stain and Annexin V single stain controls, the samples were gated to allow recognition of cells staining positive and negative for PI and Annexin V (see *figure 3.10* for an example of gating strategy). The flow cytometer software was then used to determine the percentage of neutrophils within the samples staining -ve for both PI and annexin V (i.e. viable neutrophils), +ve for annexin V (early apoptotic cells) and +ve for propidium iodide and annexin V (dead cells).

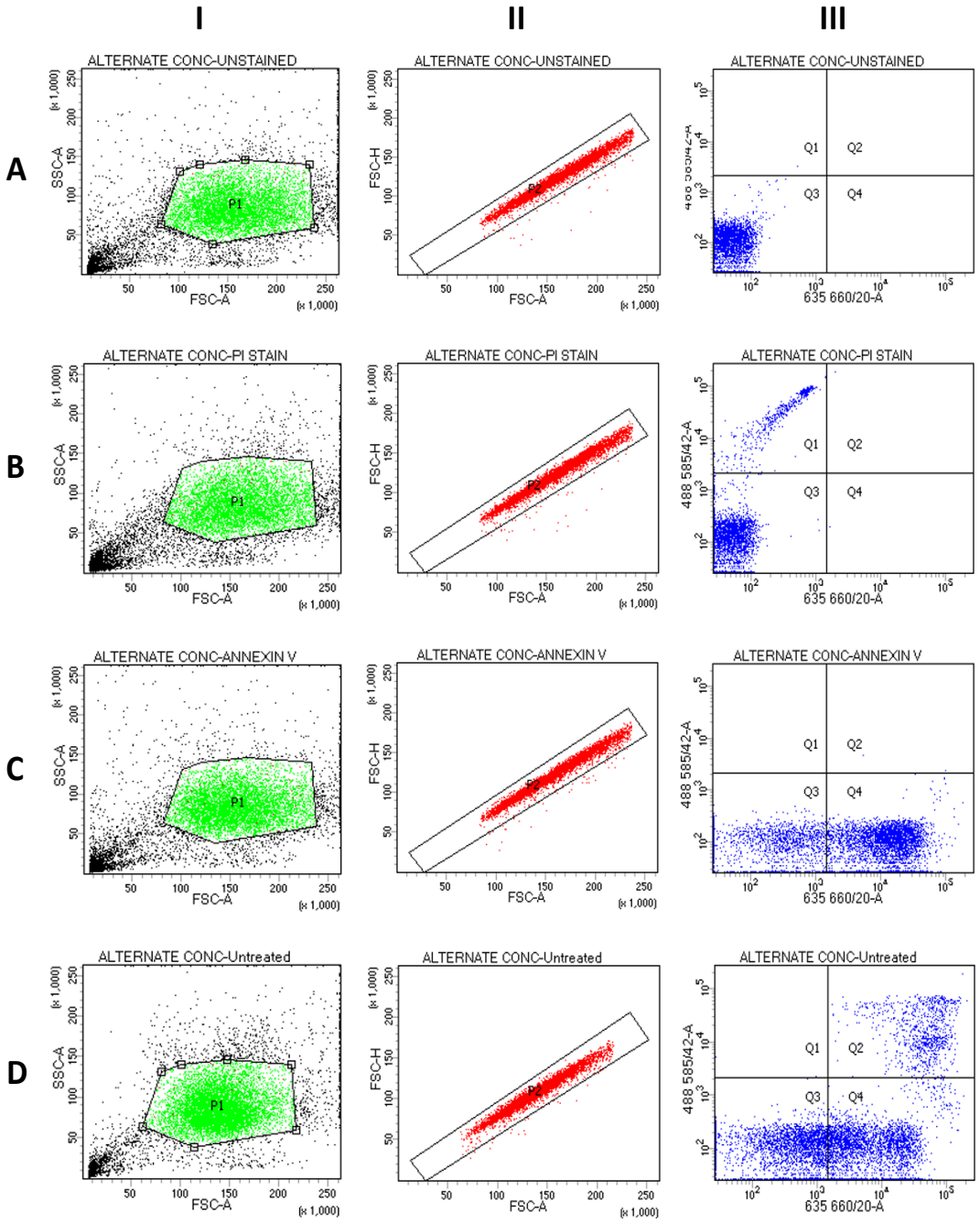

Figure 3.10 Flow cytometric gating for controls and samples assessed for viability. Rows A, B, C and D show the gating for unstained control, PI stain control, annexin V stain control and dual stained sample respectively. Column I shows gating for neutrophils on the basis of size and granularity in order to isolate neutrophils scanned by the flow cytometer (residing within gate P1). Column II shows the gating used to remove double clusters of cells which have passed through the flow cytometer (i.e. only single cells will be present in P2). Column III shows the gating for Annexin V (x-axis) and PI (y-axis), using the controls (rows A-C) the gates were set in place to determine the cut off point for staining positive for annexin V and PI, which was used to measure viability in samples such as that seen in Row D. The quadrants (Q1,2,3 and 4) on the final plots (Column 3) are used to determine the percentage of viable cells within a sample. Q1 = PI+ve/Annexin V-ve (typically only seen in PI stain control only), Q2 = PI+ve/Annexin V+ve (Apoptotic), Q3 = PI-ve/Annexin-ve (viable), Q4 = PI-ve/Annexin V+ve (early apoptosis).

3.8 Bronchiectasis and rheumatoid arthritis patient sampling

3.8.1 *Recruitment of patients and handling of samples*

A broad range of patients were recruited to take part in the study including idiopatic BR patients (n=9), BROS patiets (n=3) and a patient with BR and overlapping COPD. A broad range of phenotypical data was collected for patients including: age, gender, BMI, height, weight, number of exacerbations in previous 12 months, medical research council dyspnoea score, SQRQ score, QoL-B questionnaire score, FEV1 (plus predicted), FVC (plus predicted), FEV1/FVC, currently receiving nebulised antibiotic, long term macrolide use and other recieved medications (demographic data presented in chapter 7).

Approval for the study was granted by the North West – Greater Manchester East Research Ethics Committee (17/NW/0409) with the recruitment and processing of parents being undertaken at the Freeman hospital in Newcastle Upon Tyne, UK. 36ml of whole blood was taken from consented patients (consent and blood taken by a clinically trained research nurse) and mixed with citrate (same concentration described in 3.3.1) to prevent clotting. Blood samples were then collected and transported to the Institute of cellular medicine (Newcastle Upon Tyne, UK) for processing, with a transit time between the sites of roughly 20 minutes.

3.8.2 *Protocols used with BROS samples*

Peripheral blood neutrophils were isolated from whole blood using the Percoll gradient methodology described in chapter 3.3.2. Counts and purity assessment were then carried out as previously described to confirm the quality of the neutrophil isolation and ensure an accurate number of cells was used in each planned experiment. In total 6 of the previously described experimental methods were used to assess patient neutrophil responses; PAD activity assay, NET immunofluorescent staining, SYTOX extracellular DNA assay, phagocytosis assay, superoxide assay and neutrophil viability assay.

Healthy samples (obtained as described in 3.4.1) were also run simultaneously to patient samples, to confirm any anomalous results for patient samples were not due to a problem with the reagents/methodology on the given day. Given that these healthy volunteer samples were from a range of ages (18-55 years of age) they were viewed as a biological test for the assay rather than an accurate comparator for patient samples.

3.8.3 *Patient demographics recorded and analysis of data*

All assays and results were collected and processed in a blinded fashion, with only the clinical research staff at the Freeman hospital (whom played no part in processing of samples in the lab) knowing the demographics of the individual patients. Following collection of the final patient sample, the study became unblinded with details on demographics (e.g. patient diagnosis, age, gender) becoming available.

3.9 Use of inhibitors in experimental work

3.9.1 *Pre-treatment of neutrophils with cl-amidine*

In the thesis a large chapter of the work focused on examining the effect of the inhibitor Cl-amidine on NETosis, citrullination and general neutrophil function. Cl-amidine is an inhibitor of PAD enzymes previously used in neutrophil based research (Biron et al 2017, Kusonoki et al 2016, Knight et al 2013), which has been shown to have varying specificities, with IC50 values of 0.8µM, 6.2µM and 5.9µM reported for Cl-amidine in relation to PAD1, PAD3 and PAD4 respectively (Luo et al 2006, Knuckely et al 2010).

To establish the toxicity of Cl-amidine, neutrophils were incubated with various concentrations of the inhibitor for 4 hours at 37°C before being assessed for viability (chapter 3.7.3). Once a non-toxic concentration of Cl-amidine was established, in future experiments neutrophil were incubated with 200µM Cl-amidine for 30 minutes. Following this all experimental work using the assays previously described was carried out as stated, with a parallel set of conditions from the same individual volunteer without Cl-amidine pre-treatment.

3.9.2 *Use of LPS signalling inhibitors*

Two peptide inhibitors of separate components of the LPS signalling pathway were used in experiments: TRIF-pep and MyD88-pep. TRIF-pep is a 14 amino acid peptide first designed and described by Toschakov et al (2005), which blocks TRIF mediated signalling by blocking interaction between TRIF and the LPS receptor complex. MyD88-pep is a 26 amino acid peptide initially described by Loiarro et al (2005), which inhibits the MyD88 dependent pathway of LPS signalling by binding MyD88 and preventing homodimerization needed for the signalling process. The manufacturer of the inhibitors

(invivogen) advised incubation periods of 8 hours and beyond in cell based *in vitro* experiments, which may explain the lack of any previous work demonstrating the use and efficacy of these molecules in inhibiting LPS signalling in neutrophils (due to the technical difficulties of work requiring long incubation periods with neutrophils).

The toxicity of the two inhibitors was assessed on isolated neutrophils at 7 hours (suggested incubation time based on manufacturers recommendation description) by incubating the neutrophils (in IMDM media and 1% v/v serum) with a range of concentrations of the two inhibitors and establishing differences in viability at 7 hours using PI/ Annexin V by flow cytometry (FC) (as was done for establishing Cl-amidine viability).

After establishing concentrations of the inhibitors that had no impact on neutrophil viability but still effectively inhibited neutrophil activation (confirmed by CXCL8 ELISA of inhibited neutrophil supernatants showing decreased CXCL8 secretion in response to LPS) at 7 hours, cells were incubated in IMDM, serum and either TRIF-pep or MyD88-pep for 3 hours at 37°C. Following this incubation LPS was added to relevant wells and all conditions were incubated at 37°C for a further 4 hours. After which supernatants were collected and relevant assays (e.g. PAD activity assay) were carried out. Due to the longer periods on incubation needed for LPS inhibition some assays (e.g. superoxide detection, phagocytosis) could not be reliably carried out on these samples.

3.10 Statistical analysis

Microsoft Excel and PowerPoint 2016 were used for the collection/processing of all data and the creation of figures, with Minitab 17 being used for statistical analysis. The number of samples collected for each experiment is available in the description for each figure. A Shapiro-Wilk normality test was used to assess each data for normal distribution. Averages of results with a normal distribution were presented as means with 95% confidence intervals with stats tests used including; Pearson correlation coefficient test, paired t-test, repeated measures ANOVA and One-way ANOVA. Post hoc stats tests selected were the Dunnett's, Bonferroni and Tukey's test dependent on the type of data comparison.

Data with non-normal distribution were presented with their median value as the average and interquartile range values as the error bars on presented figure. Spearman-Rho correlational analysis was used to determine whether correlations of non-significant data was statistically significant. For all results, $p=0.05$ was set as the establishing point for establishing significance, with a p-value lower than this suggesting statistical significance.

Chapter 4: Impact of Bronchiectasis relevant molecules on healthy volunteer neutrophil mediated citrullination and NETosis

4.1 Introduction

4.1.1 *Overview*

Since their discovery in 2004, several molecular triggers of NETosis have been reported. Classically Phorbol 12-myristate 13-acetate (PMA) and calcium ionophores have been argued to be the most potent inducers of the process in neutrophils (Remijsen et al. 2011, Douda et al. 2015), with several other molecules (e.g. LPS and TNF-α) and bacterial pathogens such as *P. aeruginosa* and *H. influenzae* (both of which are relevant to BR) also suggested to induce NETosis (Guimarães-Costa et al. 2012). However, there are large differences between pieces of research documenting these molecular triggers of NETosis including the methodology design used to assess NETosis (e.g. targets used in immunofluorescent staining for NETs) and the types of cells assessed (e.g. human neutrophils, animal derived neutrophils, HL-60 cells). Furthermore, there is limited data on the impact of these disease relevant molecules in relation to citrullination which underpins NETosis and is associated with RA and possibly BROS.

Peripheral blood neutrophils obtained from healthy volunteers were incubated with several disease relevant molecules including: the Protein kinase C (PKC) agonist PMA, LPS derived from *P. aeruginosa*, the bacterial derived chemokine N-Formyl-Met-Leu-Phe (fMLP) and the rheumatoid arthritis (RA) associated TNF-α. After which a variety of methodologies were used to assess NETosis rates and citrullination to establish an accurate representation of each stimuluses ability to alter these processes. In addition, whole cell lysates (WCL) generated using bronchiectasis patient derived clinical strains of *P. aeruginosa* (strain 968333S) and *H. influenzae* (strain 2386), were also incubated with peripheral blood neutrophils, in order to assess the impact of bacterial cell remains (which would arguably be present chronically within the lungs of bronchiectasis patients) on neutrophils in regard to citrullination and NETosis *in vitro*.

4.1.2 *Hypothesis*

- Neutrophils stimulated *in vitro* with bronchiectasis disease relevant molecules and bacterial cell lysates will show signs of elevated NETosis.
- Neutrophil mediated citrullination will increase following stimulation with bronchiectasis relevant stimuli in comparison to unstimulated neutrophils.

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4.2 Results

4.2.1 *Purity of peripheral blood neutrophil isolations*

As the work throughout the thesis involved assessing the responses of peripheral blood neutrophils it was essential that the purity of each neutrophil isolation procedure was assessed. *Table 4.1* shows an example of the recorded purity of 22 neutrophil preparations carried out within a 4-month time span during the thesis. As can be seen the median purity of the neutrophil isolation procedure was 94%, with the lowest recorded purity in this period being 85%, which remained within the acceptable range for experimental work (85% or higher).

Table 4.1 Purity of neutrophil preparations carried out between December 2015 – April 2016. All neutrophil preparations in this timespan had a purity of 85% or higher, which was higher than the set arbitrary cut off point for any work involving neutrophils

Sample ID	Date obtained	Purity (%)	Sample ID	Date obtained	Purity (%)
HV01	1.12.2015	91	HV12	9.3.2016	96
HV02	14.12.2015	85	HV13	10.3.2016	88
HV03	12.1.2016	98	HV14	11.3.2016	91
HV04	10.2.2016	94	HV15	14.3.2016	89
HV05	11.2.2016	96	HV16	15.3.2016	96
HV06	12.2.2016	97	HV17	21.3.2016	97
HV07	25.2.2016	94	HV18	3.3.2016	87
HV08	1.3.2016	94	HV19	17.3.2016	90
HV09	2.3.2016	92	HV20	30.3.2016	97
HV10	7.3.2016	93	HV21	31.3.2016	95
HV11	8.3.2016	87	HV22	12.4.2016	96
Median Purity:		94.00%			
St. Dev:		3.86%			
SEM:		0.82%			

4.2.2 *Establishing neutrophil incubation times*

As neutrophils are a mature terminal cell (i.e. non-dividing), long term culturing of neutrophils and expansion of obtained peripheral blood neutrophil samples from healthy volunteers is not possible. Therefore, it is critical to establish the lifespan of neutrophils *in vitro*, in order to guide experimental design and ensure all functional assays were carried out in a time period (post-isolation) where the vast majority of neutrophils will remain largely functional and viable (i.e. not entering the early stages of apoptosis). Neutrophil viability at set time intervals (4 hours, 6 hours 8 hours and 24 hours) was recorded using flow cytometric analysis of annexin V and PI staining.

Viability was observed to drop significantly at each timepoint measured with median viabilities of 85.9%, 65.9%, 49% and 3.7% at 4, 6, 8 and 24 hours respectively (*fig 4.1a*). Correlational analysis of neutrophil viability against time showed this strong negative correlation (Spearman-Rho = -0.962) was highly significant ($p < 0.001$, f*ig 4.1b*), as expected given our understanding of neutrophil biology in relation to biological lifespan. Based on these results it was decided that functional work e.g. neutrophil incubation should attempt to remain within the 4-hour timespan post isolation where possible, as after this timespan neutrophil function will likely begin to decline and may impact functional assay results.

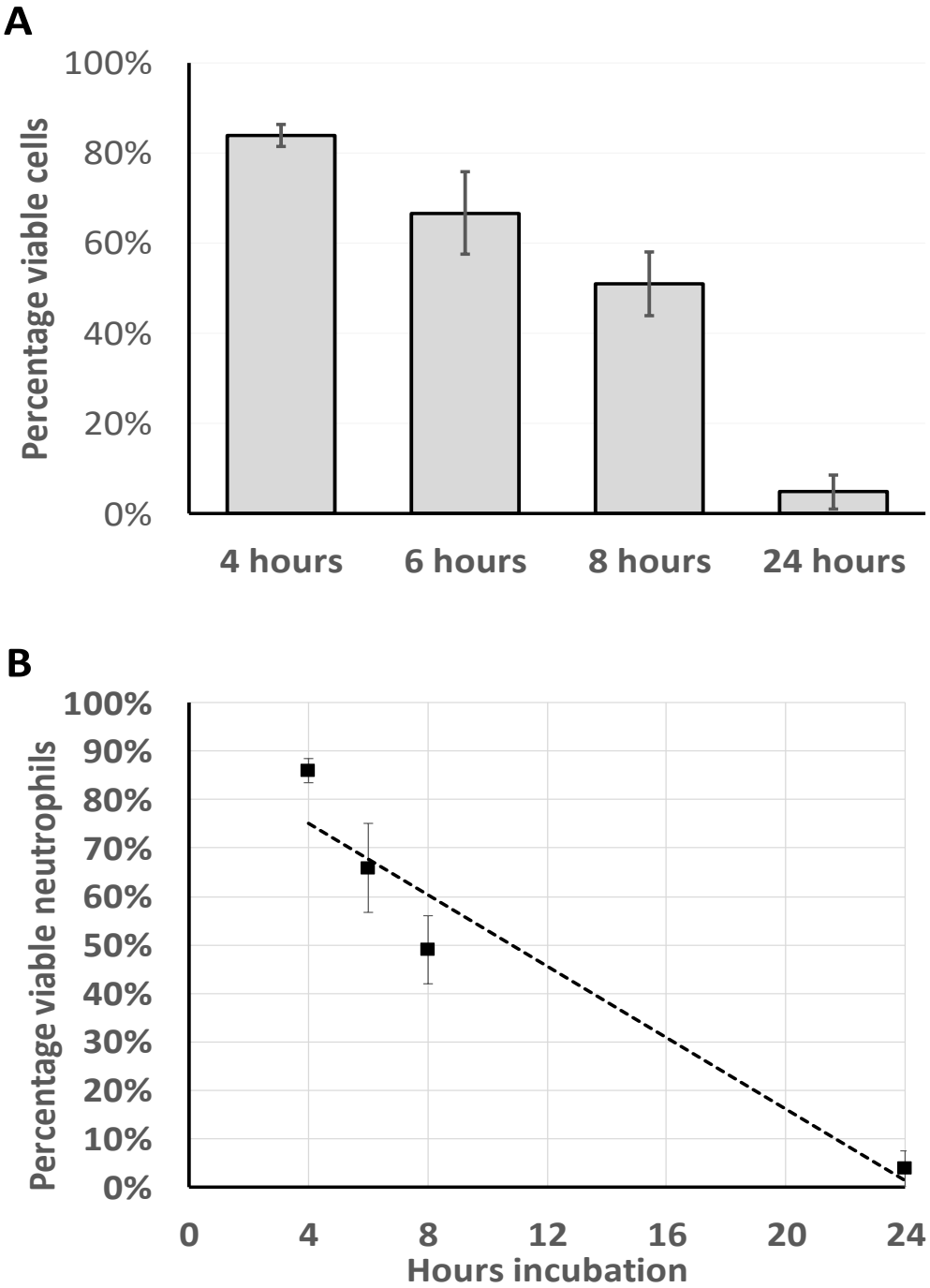

Figure 4.1 Neutrophil viability in vitro over a 24-hour timespan post-isolation from whole blood. (A) The viability for peripheral blood neutrophils decreased significantly at each observed time interval, at each observed timepoint (n=5 +/- IQR, ** $p < 0.01$, *** $p < 0.001$). (B) The decrease in viability at each timepoint was reflected in the correlation plot of the data, in which the same data showed a strong negative correlation (Spearman Rho correlation value = -0.946) that was statistically significant (+/- IQR, $p < 0.001$).

4.2.3 *Confirming biological activation of neutrophils by disease relevant PAMPs*

To confirm the stimulatory molecules and bacterial lysates were biologically active, the supernatants of neutrophils (both untreated and stimulated) were assessed for CXCL8 concentration. CXCL8 was selected based on previously published and well-established research showing that CXCL8 secretion occurs in neutrophils in response to a broad range of stimuli (Fujishima et al. 1993). CXCL8 secretion was elevated when neutrophils were treated with all selected stimuli (*Fig 4.2a*). Although all stimuli resulted in a significant increase in supernatant CXCL8 concentration, PMA was seen to result in a much higher increase in CXCL8 release than other stimuli. However, when observing the differences in supernatant CXCL8 concentration between unstimulated neutrophils and those stimulated with other molecules (excluding PMA), a 8-fold, 3-fold, 7- fold, 9-fold and 8-fold increase in CXCL8 concentration was seen in regards to untreated neutrophils for LPS, fMLP, TNF-α, WCL *P. aeruginosa* and WCL *H. influenzae* respectively (*Fig 4.2b*).

Therefore, all of the tested stimuli successfully triggered CXCL8 secretion and by extension activating the isolated neutrophils *in vitro*. Once this was confirmed, the stimuli (at the established concentrations) were used in the following experiments to assess NETosis rates and citrullination. It is important to note that no inference is made on the relative potency of each stimulus, as dose response curves for each stimuli were not conducted.

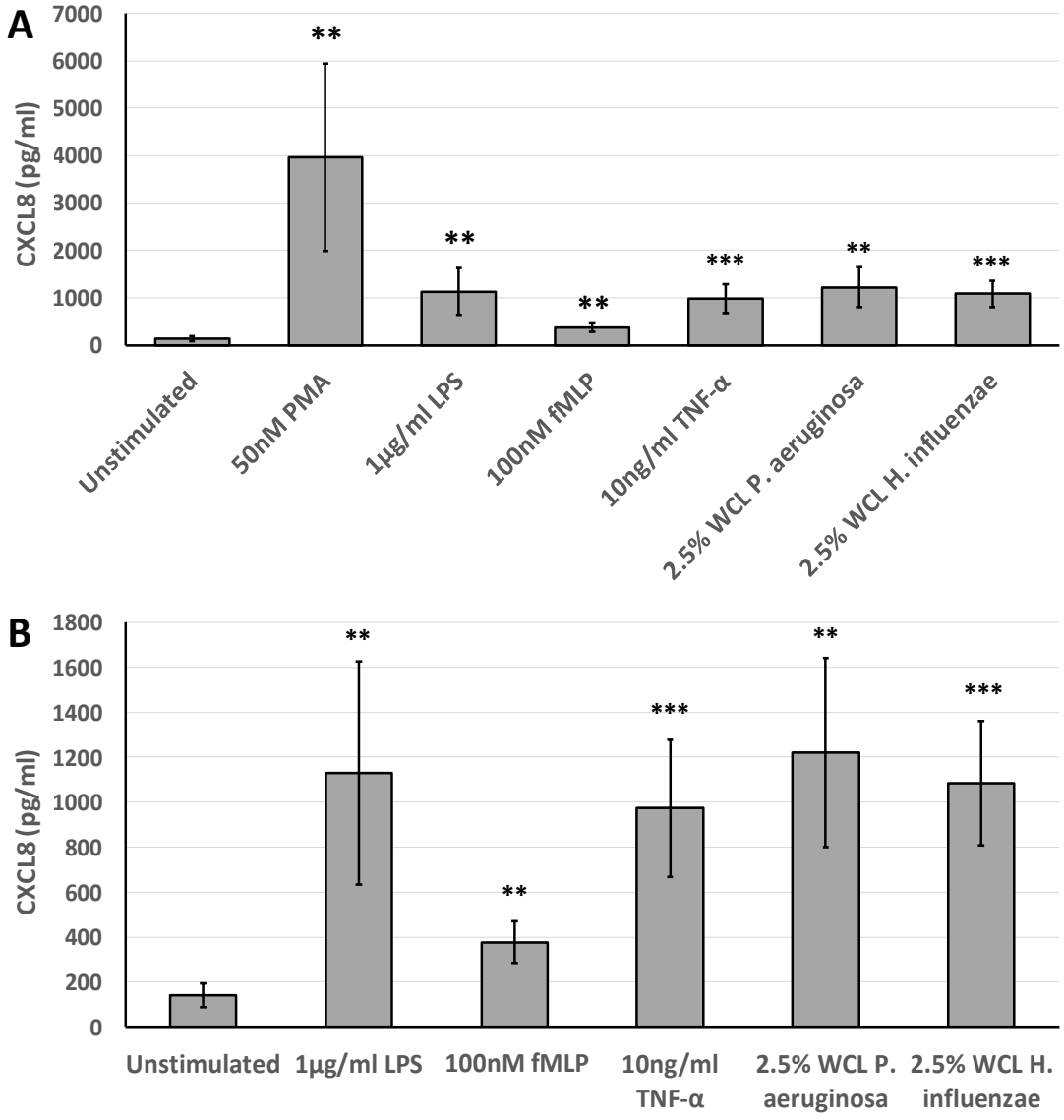

Figure 4.2 CXCL8 concentration in neutrophil treated with bronchiectasis and rheumatoid arthritis relevant stimuli. (A) CXCL8 concentration was significantly higher than untreated in every condition, with PMA showing the largest increase. Graph B shows the same data seen in graph A, with the PMA condition results being excluded to demonstrate the large differences between the CXCL8concentration for neutrophils stimulated with the other molecules/lysates and untreated neutrophils (n=10, mean +/- 95% CI, ** $p < 0.01$, *** $p < 0.0001$)

4.2.4 *NET immunofluorescent staining*

Before assessing NETosis rates in response to tested PAMPs and lysates, the immunofluorescent assay was first validated by a dose response experiment using PMA. As PMA is an established inducer of NETosis, it is expected that neutrophils incubated with increasing concentrations of PMA should in response show higher rates of NETosis. When left untreated or stimulated with 25nM, 50nM and 100nM PMA for four hours and assessed for the presence of NETs with immunofluorescent staining there is a significant positive correlation ($p < 0.001$, Pearson correlation = 0.921) between PMA concentration and the number of NETs detected (*Fig 4.3a*), with the number of detected NETs increasing significantly between each increase in PMA concentration (*Fig 4.3b*). Supporting the assumption that the staining counts does correspond to NETosis.

Immunofluorescent staining was then used to assess the impact of the selected stimulatory molecules on isolated neutrophil NETosis rates *in vitro*. After 4 hours incubation, 4% of unstimulated neutrophils were recorded as undergoing NETosis, identical to the rate seen in fMLP stimulated neutrophils. PMA, LPS, TNF-α, WCL *P. aeruginosa* and WCL *H. influenzae* all induced a higher rate of NETosis (53%, 37%, 18%, 29% and 24% respectively), with all conditions, excluding *H. influenzae*, resulting in statistically significant increase in NETosis (*Fig 4.4*). This support previous work identifying PMA, LPS and TNF-α as inducers of NETosis (although TNF-α as an initiator of NETosis has previously only been demonstrated in HL-60 cells) (Guimarães-Costa et al. 2012, Wang et al. 2009). Live *P. aeruginosa* has been shown to induce NETosis in mast cells, however our results suggest that pathogen associate molecular patterns contained in whole cell lysates of the bacteria alone can also induce NETosis rates in human neutrophils (von Köckritz-Blickwede et al. 2008).

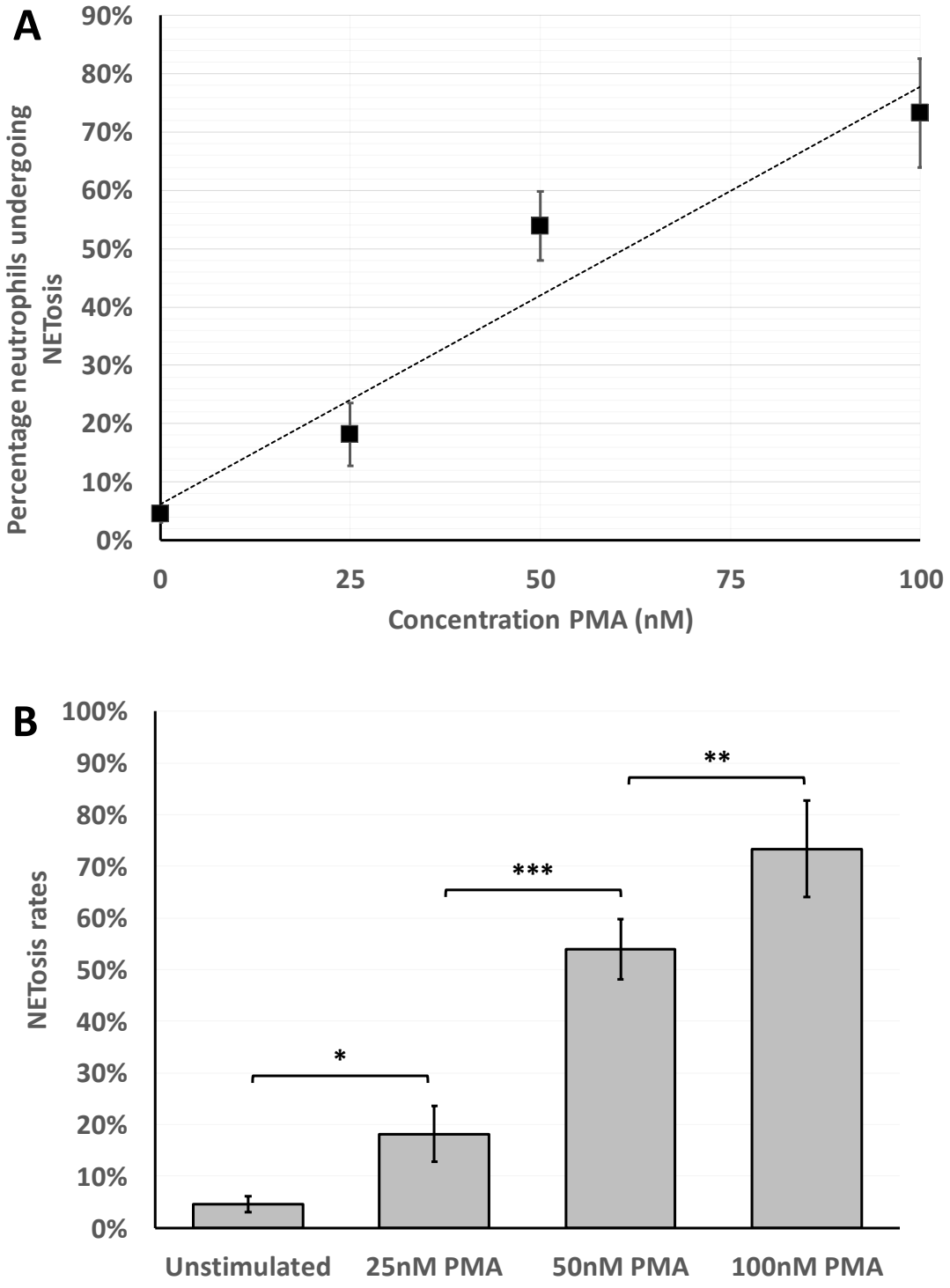

Figure 4.3 Increasing concentrations of PMA on NETosis rates assessed by immunofluorescent microscopy. (A) The percentage of neutrophils undergoing NETosis showed a significant positive correlation with PMA ($p < 0.001$, Pearson correlation = 0.921). (B) Comparison of each incremental increase in PMA concentration (25, 50 and 100nM PMA) showed a significant increase in NETosis rates between each increase (n=6 +/- 95% CIs, * $p < 0.05$, ** $p<0.01$, *** $p < 0.001$).

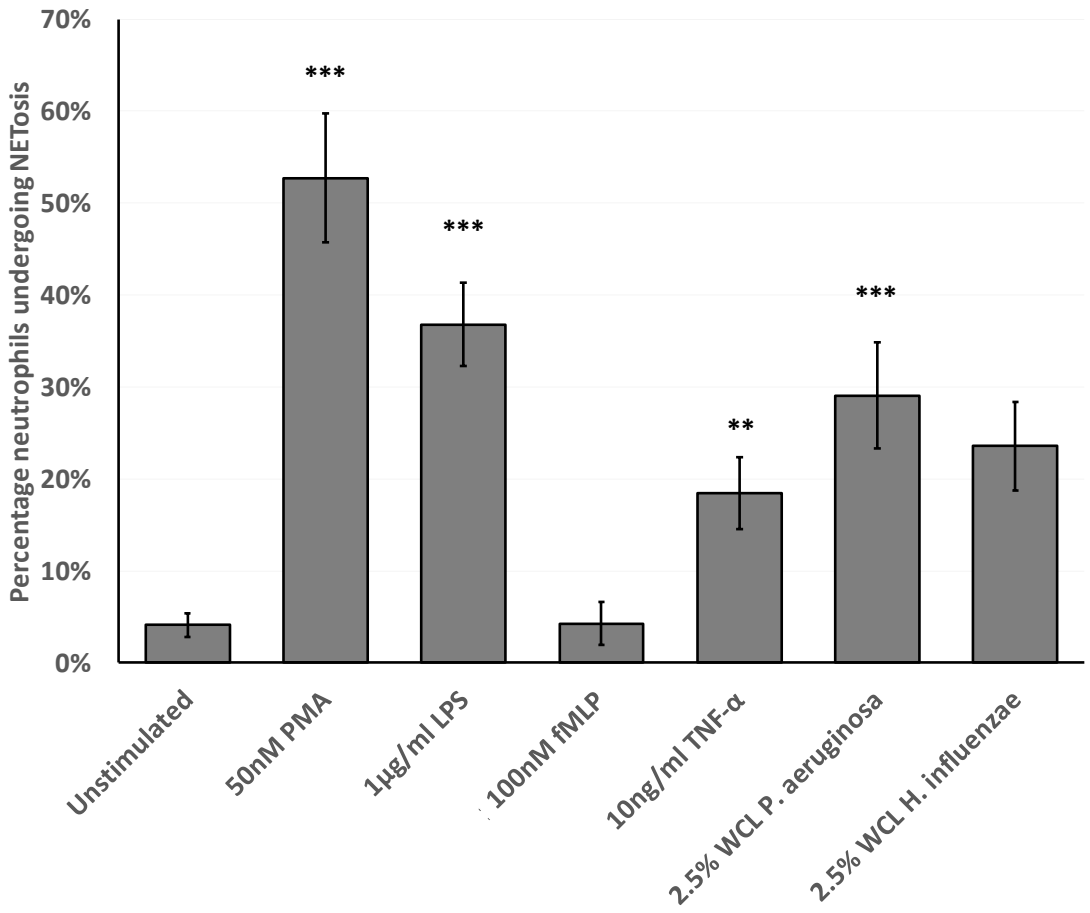

Figure 4.4 Immunofluorescent microscopy determined NETosis rates in response to bronchiectasis and rheumatoid arthritis relevant stimuli. All conditions except fMLP showed an increase in the percentage of neutrophils that had underwent NETosis, however only treatment PMA, LPS, TNF-α and WCLs of P. aeruginosa resulted in a significant increase in NETosis rates (n=11 +/- 95% CIs, ** $p < 0.01$, *** $p < 0.001$).

4.2.5 *Supernatants from stimulated neutrophils have higher levels of PAD activity but no corresponding increase in PAD4 concentration*

Using a PAD activity assay, the levels of PAD activity in the supernatant of neutrophils incubated for four hours with/without stimulation was assayed (*Fig 4.5*). PMA, LPS, fMLP, TNF-α and WCL *P. aeruginosa* treatment all resulted in an increase in PAD enzyme activity for the supernatant, with PMA showing the largest increase in enzyme activity (3-fold increase), with this increase for all of these conditions excluding fMLP being statistically significant. WCL of *H. influenzae* appeared to have no significant effect on extracellular PAD enzymatic activity compared to untreated, however further repeats would be useful to confirm this.

Using a PAD4 ELISA to measure the concentration of PAD4 in neutrophil supernatants incubated under the given conditions for 4 hours showed opposing results. As an increase in citrullination was seen in supernatants treated with PMA, LPS, and WCL *P. aeruginosa* it is expected that there may be a corresponding increase in PAD4 concentration in stimulated supernatants. As seen in *Fig 4.6*, there were slight differences in the supernatant PAD4 concentration for all conditions however there was no significant difference between the conditions.

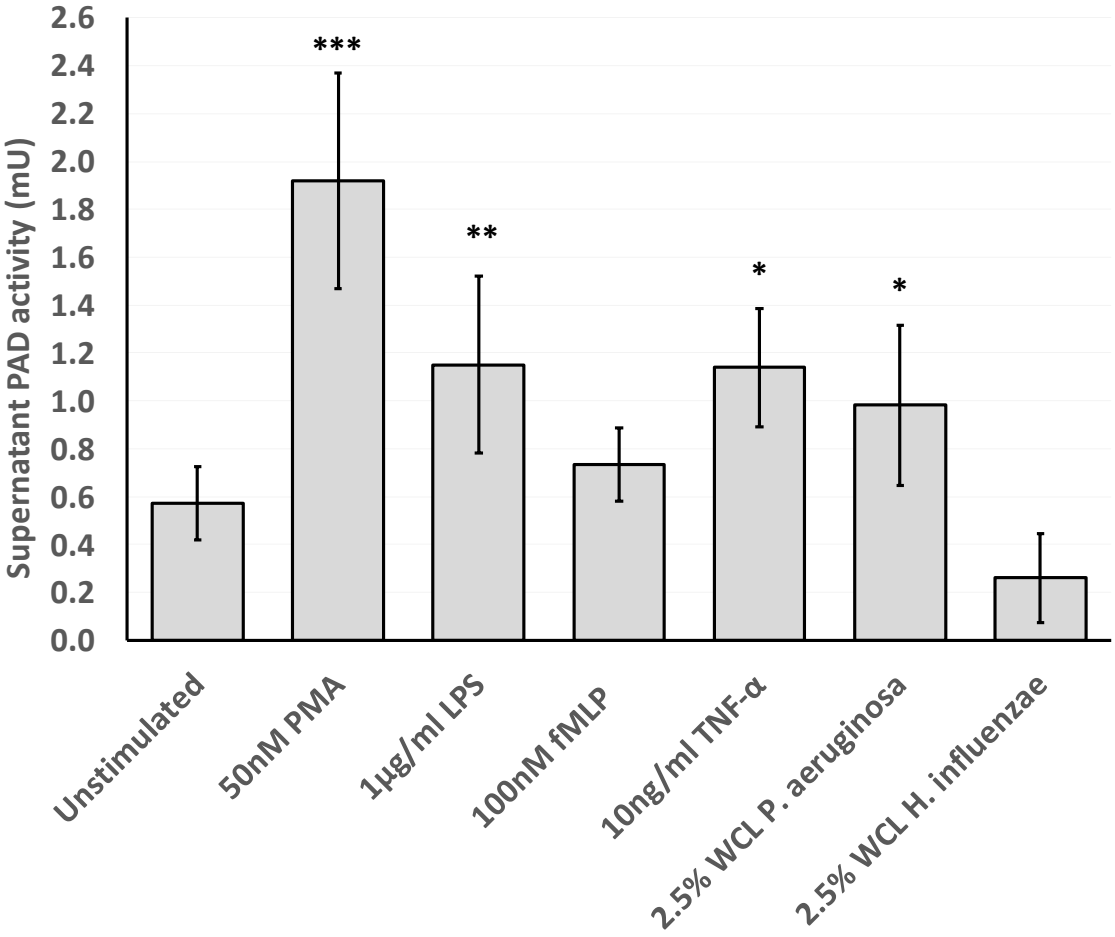

Figure 4.5 PAD activity rates in supernatants from unstimulated and stimulated peripheral blood neutrophils. Supernatant PAD enzyme activity was significantly increased in PMA (n=16), LPS (n=16), TNF-α (n=10) and WCL P. aeruginosa (n=16) in comparison to untreated (n=16). fMLP (n=16) also showed an increase in PAD activity but this was not shown to be significant. WCL H. influenzae (n=6) showed a decrease in PAD activity, which also did not reach statistical significance (+/- 95% CIs * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

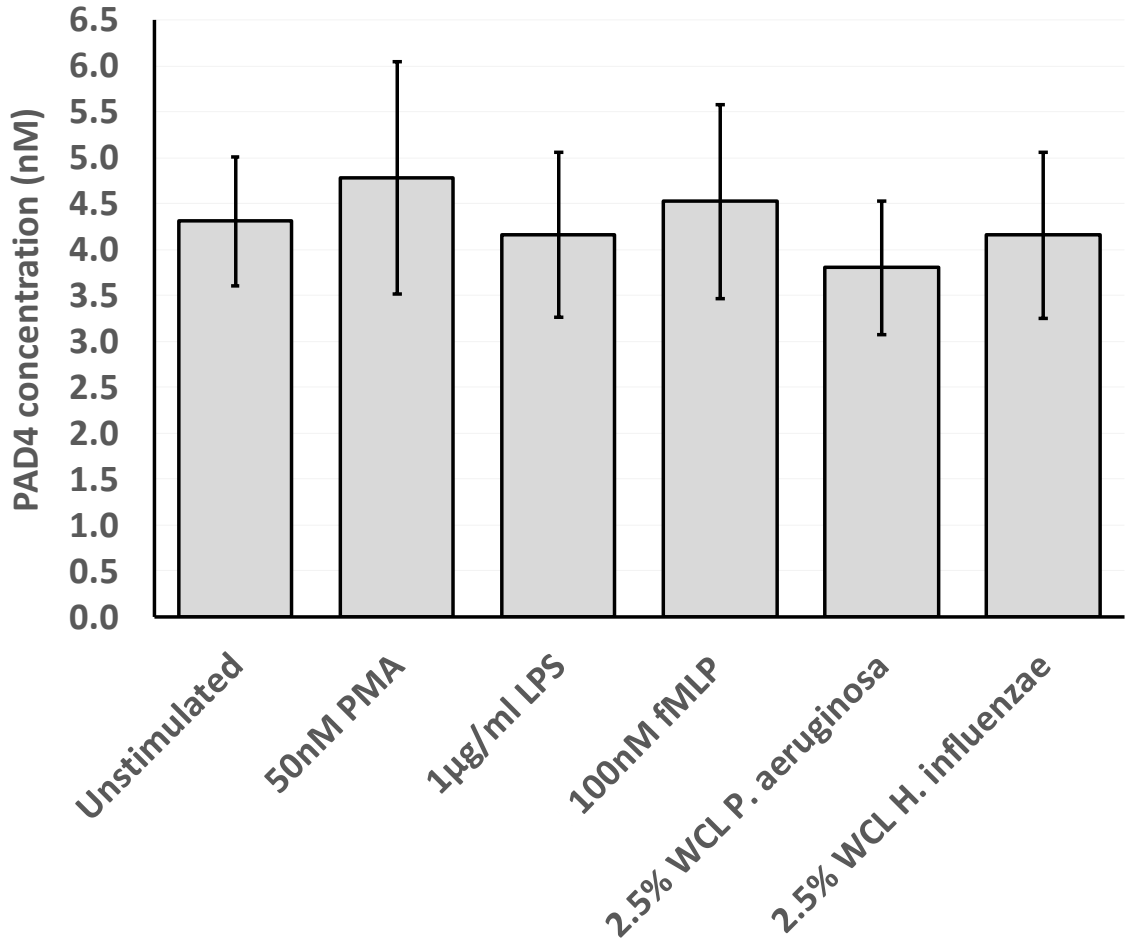

Figure 4.6 PAD4 concentration in supernatants from unstimulated and stimulated peripheral blood neutrophils. Small differences in PAD4 concentration was observed for the tested stimuli however no statistically significant difference was seen in comparison to untreated neutrophil supernatants (n=7, +/- 95% CIs).

4.2.6 *No difference in supernatant PAD activity following stimulation with differing strains of Pseudomonas aeruginosa*

As WCLs of of *P. aeruginosa* (strain 968333S, a bronchiectasis patient derived strain) was shown to increase extracellular PAD activity, the impact of other non-bronchiectasis *P. aeruginosa* strains on neutrophil extracellular PAD activity was assessed. WCLs for three additional strains were assessed for supernatant PAD activity and compared to the previously used 968333S strain and unstimulated neutrophils; PA01 (genome sequenced lab strain), 57931PA (COPD patient derived clinical strain) and DK2 (cystic fibrosis clinical strain).

Mean extracellular PAD activity was higher in neutrophils stimulated with each of the *P. aeruginosa* strains, none of the results were significantly higher than unstimulated neutrophil supernatants (*Figure 4.7*). Although small differences in mean supernatant PAD activity were seen between the four strains the 95% confidence intervals for each condition showed a great deal of overlap, with no significant difference between any of the strains being detected.

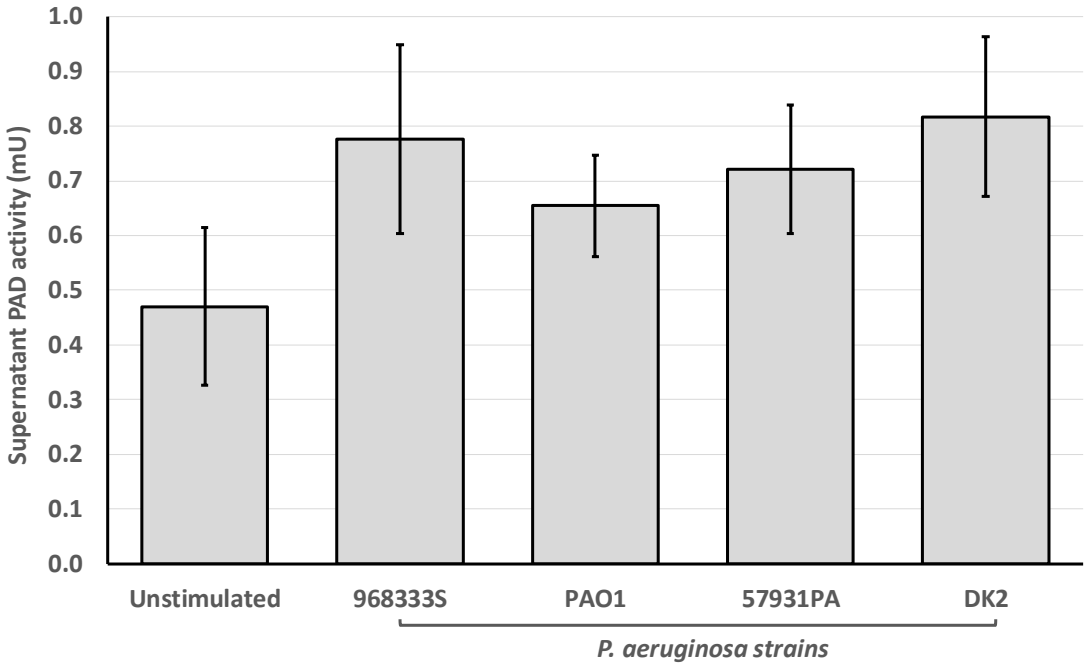

Figure 4.7 PAD activity in supernatants from neutrophils stimulated with whole cell lysates from a range of Pseudomonas aeruginosa strains. Stimulation with each of the 4 WCLs resulted in an increase in mean PAD activity. No significant differences were detected in supernatant PAD activity between the various P. aeruginosa WCLs (n=5, +/- 95% CI).

4.2.7 *Extracellular DNA concentration is higher in neutrophils stimulated with Bronchiectasis relevant PAMPs*

As extracellular DNA release is a key feature of NETosis, extracellular DNA release in response to incubation with the selected PAMPs/lysates was assessed by measuring emitted fluorescence when incubated with SYTOX (a DNA binding fluorescent dye). To validate the assay two experiments were carried out. Firstly, increasing numbers of neutrophils (ranging from 10,000 to 250,000) were incubated for four hours before the addition of SYTOX. As seen in *Fig 4.8* there was a strong positive correlation (Pearson correlation = 0.951, Spearman Rho correlation = 0.962), which was statistically significant ($p < 0.001$). This supports the conclusion that any detected fluorescence signal within the SYTOX treated wells is due to the presence of neutrophils rather than other contaminates or variables.

A second validation experiment was also carried out to confirm the fluorescence detected was due to the SYTOX binding DNA rather than any other present nucleic acids (e.g. RNA). To confirm this, neutrophils were incubated for 4 hours in the absence or presence of LPS (1µg/ml). After this incubation period DNase I (RNase free) or media was added to each of the conditions. *Fig 4.9* shows the fluorescence values for the various experimental conditions in relation to neutrophils untreated by LPS and DNase I (which is set as the baseline value of 100%). In each of the DNase I treated samples a large drop in fluorescence was seen, with the LPS treated neutrophil showing a significant decrease in fluorescence when treated with DNase I. This confirms that the fluorescence detected within the wells containing neutrophils is largely due to the presence of DNA rather than other nucleic acids not implicated in NETosis.

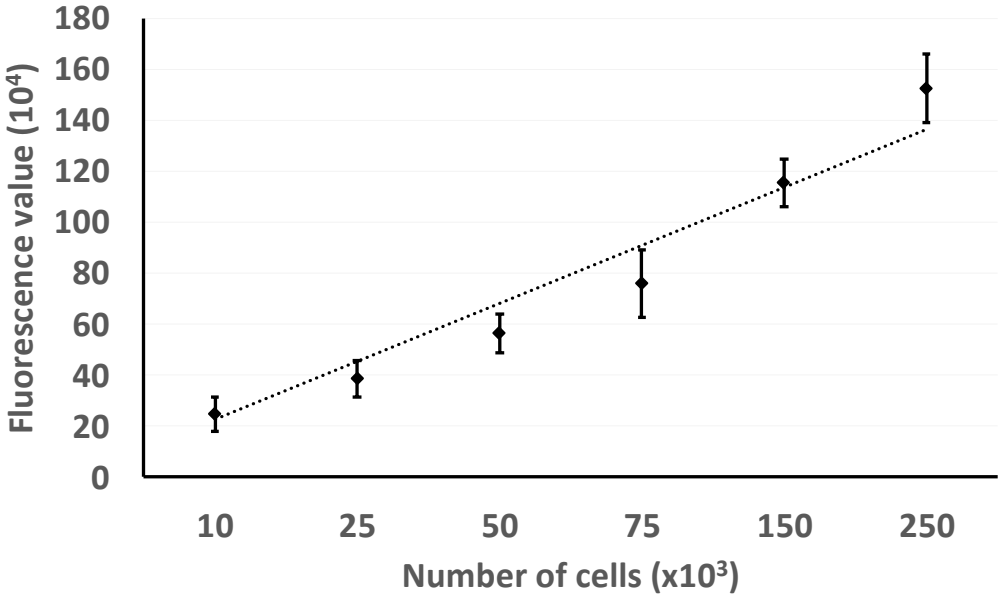

Figure 4.8 Correlation between the number of neutrophils and SYTOX dependent fluorescence. A strong positive correlation is clearly visible with a Pearson correlation value of 0.951 detected. This correlation was confirmed to be statistically significant ($p < 0.001$, n=7, +/- 95% CI).

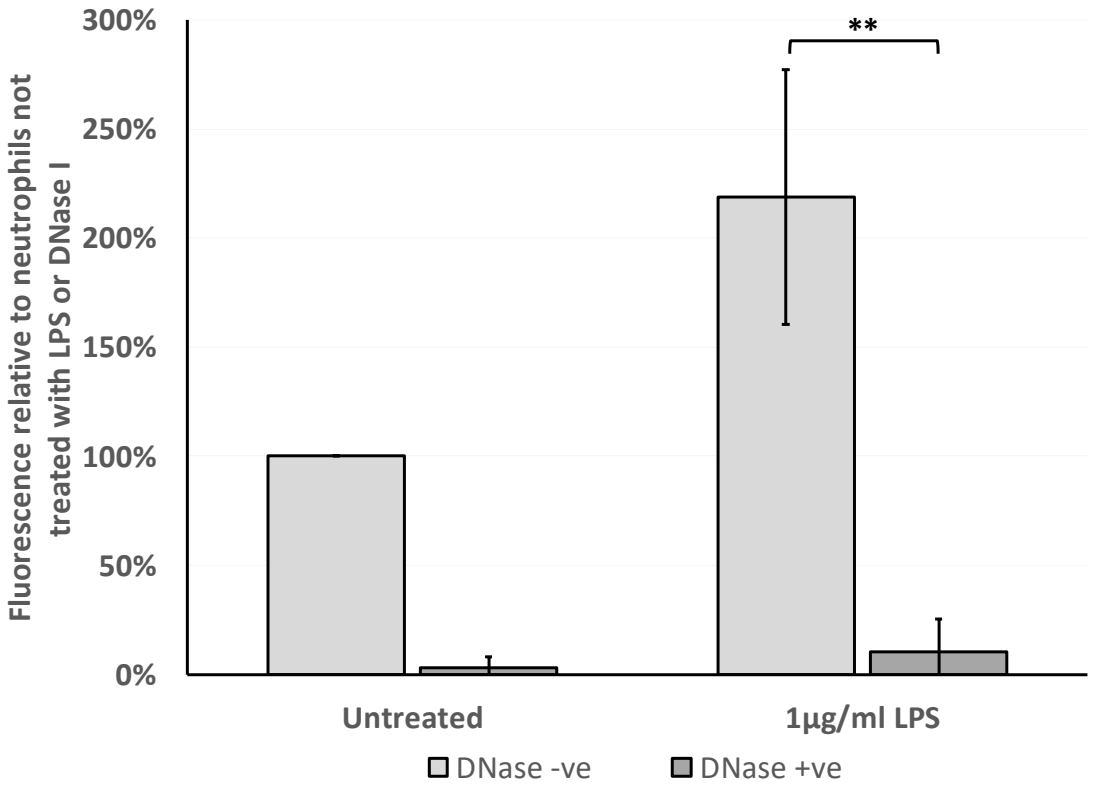

Figure 4.9 Fluorescence of untreated and LPS stimulated neutrophils in the absence and presence of DNase I. SYTOX dependent fluorescence was measured using neutrophils untreated by DNase I or LPS as a baseline 100% value (with 0% representing the fluorescence value recorded for the blank condition). Treatments with DNase I resulted in a decrease in observed fluorescence (for both unstimulated and LPS stimulated neutrophils). The decrease in fluorescence seen between the "LPS+ve DNase-ve" and "LPS+ve DNase+ve" treated samples was shown to be statistically significant (n=5, 95% CI ** $p < 0.01$)

Once the SYTOX based assay was validated, the amount of extracellular DNA released was determined by measuring the SYTOX dependent fluorescence in each of the conditions, with the fluorescence value of untreated neutrophils being assigned as the baseline (i.e. 100%) value. The relative fluorescence for PMA and LPS were the only conditions with a significant increase ($p < 0.001$ and $p < 0.01$ respectively). There was no difference between the baseline unstimulated and fMLP in fluorescence. TNF-α showed a slight decrease in fluorescence compared to untreated neutrophils, however this was not statistically significant (*Fig 4.10*). These results suggest that of the four stimuli only PMA and LPS cause an increase in extracellular DNA, which corresponds with the previous results of immunofluorescent NET counts. TNF-α however should no increase in extracellular DNA, whereas NET counts suggested an increase in NETosis rates in neutrophil stimulated by the molecule (*Fig 4.4*).

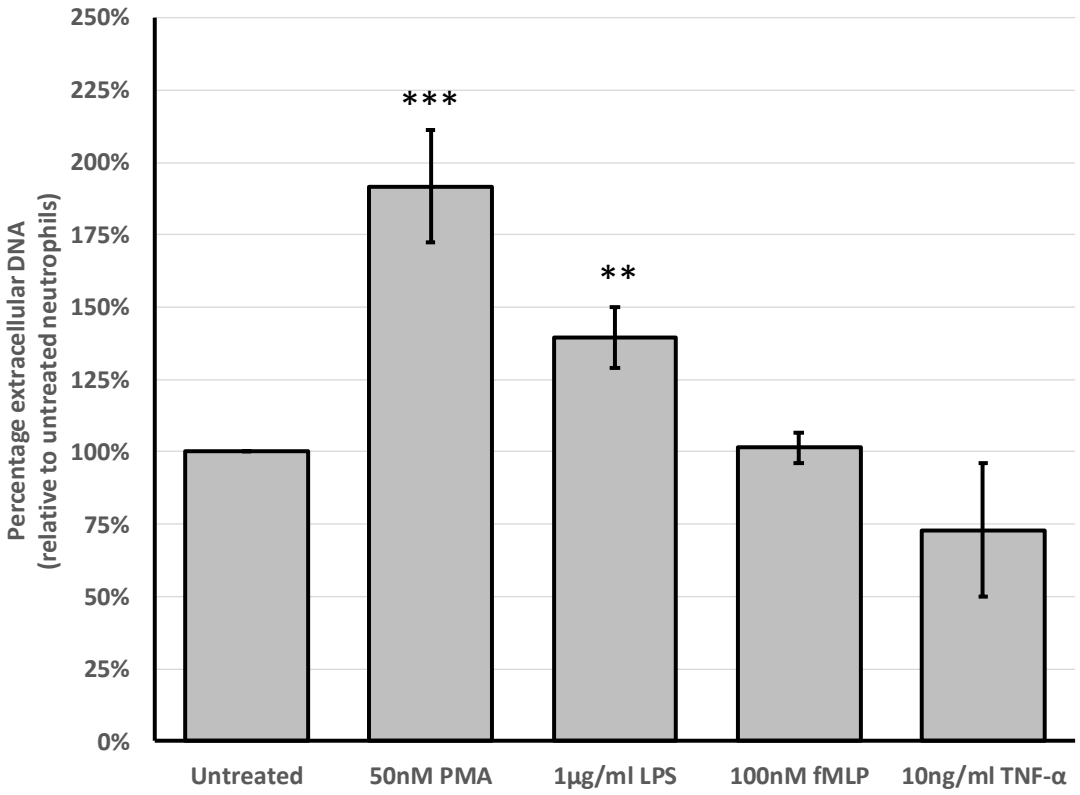

Figure 4.10 Extracellular DNA release in response to various bronchiectasis and rheumatoid arthritis stimuli. PMA and LPS both showed a significant increase in relative fluorescence, suggesting higher extracellular DNA release. No significant change in fluorescence was seen in neutrophils treated with fMLP and TNF-α (n=6 +/- 95% CI, ** $p < 0.01$, *** $p < 0.001$).

4.2.8 *Increased NETs in supernatants of neutrophils treated with LPS and PMA*

Supernatants from neutrophils incubated in the absence or presence of the various bronchiectasis and rheumatoid arthritis stimuli were assessed for the presence of NETs using a histone-elastase complex ELISA assay. As there is no agreed method for quantifying the number of NETs within a soluble sample, the samples tested by the ELISA were semi-quantified using a biological standard confirmed to contain high amounts of NETs (provided and confirmed by Prof. James Chalmers, Dundee University, UK). This allowed each sample to be assigned an arbitrary unit (in relation to the biological standard) allowing comparison between samples ran on different plates.

All tested stimulatory conditions showed some increase in the amount of NETs detected within the supernatant (*Fig 4.11*). Only PMA, LPS and TNF-α had a significant increase in comparison to untreated neutrophils ($p < 0.001$, $p < 0.001$ and $p < 0.05$ respectively). This suggests that there are a greater number of non-adherent NETs present in neutrophil culture mediums treated with these samples, which may also be an indicator of the greater rate of NETosis occurring in the neutrophil population in response to these stimuli.

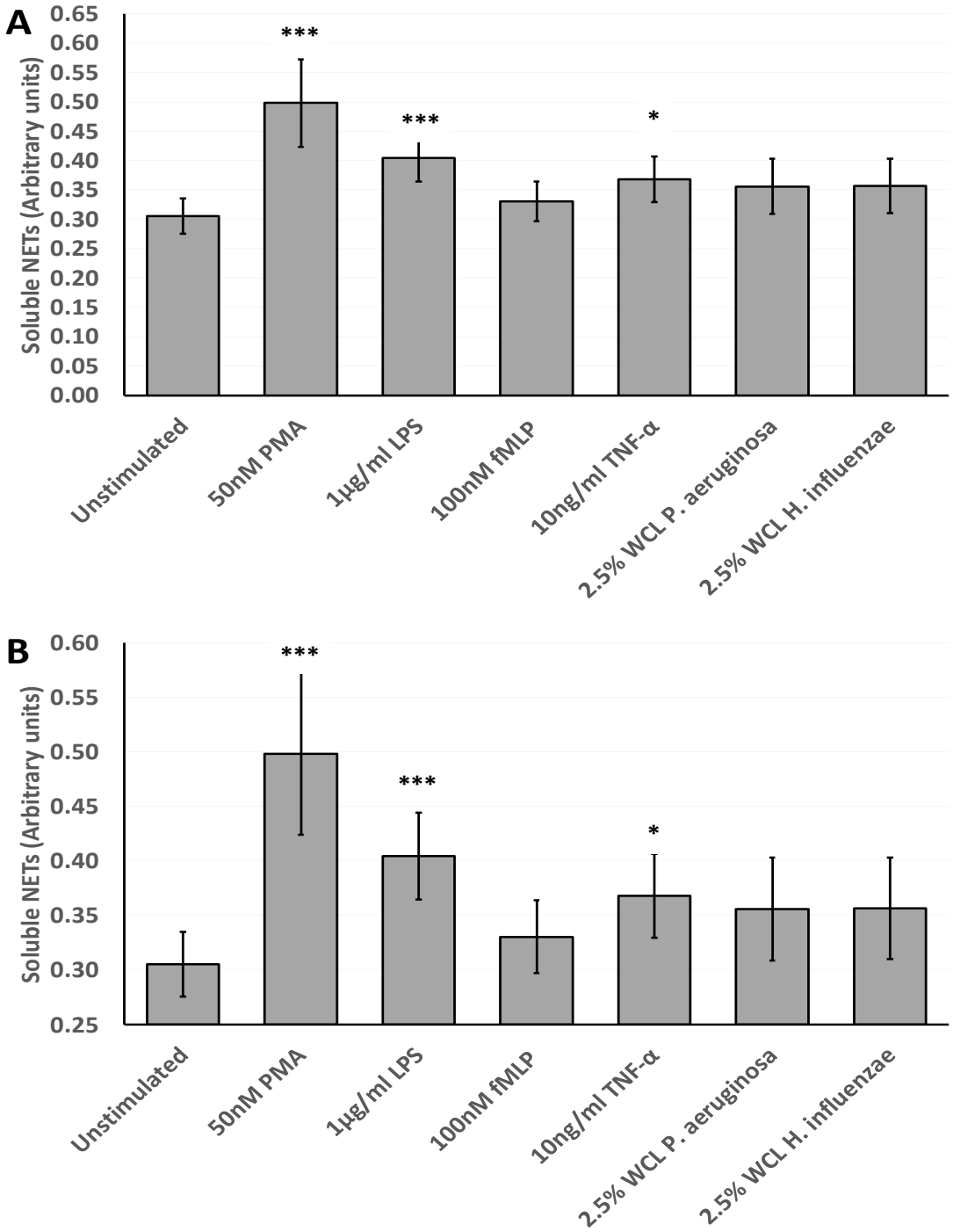

Figure 4.11 NETs present in supernatants of stimulated and unstimulated peripheral blood neutrophils. (A) The relative amount of NETs in the supernatants was elevated in all conditions, however only PMA, LPS and TNF-α were significantly higher than unstimulated neutrophil supernatants. Graph B shows the same data set as A, only with the y-axis set to a baseline value of 0.25 in order to more clearly demonstrate the differences between the conditions and little overlap of error bars (n=16 +/- 95% CI, * $p < 0.05$, *** $p < 0.001$).

4.2.9 *Elastase concentration and activity is elevated in stimulated neutrophils*

Neutrophil elastase is a key component of the NETosis pathway and is commonly present within the structure of fully formed NETs (Papayannopoulos et al 2010). Neutrophil elastase concentration and activity was measured in neutrophil culture supernatants to serve as an additional marker for the presence of NETs in supernatants.

The concentration of free elastase (i.e. elastase free in neutrophil supernatant and not bound to formed NETs) was measured using a "Human PMN Elastase ELISA Kit" (Abcam: ab119553). An increase in free elastase concentration was seen in all of the stimulatory conditions, with at least a 4-fold increase (compared to unstimulated) in free elastase being detected for all conditions (*Fig 4.12*). Despite this increase for all conditions, only PMA, TNF-α, *P. aeruginosa* and *H. influenzae* treatment resulted in a significant increase in free elastase concentration in neutrophil supernatants.

Elastase activity in neutrophil supernatants was assessed using an "in house" assay protocol developed by Prof. James Chalmers (Dundee University). Elastase activity determined using the protocol produced results which conflicted with the general increase in supernatant elastase concentration (*Fig 4.13*). Stimulation with LPS, fMLP, WCLs *P. aeruginosa* and WCLs *H. influenzae* resulted in a 2-3 fold increase in supernatant elastase activity compared to unstimulated (with only LPS resulting in a significant increase in elastase activity). PMA and TNF-α treated neutrophils showed a greater increase in supernatant elastase activity (8-fold increase), both of which were statistically significant. In general, the assay results showed wide confidence intervals.

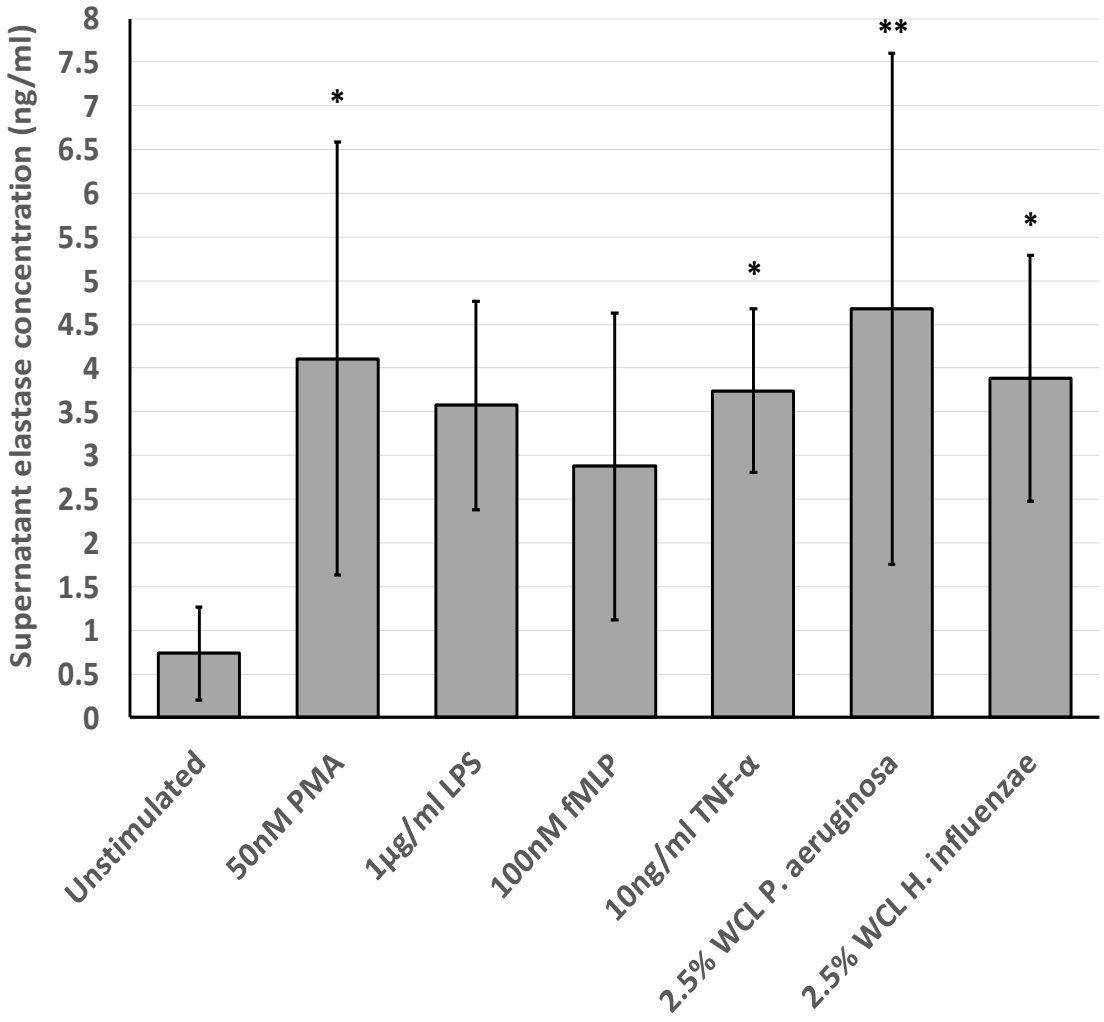

Figure 4.12 Neutrophil free elastase concentration in the supernatants of stimulated neutrophils. All conditions showed an increase in supernatant free elastase concentration in comparison to unstimulated neutrophils. PMA, TNF-A, P. aeruginosa and H. influenzae WCLs all caused a significant increase in extracellular elastase concentration (n=7 95% CIs, * $p < 0.05$, ** $p < 0.01$).

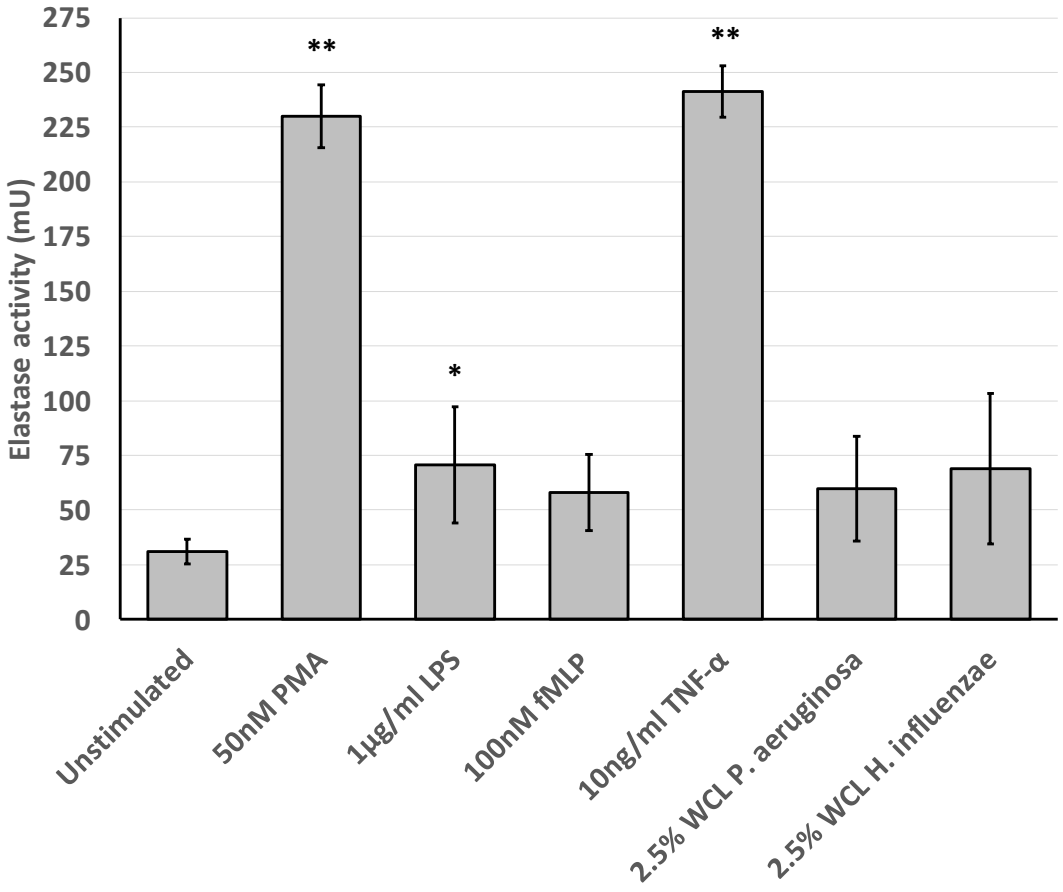

Figure 4.13 Elastase activity in *in vitro* neutrophil supernatants. All conditions resulted in some increase in mean supernatant elastase activity in comparison to unstimulated neutrophils. Only LPS, PMA and TNF-α resulted in a statistically significant increase in elastase activity (n=7 95% CIs, * $p < 0.05$, ** $p < 0.01$).

4.2.10 *Summary*

The bronchiectasis and rheumatoid arthritis relevant stimuli were confirmed to be biologically activate when applied to isolated peripheral blood neutrophils *in vitro.* Immunofluorescence (IF) counts of adherent NETs suggested that PMA, LPS, TNF-α and WCL of *P. aeruginosa* all caused a significant increase in the presence of adherent NETs *in vitro*. This pattern of results was reflected in the relative number of non-adherent NETs (NET ELISA results) in neutrophil culture supernatants, however the number of soluble NETs in WCL of *P. aeruginosa* treated neutrophils was not significantly higher.

Extracellular DNA measurements of selected stimulatory conditions (PMA, LPS, fMLP and TNF-α) suggested PMA caused the highest increase in extracellular DNA, followed by LPS, which is similar to the results for IF counts of adherent NETs and the measurements of soluble NETs. Contradictory to the results in the other NET measurement assays there was no observed increase in extracellular DNA for TNF-α stimulated neutrophils.

Assays of neutrophil elastase, as a marker for NETosis produced conflicting results. Supernatant elastase concentration was increased for all conditions; however activity was only greatly elevated in supernatants of neutrophils incubated with PMA and TNF-α.

Supernatants of PMA, LPS, TNF-α and WCL of *P. aeruginosa* treated neutrophils all showed a significant increase in the amount of PAD enzyme activity, which parallels the results seen for the IF adherent NET counts and soluble NET measurements. Unexpectedly there was no significant increase in PAD4 supernatant concentration seen in these or any other stimulatory conditions.

4.3 Discussion

4.3.1 *NETosis rates in stimulated neutrophils*

Of all tested stimuli, PMA consistently resulted in the highest rates of NETosis (IF NET counts, supernatant NET ELISA, extracellular DNA detection). Given this molecules widespread use as a positive control for inducing NETosis this was expected and supports the assays used are successfully measuring NETosis. IF NET stains of neutrophils stimulated with 50nM PMA for four hours resulted in a mean NETosis rate of 52.7% (95% CI: 45.7%, 59.7%), which was significantly higher than unstimulated neutrophil NETosis rates. Similar work by Brinkmann et al (2013) reported NETosis rates in two human donor samples (determined by IF staining) of 40% and 80% when incubated with 50nM PMA, however this is of limited use for comparison given the low sample size and difference in incubation time (6 hours as opposed to 4 hours). Contradictory to our results, Gupta et al (2014) reported a NETosis rate (also using IF microscopy) of near 100% within 3 hours when stimulating human neutrophils with 50nM of PMA. This large difference between results highlights the difficulty of comparing published data on NETosis rates.

The similar results presented in this chapter for IF staining of adherent NETs and the NET ELISA used to assess neutrophil supernatant implies that of the tested stimuli (at the concentrations used) PMA, LPS and TNF-α all resulted in increased numbers of NETs formed *in vitro.* These data confirm previously published studies showing these molecules as inducers of NETosis (Fuchs et al 2007). Whilst extracellular DNA was higher in PMA and LPS, no increase was seen in TNF-α however this could be attributed to the lower specifictiy (comparative to immunofluorescence and ELISA) of the assay for detecting NET dependent increases in extracellular DNA concentration *in vitro*.

Incubation of healthy neutrophils with WCLs of *P. aeruginosa* also resulted in a significant increase in adherent NET IF microscopy counts (but no significant increase in the NET ELISA results). As IF microscopy counts are a fully quantitative method of measuring NETosis (as opposed to the semi-quantitative method used in ELISA analysis) that is well established in the literature, this is still ample evidence to suggest *P. aeruginosa* lysates may trigger NETosis. Previously the literature has only reported the ability of live *P. aeruginosa* bacteria to trigger NETosis (Guimarães-Costa et al. 2012), the

results in this thesis suggest that even once *P. aeruginosa* is killed and heavily degraded (lysis and proteolytic degradation leaving a LPS rich fraction) the lysate solution can still induce NETosis *in vitro* at the given concentration (2.5% v/v).

Neutrophils stimulated by fMLP and WCLs of *H. influenzae* showed no significant difference in the number of detected NETs compared to unstimulated neutrophils. As fMLP is a simple chemokine that recruits neutrophils towards a site of infection then it is expected the molecule would have little (or no) impact on NETosis rates, as activation of NETosis would theoretically be most beneficial after reaching the recruitment site (where other molecules, e.g. LPS, will be present). WCLs of *H. influenzae* could be expected to induce NETosis given that live *H. influenzae* have been reported to cause NET formation (Guimarães-Costa et al. 2012) and as WCLs of *P. aeruginosa* was shown to promote NETosis in these results. A potential explanation of the difference between the two WCLs could be the content within the lysates. It is possible the *P. aeruginosa* WCLs contain higher relative amounts of a given immunostimulatory molecule which is resistant to degradation (e.g. LPS) than the *H. influenzae* WCLs.

4.3.2 *Use of elastase as a measure of NETosis*

The use of neutrophil elastase activity/concentration was initially planned to be used in a similar manner to that of extracellular DNA release (i.e. as a secondary assessment of NETosis to confirm results using the more accurate/valid protocols of IF NET counts and NET ELISA). Although the commercial ELISA kit used to assess elastase concentration is well validated, the in-house activity assay is not widely used. Furthermore it was found that the reagents used in the assay (which changes absorbance when cleaved) was also reactive to other neutrophil derived proteases (e.g. cathepsin G), meaning the assay is likely less an indicator of elastase activity but rather broad neutrophil protease activity.

Furthermore, neutrophil elastase release is a commonly seen occurrence in neutrophil degranulation in vitro in response to stimulation as a host defence mechanism, meaning the validity of using this molecule to assess NETosis rates in vitro comes under great scrutiny. Therefore, the results collected in relation to elastase concentration and activity should be interpreted with caution as a marker of NETosis (supported by the clear differences in elastase concentration/activity in comparison to measure NET rates show in this chapter).

4.3.3 *PAD mediated citrullination in response to disease relevant stimuli*

Supernatant PAD activity was significantly higher following stimulation with PMA, LPS, TNF-α and WCL *P. aeruginosa*, which were also indicated to promote NETosis *in vitro*. As citrullination is a central feature to NETosis, and as NETosis results in the release of intracellular contents (e.g. possibly including PAD enzymes), this increase in PAD activity in the enzyme activity could be a result of increased NETosis rather than secretion of activated PAD enzymes. As there is limited information available, whether this increase in extracellular PAD activity would have any substantial impact on the modification of self-antigens cannot be determined. However, the increase in extracellular PAD activity in response to these stimuli does suggest that bronchiectasis disease relevant stimuli may support the production of a chronic pro-citrullination environment.

Unexpectedly the PAD4 detected by ELISA in supernatants did not show a similar result with no significant increase in PAD4 concentration noted. This could be explained by the given stimulatory conditions (PMA, LPS, TNF-α and WCL *P. aeruginosa*) resulting in greater activation of PAD4 (e.g. by protein-protein interaction) which increased activity prior to release by NETosis. This is potentially feasible given publications reporting a 10,000-fold increase in PAD4 activity when activated by Ca^{2+} (Knuckley et al. 2011). However, a paper by Zhou et al (2017) suggested that whilst neutrophil supernatants showed PAD activity (confirmed by citrullination of extracellular histones and fibrinogen) it was PAD2 that was predominantly secreted to the extracellular medium and not PAD4. This means the increase in PAD activity for the supernatant samples seen in this chapter could be the result of increased release of PAD2.

Whilst this would explain the presence of citrullination in the absence of any change in PAD4 concentration, there is still some question as to why PAD4 was detected in supernatants from unstimulated neutrophils (which had comparatively low NETosis rates). Zhou et al (2017) also reported that biologically active PAD4 is expressed on the surface of neutrophils (which they confirmed was not the result of neutrophils binding secreted soluble PAD4) and that the enzyme may be released following cell death. As the supernatants studied in this chapter were cell free this rules out the potential for cell membrane anchored PAD4 to be the cause of the observed increased PAD activity. It is possible however, that the isolation procedure may result in the activation/death of a

proportion of neutrophils releasing PAD4 to the extracellular medium (explaining the detected PAD4 in all neutrophil supernatants).

Although there was no other assay used to establish citrullination rates, due to the difficulty in precisely measuring citrullination in biological samples, it is possible that the IF staining results could also serve as an indicator of citrullination activity. As one of the stains used in the IF staining was targeted against citrullinated-histone all counts of NET+ve structures were neutrophils with citrullinated histones. Therefore, the increase in NETosis rates for PMA, LPS, TNF-α and WCL *P. aeruginosa* treated neutrophils could be viewed as an indicator of increased intracellular histone citrullination. This parallels the results seen for supernatant PAD activity and suggests that in addition to increasing extracellular PAD activity the stimuli may also increase intracellular PAD activity (i.e. primarily histone citrullination).

4.3.4 *No difference in PAD activity following stimulation by differing strains of P. aeruginosa*

The results suggested that when neutrophils were stimulated with WCLs generated from four separate strains of *P. aeruginosa* there was a consistent increase in extracellular PAD activity between the strains, but no difference in PAD activity elicited across the strains used. Given the WCLs were created by lysis followed by destruction of bacterial proteins/DNA this is to be expected as there may theoretically be very little separating the WCLs of the four strains in terms of content. Therefore, this result cannot conclusively show there is no difference in response of neutrophils (in relation to citrullination) to the differing bacterial strains. To establish this, further study would likely need to be carried out involving the incubation of isolated neutrophils with viable bacterial strains rather than WCLs (or sonicated but undigested bacteria).

4.3.5 *Differences in response to stimuli*

Throughout this chapter there was a consistent trend of the stimuli producing differing levels of responses in relation to NETosis and citrullination. i.e. PMA producing the largest increase, followed by LPS and with the other stimuli (TNF-α and WCLs of *P. aeruginosa)* following. This raises a question of why there is such a consistent difference in the responses to the different stimuli. It is likely this is a combination of the biochemical properties of each individual stimulus in relation to neutrophil function and

a dose-response effect (i.e. Increased dosage of LPS may produce results similar to 50nM PMA), which was not assessed in this body of work.

4.3.6 *Conclusion*

Collectively the results presented in this chapter suggest that at the tested concentrations; PMA, LPS. TNF-α and WCL *P. aeruginosa* all caused an increase in NETosis rates and PAD activity in the extracellular environment. These stimuli are either known to be present in the bronchiectasis airway (TNF-α, LPS and Pseudomonas) or activate pathways biologically plausible as involved in bronchiectasis (PMA) This supports the hypothesis underpinning this work, which suggests that infection/inflammatory stimuli commonly seen in bronchiectasis promote NETosis and may help establish a pro-citrullination extracellular environment.

Chapter 5: Assessing the LPS signalling pathway and its role in neutrophil citrullination and NETosis

5.1 Introduction

5.1.1 *Overview*

The previous chapter showed that a wide variety of infection/inflammation related molecules promote NET formation and citrullination *in* vitro. These include PMA, LPS and TNF-α which have all been previously suggested to be inducers of NETosis (Remijsen et al. 2011, Douda et al. 2015, Gupta et al 2014, Guimarães-Costa et al. 2012). A large amount of published work attempting to determine the signalling mechanisms linking stimulation by these molecules to NETosis and/or citrullination has focused on PMA.

As PMA is a protein kinase C (PKC) activator and a potent inducer of NETosis, along with observations that chronic granulomatous disease patients whom have faulty NADPH oxidase pathway function (which is activated by PKC via the MAPK-ERK pathway) cannot form NETs in response to PMA, it has become gradually accepted that PKC and NADPH oxidase plays a central role in NETosis (Branzk et al 2013, Fuchs et al 2007). A paper by Neeli et al (2013) examined the relationship further, suggesting that the PKCζ isoform was specifically associated with increased histone citrullination and NETosis when stimulated. Given the broad effects of PKCζ activation of a variety on cell function, including NADPH oxidase formation (Dang et al 2001), it is plausible that the isoform may also promote processes such as histone citrullination and NETosis.

Whilst the research has focused on the PMA pathway in citrullination and NETosis, an unaddressed aspect of the research is the molecular mechanism by which the other stimuli shown to induce citrullination and/or NETosis (e.g. LPS and TNF-α). As both the LPS and TNF-α signalling pathways have been shown to lead into the MAPK-ERK pathway, and subsequently activate NADPH oxidase (*Figure 5.1*), it's plausible that this may be the route by which such a broad range of stimuli can promote citrullination and NETosis.

This chapter aimed to evaluate the LPS signalling pathway in relation to citrullination and NETosis, which despite its biological and clinical relevance has been understudied.

Peptide inhibitors of myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) were assayed for their impact on citrullination and NETosis rates from healthy volunteer peripheral blood neutrophils *in vitro*.

MyD88 and TRIF are both adaptor proteins playing a role in the first stages of the LPS signalling pathway. As shown in *figure 5.2,* the pathway is believed to divert largely at the first stage of the signalling cascade, with the TRIF dependent pathway leading into transcriptional changes by activation of IRF3 and the MyD88 dependent pathway leading to the activation of the MAPK-ERK pathway, with NFκB activation being mutual to both pathways.

This work will establish if there are any distinct differences in citrullination and NETosis between the two arms of the TLR4 signalling pathway, which will provide a foundation for future work examining the TLR4 signalling pathway in citrullination/NETosis. As the MyD88 pathway is believed to involve the activation of the MAPK-ERK pathway (and NADPH oxidase) it is expected that inhibition of MyD88 will significantly impact citrullination/NETosis. Given our current understanding of the LPS signalling there is no reason supported by published literature to suspect that TRIF inhibition will have any impact on citrullination/NETosis.

5.1.2 Hypothesis

Inhibition of MyD88 function in neutrophils will significantly reduce NETosis rates and/or PAD activity, whereas TRIF inhibition will have no impact on either feature.

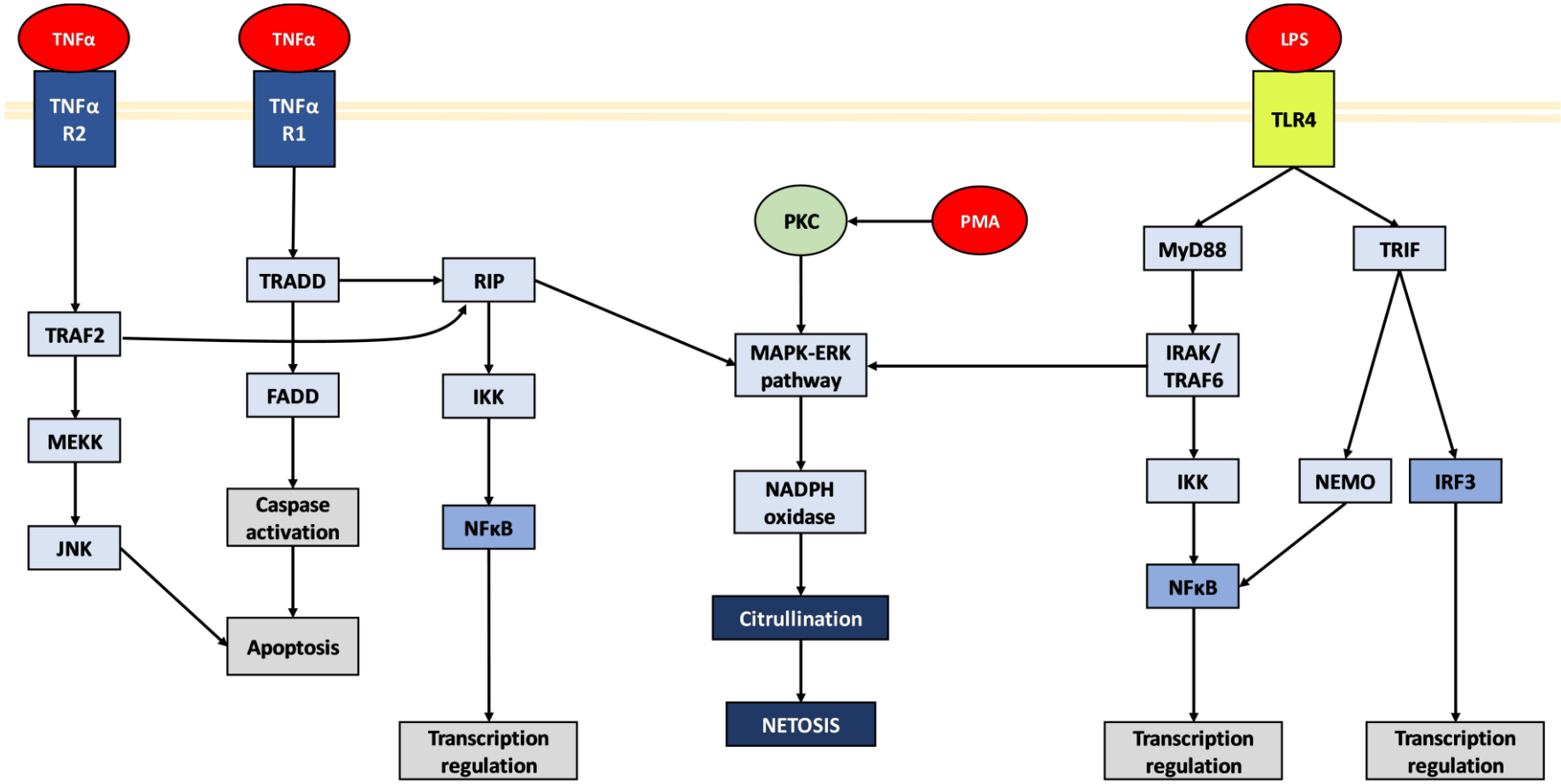

Figure 5.1 Schematic overview of proposed pathways connecting TNF-α and LPS signalling to PAD activation and NETosis. Both the TNF-α and LPS signalling pathway lead into activation of the MAP/ERK pathway, which subsequently activates protein kinase C (the target of PMA and a component of NETosis). Figure produced using information provided by Guo et al (2010), Catteneo et al (2013), Faustman et al (2013), Cicha et al (2015) and Kiziltaş et al (2016).

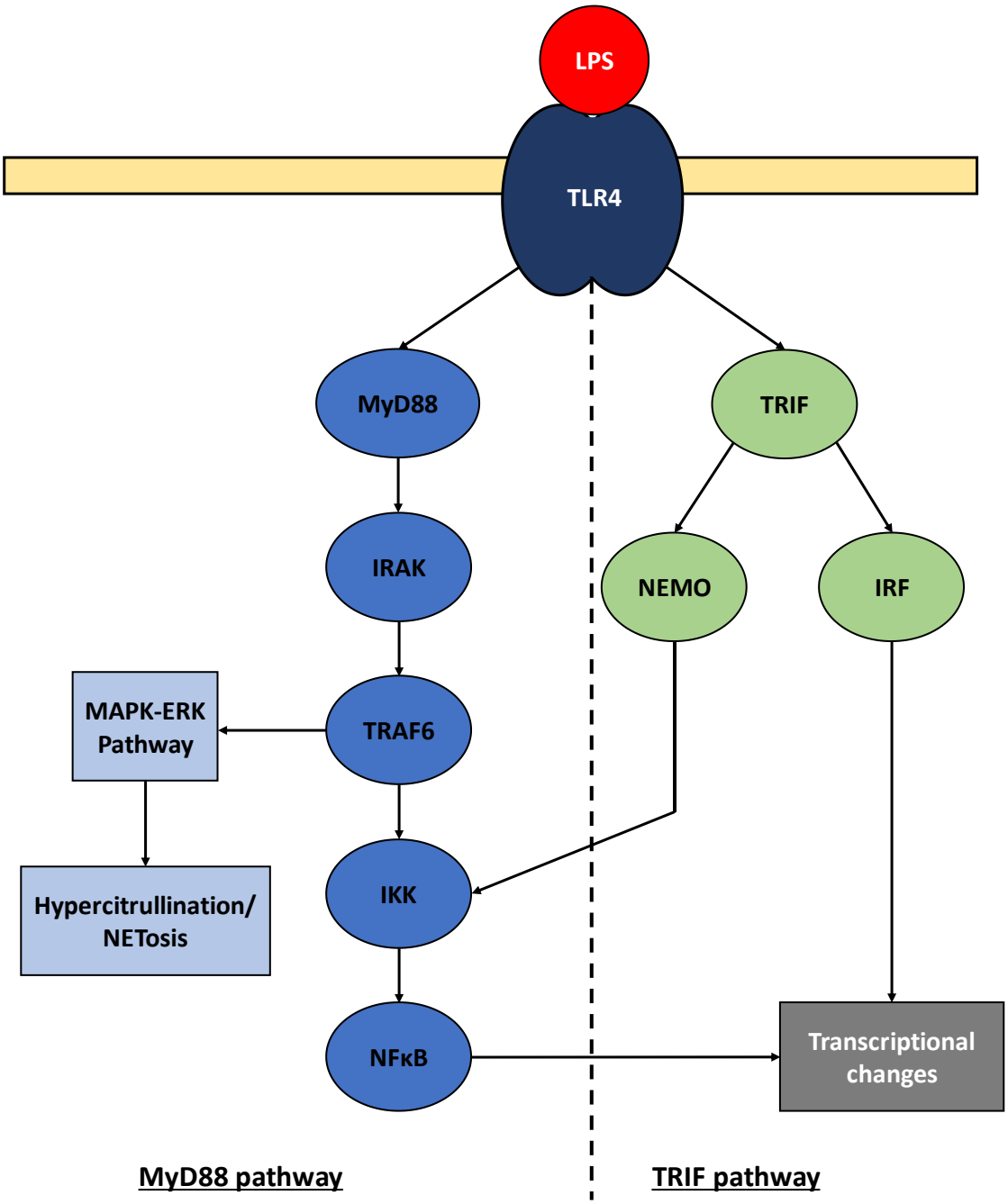

Figure 5.2 Separation of LPS signalling into MyD88 and TRIF dependent pathways. Following binding of LPS to the toll like receptor 4 (TLR4), the adaptor proteins MyD88 and TRIF are both activated. This leads to two separate signalling cascades one dependent of MyD88 signalling and one dependent of TRIF. Notable differences between the two include the activation of the MAPK-ERK pathway (MyD88 dependent), which may be implicated in hypercitrullination and NETosis (via activation of NADPH oxidase), and the activation of the transcriptional factor IRF (TRIF dependent). There is some expected crossover of the pathways with both pathways leading to the activation of the broad targeting transcriptional factor NFκB (adapted from Guo et al 2010, Piras et al 2014 and Kiziltaş et al 2016).

5.2 Results

5.2.1 *TRIF and MyD88 inhibitors on neutrophil viability*

A broad concentration range of two peptide inhibitors of TRIF and MyD88 (TRIF-pep and MyD88-pep) were selected and assayed for their impact on neutrophil viability after 7 hours incubation. 7 hours was selected as an incubation time as all experiments for this work would involve 3 hours pre-treatment with TRIF-pep/MyD88-pep followed by 4 hours stimulation with LPS.

The results for TRIF-pep treated neutrophils suggest that neutrophil viability only showed a significant change in comparison to untreated neutrophils, when concentration reached 40µM or higher (*Figure 5.3*). There was an observed decrease in mean viability for neutrophils incubated with 35µM TRIF-pep, however this was not shown to be statistically significant. As the sample size for this experiment was limited it is possible that with a higher number of samples 35µM TRIF-pep may impact viability, because of this 30µM TRIF-pep was selected as the concentration for all experiments involving TRIF inhibition.

MyD88-pep treated neutrophils showed a significant decrease in mean viability at 75µM and 100µM concentrations (*Fig 5.4*). Neutrophil viability appeared unaffected when incubated for 7 hours with 50µM MyD88-pep, for this reason this concentration was selected for all experiments requiring inhibition of MyD88.

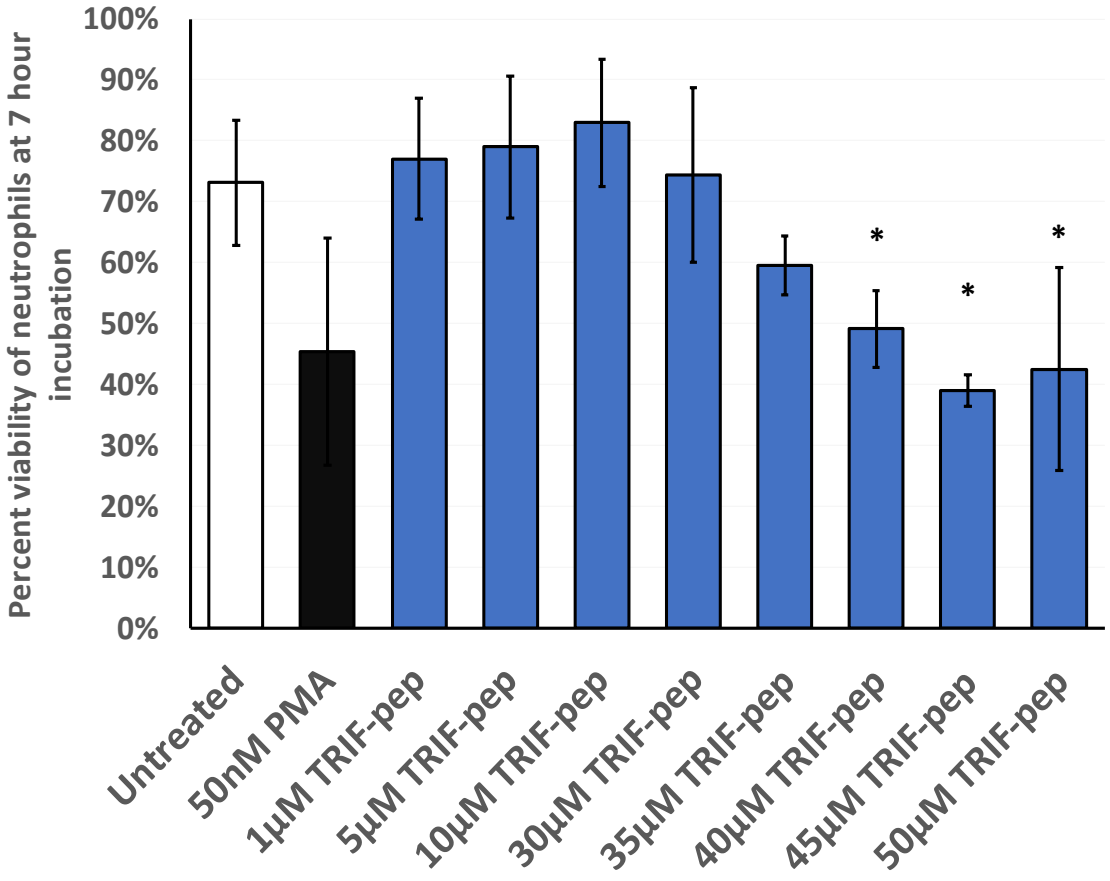

Figure 5.3 The impact of various TRIF-pep concentrations on neutrophil viability. Viability for neutrophils treated with a range of TRIF-pep concentrations and 50nM PMA (as a positive control) was assessed after 7 hours incubation. Viability significantly decreased at concentrations exceeding 40µM. A decrease in mean viability was observed at 35µM TRIF-pep, however this was not significant (n=4, * $p < 0.05$).

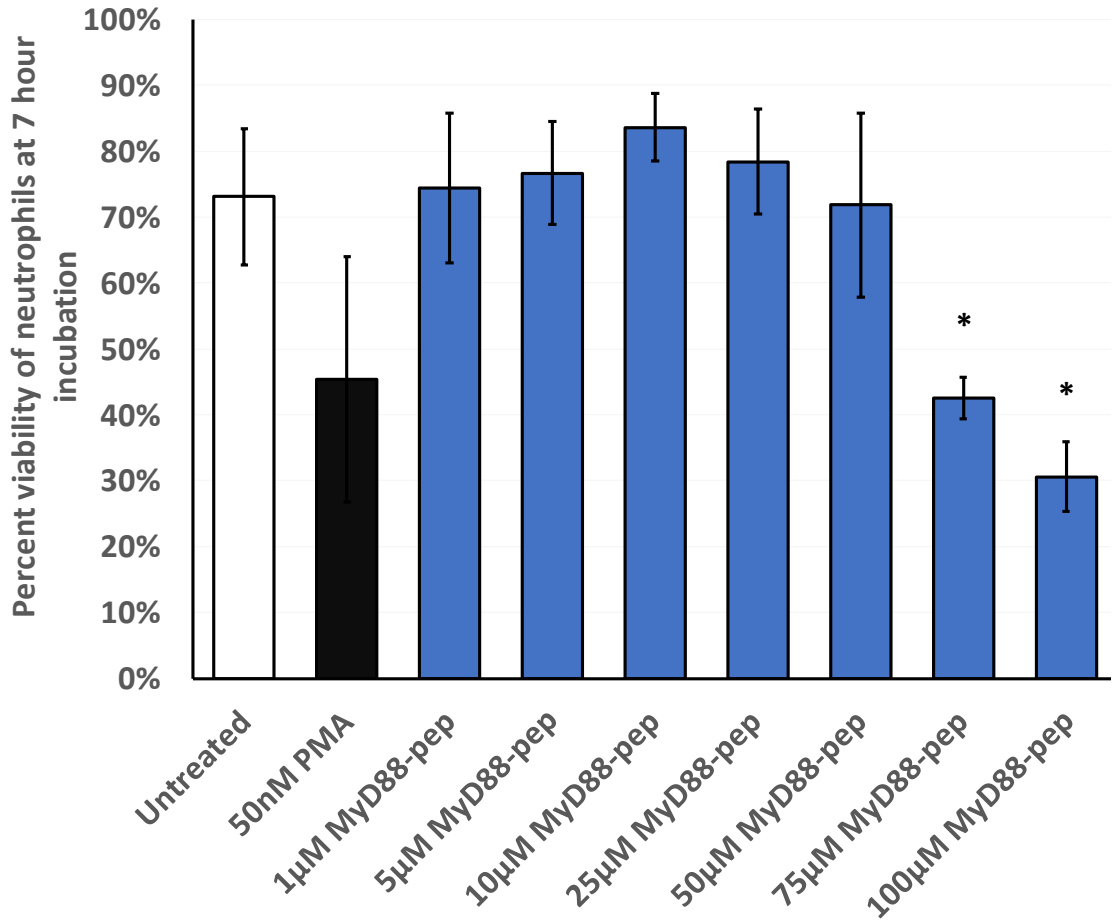

Figure 5.4 The impact of various MyD88-pep concentrations on neutrophil viability. Viability for neutrophils treated with a range of MyD88-pep concentrations and 50nM PMA (as a positive control) was assessed after 7 hours incubation. 75μM and 100μM MyD88-pep resulted in a significant decrease in neutrophil viability (n=4, * $p < 0.05$).

5.2.2 *Selected TRIF-pep and MyD88-pep concentrations inhibit neutrophil activation by LPS*

In order to confirm the TRIF-pep and MyD88-pep were active the ability of the inhibitors to successfully limit LPS stimulation of neutrophils was assayed. As NFκB activation occurs in both the MyD88 and TRIF pathway, and given CXCL8 production/secretion is dependent on activation of the transcription factor (Wang et al 2010), CXCL8 secretion was used as a marker of neutrophil activation by LPS.

Figure 5.5 shows the CXCL8 secretion of neutrophils either uninhibited or incubated with TRIF-pep or MyD88-pep prior to stimulation with LPS. The mean supernatant concentration of CXCL8 in uninhibited neutrophils was determined to 768.96pg/ml (95% CI: 550.28, 987.55). A significant decrease in supernatant CXCL8 concentration was seen for both TRIF-pep and MyD88-pep treated neutrophils, with means of 470.41pg/ml (95% CI: 263.63, 677.20) and 476.79pg/ml (95% CI: 162.92, 790.65) respectively. This shows CXCL8 secretion by neutrophils in response to LPS stimulation was significantly lower following incubation with the inhibitors, suggesting they are successfully inhibiting the signalling pathway as expected.

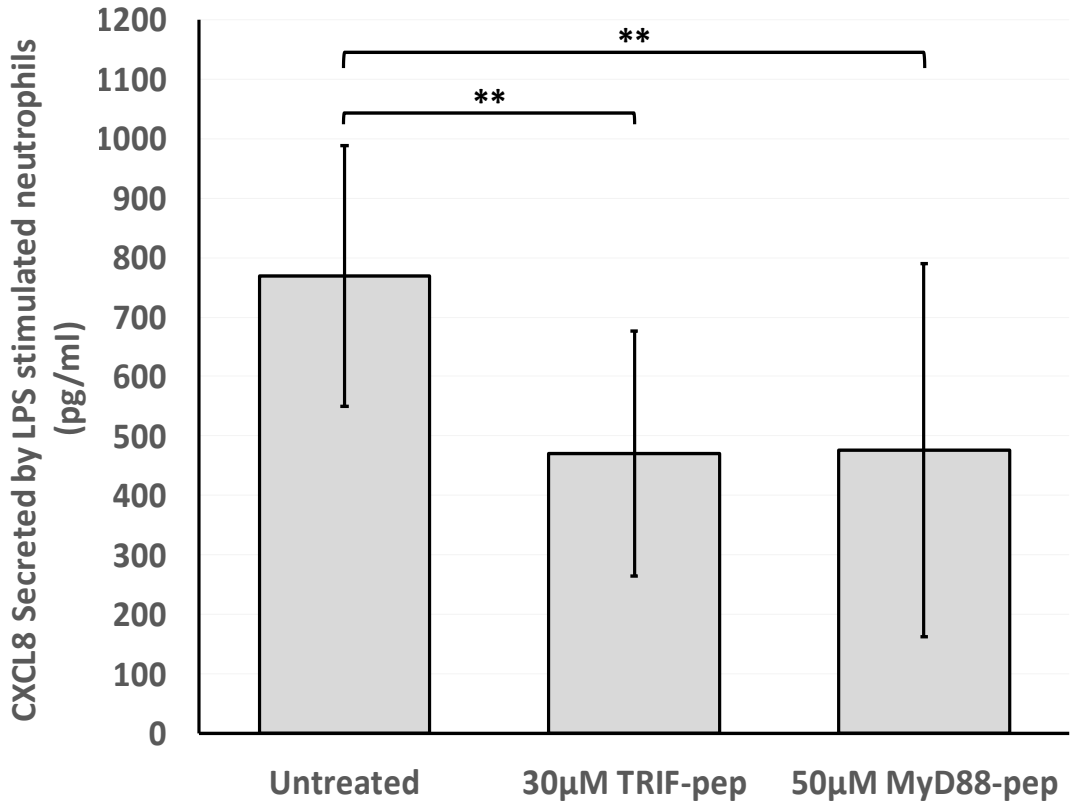

Figure 5.5 Supernatant CXCL8 concentration in TRIF-pep and MyD88-pep treated neutrophils. Significant decreases in supernatant CXCL8 concentration were observed for neutrophils inhibited with TRIF-pep and MyD88-pep prior to LPS stimulation (n=5, ** $p < 0.01$).

5.2.3 *Extracellular PAD activity unchanged by TRIF or MyD88 inhibition*

Supernatants of stimulated and inhibited neutrophils were assessed for extracellular PAD activity as in the previous chapter. Mean PAD activity in supernatants of uninhibited neutrophils stimulated with LPS (1.35mU) was higher than the supernatants of neutrophils incubated with TRIF-pep and MyD88-pep (0.98mU and 0.77mU respectively) (*Fig 5.6*).

Neither of the decreases in supernatant PAD activity when neutrophils were incubated with TRIF-pep or MyD88-pep were found to be statistically significant, however this could be attributed to the large spread of the data visible in the large 95% confidence intervals for each condition. From these results there is no conclusive impact of the inhibitors on extracellular PAD activity in neutrophils *in vitro*.

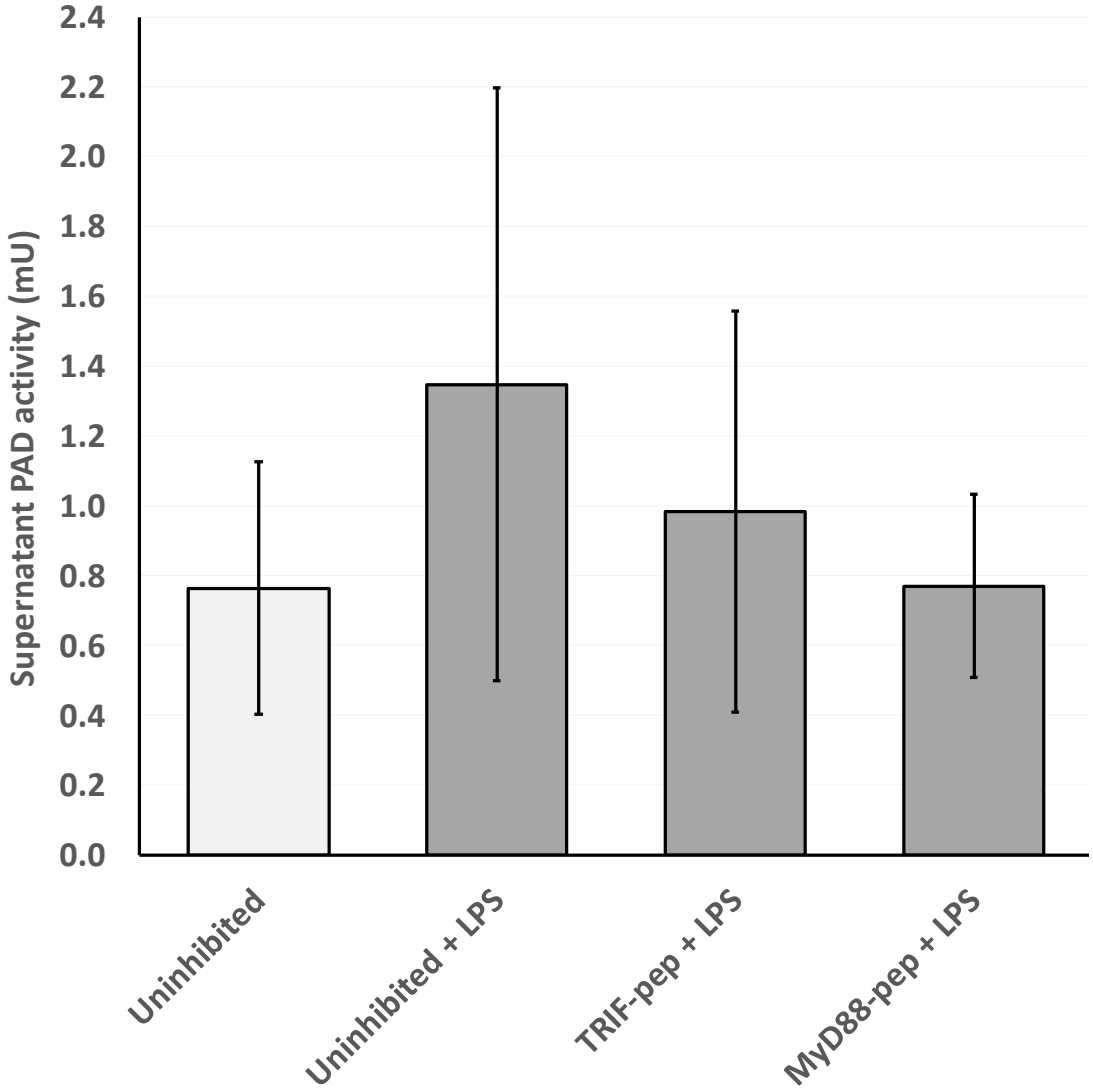

Figure 5.6 Extracellular PAD activity in neutrophils pre-treated with TRIF-pep and MyD88-pep. Although supernatant PAD activity appeared to increase in uninhibited neutrophils following LPS stimulation, and mean PAD activity decreased when LPS stimulated neutrophils were pre-treated with TRIF-pep and MyD88-pep, no statistically significant differences were found (n=8).

5.2.4 *inhibition of TRIF and MyD88 decreased extracellular DNA and supernatant NET concentration*

The effect of TRIF and MyD88 inhibition on *in vitro* NETosis rates was first examined using two assays described in the previous chapter; extracellular DNA release and NET ELISA measurements of non-adherent free NETs in neutrophil supernatants.

Extracellular DNA release (detected by SYTOX staining) was measured for all stimulatory/inhibitory conditions, with the SYTOX dependent fluorescence value of unstimulated and uninhibited neutrophils being used as the baseline (i.e. 100%) value for measuring extracellular DNA release in other conditions. *Figure 5.7* shows that relative extracellular DNA release for uninhibited neutrophils increased following LPS stimulation (to 276.29%, CI: 162.27%, 390.32%). LPS stimulated neutrophils pre-treated with TRIF-pep and MyD88-pep had a significant decrease in relative extracellular DNA (93.18, 108.93%), to similar levels of the baseline value seen in neutrophils not stimulated with LPS. These results suggest that inhibition of both TRIF and MyD88 signalling had a significant impact on extracellular DNA release and possibly NETosis rates in LPS stimulated neutrophils.

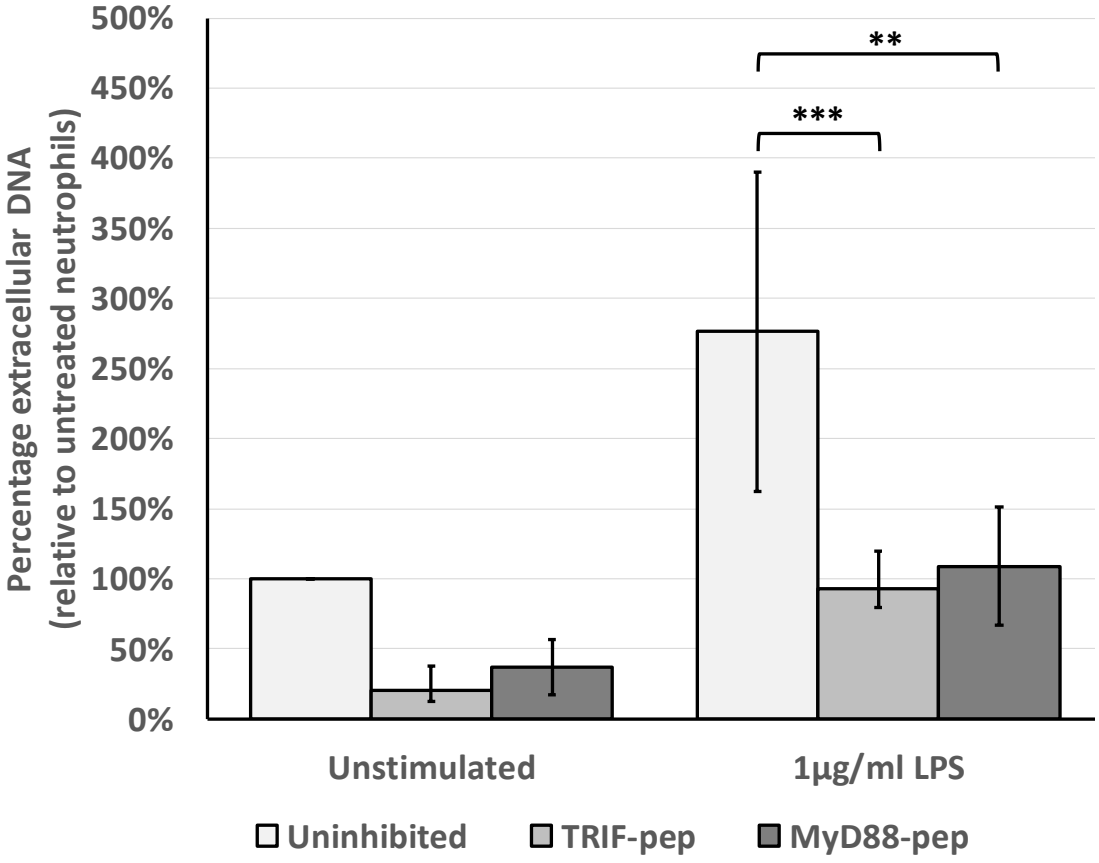

Figure 5.7 Extracellular DNA release in unstimulated and LPS stimulated neutrophils following inhibition of TRIF and MyD88. In both unstimulated and LPS stimulated neutrophils, pre-treatment with TRIF-pep and MyD88-pep resulted in a decrease in relative extracellular DNA release. However, only LPS stimulated neutrophils showed a statistically significant decrease in extracellular DNA release following incubation with both TRIF-pep and MyD88-pep (n=6, ** $p < 0.01$, *** $p < 0.001$).

NET ELISA based detection of non-adherent NETs within neutrophil supernatants showed similar results (*Fig 5.8A*). The relative arbitrary units (AU), indicating the amount of NETs in supernatant, was higher for neutrophils stimulated with LPS in comparison to unstimulated neutrophil supernatants (0.328 and 0.405 AU respectively). TRIF-pep and MyD88-pep treatment were both shown to decrease mean AU values for both unstimulated and LPS stimulated neutrophil supernatants, however only LPS stimulated neutrophils incubated with the inhibitors showed a significant decrease in mean supernatant AU. This supports the extracellular DNA results in concluding that TRIF and MyD88 inhibition both have an impact on NETosis rates.

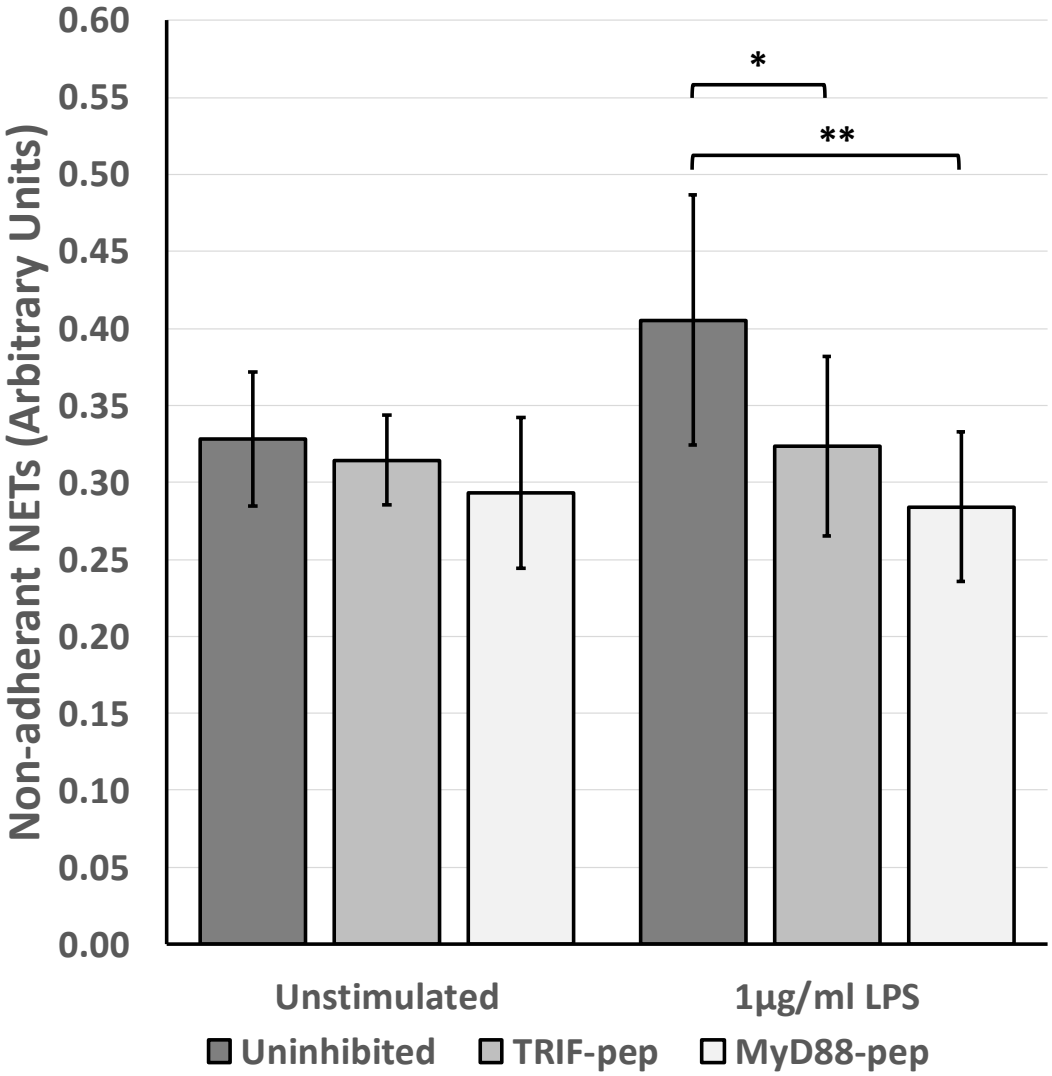

Figure 5.8A NET supernatant ELISA results for LPS stimulated neutrophils incubated with TRIF and MyD88 inhibitors. Pre-treatment of LPS stimulated neutrophils with the peptide inhibitors resulted in a significant decrease in the mean supernatant AU values, indicating decreased presence of non-adherent NETs present within supernatant (n=8, * $p < 0.05$, ** $p < 0.01$).

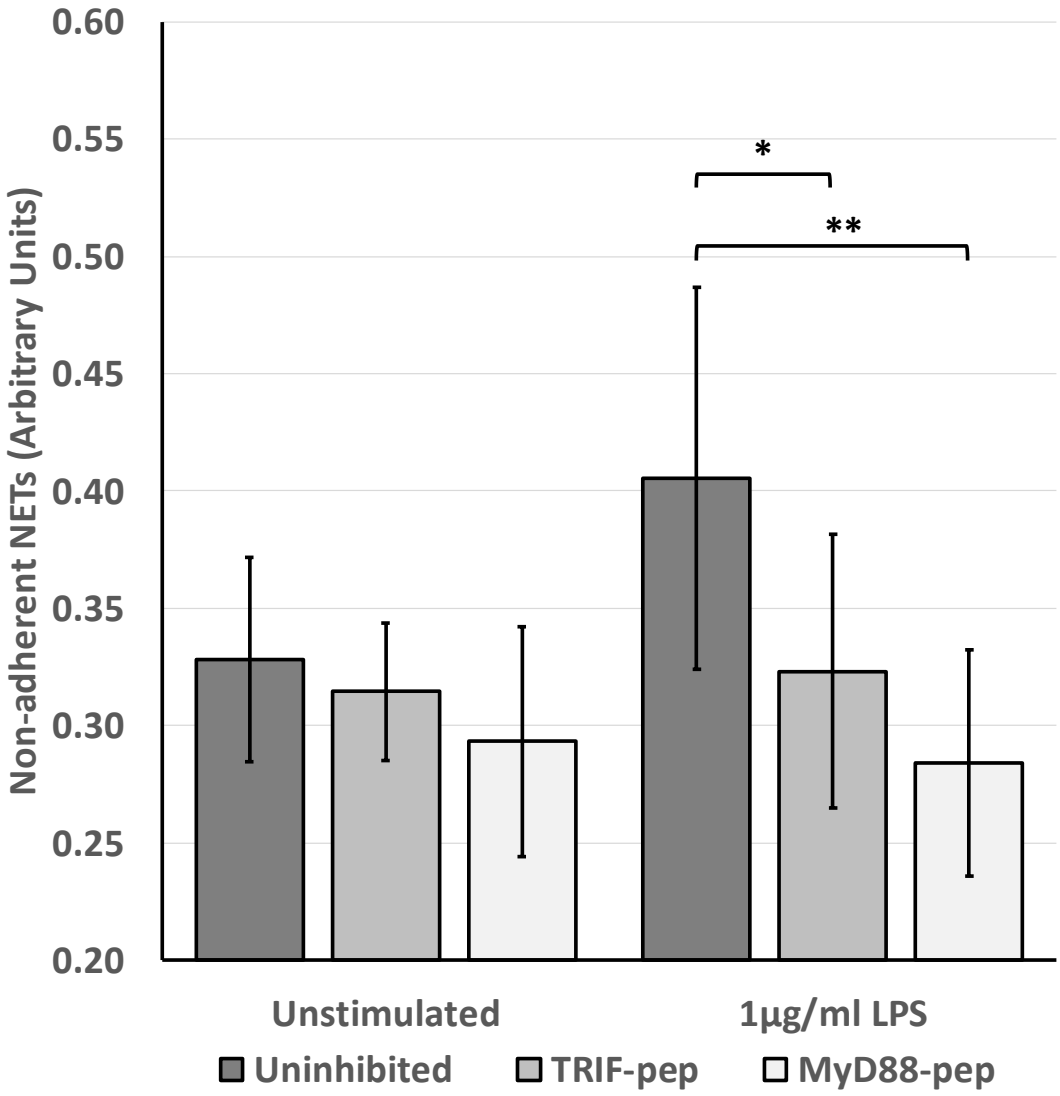

Figure 5.8B NET supernatant ELISA results for LPS stimulated neutrophils incubated with TRIF and MyD88 inhibitors with baseline. This graph shows the same data seen in figure 5.8A, with an arbitrary baseline value of 0.2 AU set for the y-axis, to better demonstrate the differences between the means following inhibition of TRIF and MyD88 (n=8, * $p < 0.05$, ** $p < 0.01$).

5.2.5 Assessment of adherent NET formation following TRIF and MyD88 inhibition

In the previous chapter immunofluorescent (IF) staining of NETs and microscopy counts was used to assess NETosis rates with regards to the formation of adherent NETs. A concern with using this assay in assessing adherent NET formation in TRIF/MyD88 inhibited samples was that the inhibitors may impact the ability of neutrophils to remain adhered to the glass coverslips, potentially impacting NETosis counts.

To address this concern, unstimulated and LPS stimulated neutrophils were incubated for 7 hours, either with or without pre-treatment with TRIF-pep or MyD88-pep. Samples were then fixed and IF stained (as previously described in chapter 3.6.4). The mean number of cells present within a single field of view (x20mag) was then determined for all conditions. As shown in *figure 5.9*, although some decrease was seen in the mean number of adherent LPS stimulated neutrophils when pre-treated with TRIF-pep and MyD88-pep, this was not statistically significant. Furthermore, this pattern was not reflected in unstimulated neutrophils. This suggests that the TRIF/MyD88 inhibitors did not significantly impact adherence rates of neutrophils *in vitro* and should therefore not impact IF count results.

IF NET counts (*Fig 5.10*) showed that LPS stimulated neutrophils had an adherent NET rate of 39.3% (95% CI: 31.9%, 46.6%). The NET rates in LPS stimulated neutrophils pre-treated with TRIF-pep and MyD88-pep was significantly lower (24.6% and 22.6% respectively). Unstimulated neutrophils also showed a decrease in adherent NET rates when incubated with TRIF-pep and MyD88-pep, however this was not statistically significant.

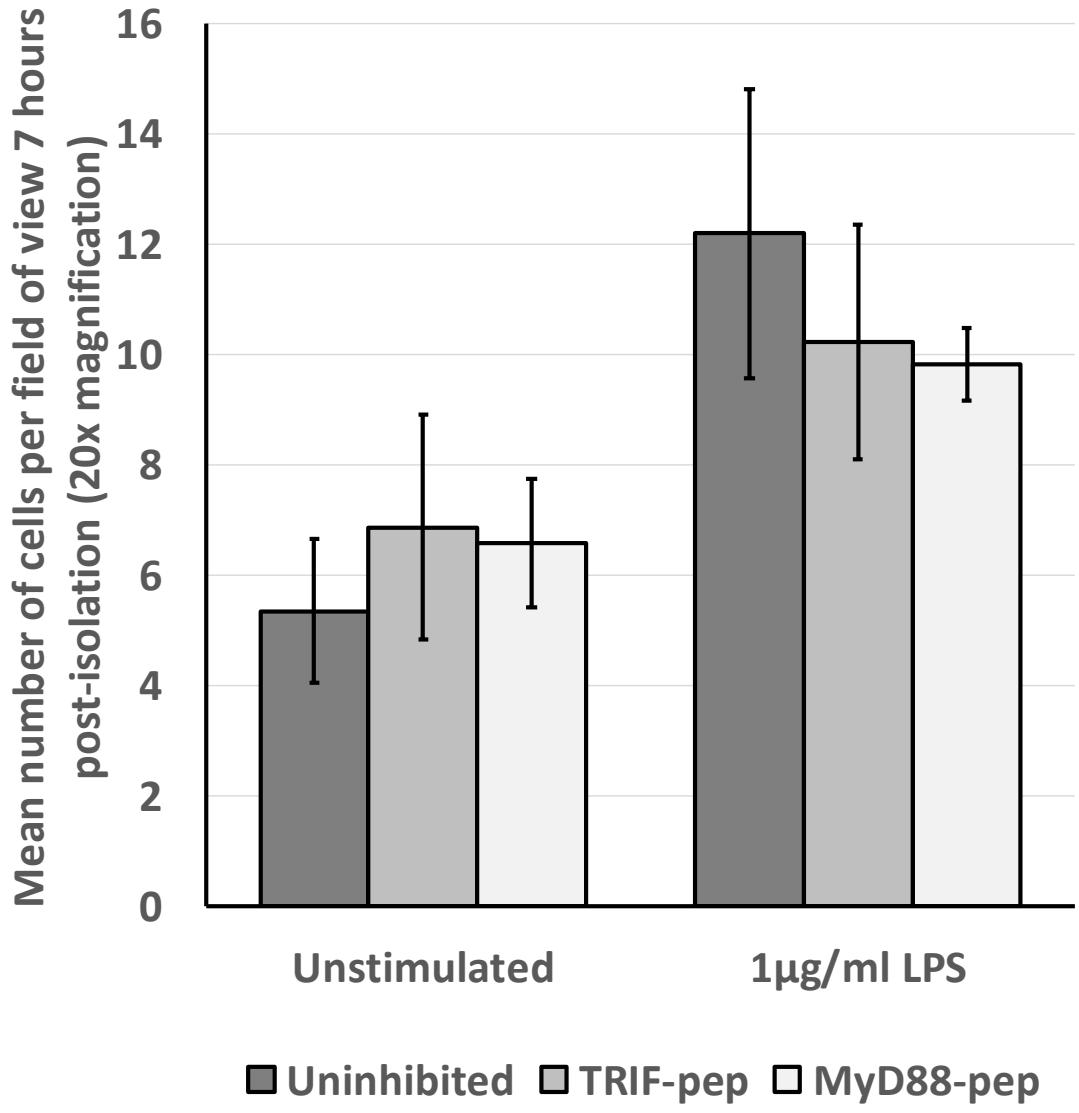

Figure 5.9 Mean number of adherent neutrophils following LPS stimulation and pre-treatment with TRIF and MyD88 inhibitors. Despite an observed decrease in the mean number of adherent cells for LPS stimulated neutrophils when incubated with TRIF-pep and MyD88-pep, no significant difference was observed suggesting inhibition of the signalling proteins did no impact 7-hour adhesion rates of neutrophils to glass coverslips in vitro (n=8).

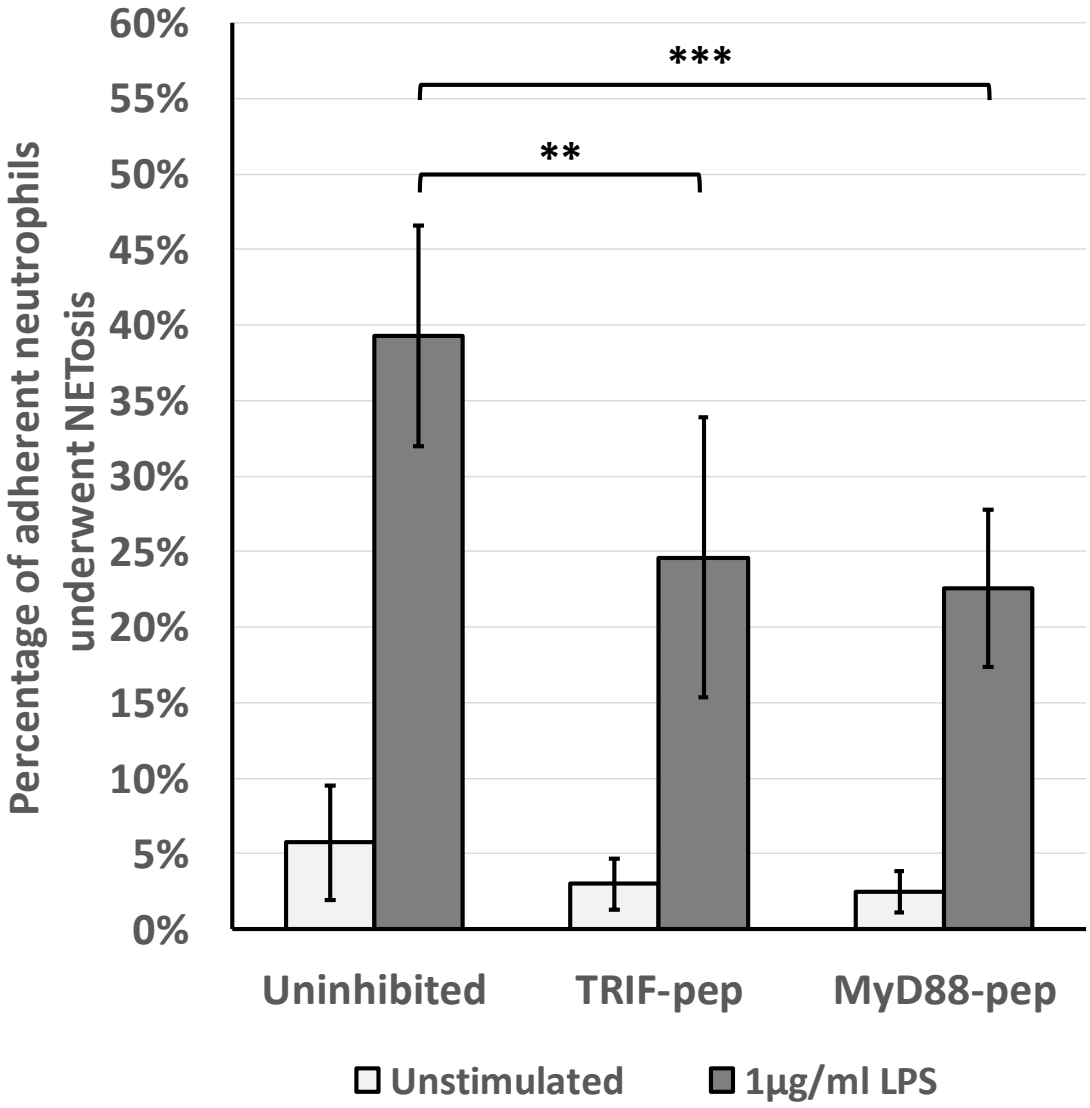

Figure 5.10 NETosis rates in LPS stimulated neutrophils pre-treated with TRIF-pep and MyD88-pep determined by immunofluorescent microscopy. The number of NETs detected significantly decreased when LPS stimulated neutrophils were incubated with TRIF-pep and MyD88-pep (n=8 ** $p < 0.01$, *** $p < 0.001$).

5.2.6 *Summary*

The results of this chapter collectively show that the selected concentrations of TRIF-pep and MyD88-pep both suppressed LPS induced CXCL8 secretion by neutrophils, which supports the inhibitors are having their intended effect on LPS signalling. No difference in extracellular PAD activity was detected following inhibition of TRIF and MyD88, however all assays measuring NETosis rates collectively suggest that both MyD88 and TRIF inhibition significantly decreased the formation of NETs *in vitro*

5.3 Discussion

5.3.1 *Inhibition of LPS signalling on extracellular citrullination*

The observed decrease in mean supernatant PAD activity was not shown to be statistically significant for TRIF or MyD88 inhibited neutrophils. However, given the large confidence intervals within each data set it is possible this lack of significance is due to low sample size. Repeating the experiment with a larger number of samples would reduce the deviation within the data set, giving a more accurate estimation of the true mean supernatant PAD activity levels in each condition and therefore allow valid statistical testing to be carried out to determine if any true difference in PAD activity occurred following TRIF and MyD88 inhibition. However, with the data set presently available no conclusions can be made on the impact of inhibition of TRIF/MyD88 on neutrophil citrullination.

5.3.2 *Interpretation of LPS signalling inhibition on NET formation*

Results for extracellular DNA release, supernatant NET concentration and adherent NET counts consistently showed a significant decrease in NETs for both MyD88 and TRIF inhibited neutrophils. The consistency in the results between each of the methods strongly supports the conclusion that inhibition of TRIF and MyD88 had similar inhibitory effects on NETosis rates by stimulated neutrophils. This conclusion opposes the hypothesis underpinning this work, which predicted only MyD88 inhibition would impact NETosis rates, and instead suggests that TRIF dependent signalling, which does not activate the MAPK-ERK pathway and NADPH oxidase, also plays a role in the induction of NETosis. As PKC, MAPK-ERK and NADPH oxidase activation are one of the few molecular events clearly implicated in NETosis, this could indicate that other molecules in the LPS signalling pathway, particularly those linked to TRIF dependent signalling (e.g. NEMO) may play a role in NETosis. To confirm or refute this in future work, a sensible follow up experiment would involve using the established methods to assess if inhibitors of proteins within the TRIF dependent signalling pathway impact NETosis, or another approach may be to determine if neutrophils from patients with TRIF-dependent signalling protein deficiencies undergo NETosis following stimulation.

Despite the results showing a consistent decrease in predicted NETosis for TRIF and MyD88, there was a difference in the relative levels of inhibition for the three methods

of NET measurement. Extracellular DNA and supernatant NET concentration in TRIF and MyD88 inhibited LPS stimulated neutrophils were observed to decrease to near baseline values (i.e. those seen in unstimulated neutrophils), implying complete inhibition of LPS induced NETosis was occurring. IF counts of adherent NETs however only showed a partial decrease in NETosis for LPS stimulated neutrophils following inhibition (40% in uninhibited neutrophils to 25% and 22.5% in TRIF and MyD88 inhibited neutrophils respectively, with unstimulated neutrophils showing a NETosis rate of 6%). Of the utilised assays, NET counts by IF microscopy is arguably the most valid and accurate measurement of NETosis rates (due to its quantifiability and use of NETosis specific markers such as citrullinated histones, see discussion chapter, 8.3.1), which gives stronger support to the conclusion that only partial inhibition of NETosis is occurring when neutrophils are pre-treated with TRIF-pep and MyD88-pep.

A partial decrease in NETosis rates when incubated with TRIF-pep and MyD88-pep could be explained by a dose response issue with the peptide inhibitors (i.e. higher concentrations would give stronger inhibition of NETosis), however given that higher concentrations were suggested to negatively impact neutrophil viability stronger inhibition of TRIF and MyD88 by tested peptide inhibitors may not be possible. Another interpretation of the partial inhibition of NETosis by TRIF-pep and MyD88-pep is that the previous conclusion (i.e. the TRIF pathway is also implicated in NETosis) is correct. Assuming complete inhibition of TRIF and MyD88 was occurring following treatment with TRIF-pep or MyD88-pep, then the observed partial inhibition of NETosis would suggest that NET formation was still possible by signalling via the uninhibited pathway (e.g. partial inhibition of NETosis following MyD88-pep treatment, suggests TRIF signalling induced NETosis).

To assess this interpretation, future work building on these results would include an experiment involving incubation of neutrophils with TRIF-pep and MyD88-pep simultaneously (after confirming no negative impacts on cell viability) then determining NETosis rates by IF microscopy. Providing both pathways (TRIF and MyD88) are implicated in NETosis, it is expected this experiment would result in complete inhibition of NETosis rates when treated with TRIF-pep and MyD88-pep simultaneously, and only partial inhibition (as seen in this chapter) when treated with each inhibitor individually.

5.3.3 *Limitations of LPS pathway MyD88 inhibition results*

A central criticism with the interpretation of the results detailed in this chapter is the potential oversimplification of the cell signalling pathway. In most biological signalling pathways, signalling proteins are often observed to interact with a large variety of other proteins within the pathway resulting in cross-talk which complicates isolating individual elements of a pathway. Therefore, it is questionable whether TRIF signalling is truly isolated from the MyD88 dependent pathway and has no impact on activation of the MAPK-ERK pathway and NADPH oxidase activation.

The role of the MAPK-ERK pathway and PKC activation in LPS signalling could be assessed in future work by using specific inhibitors of the various proteins within this pathway, as previously done in other published work (Neeli et al 2013, Schuh et al 2009), on isolated neutrophils with NET formation rates assessed as shown in this work. A potential problem with this methodology is the prolonged period of incubation often suggested for the inhibitors, is not always feasible given the isolated neutrophil lifespan. A solution to this difficulty may be to instead use neutrophils derived from animals pre-treated with the inhibitors, however this presents additional ethical and technical challenges, particularly with translating the results to human biology. One alternative would be study neutrophils from patients with genetic defects in the pathways of interest

5.3.3 *Conclusion*

Despite the inability to draw conclusions on the relationship between PKC and MAPK-ERK on NETosis the results presented do allow the conclusion that both the TRIF and MyD88 signalling proteins (and their respective signalling pathways) are implicated in the formation of NETs. These data provide a starting point for future work determining which aspects of TLR4 mediated cell signalling are implicated in NETosis. This may provide a greater understanding of the molecular mechanisms underpinning the formation of NETs in response to infection.

Chapter 6: Examining the effect of Cl-amidine on NETosis, citrullination and neutrophil function

6.1 Introduction

6.1.1 *Overview*

NETosis and citrullination have been implicated in microbial infection (Branzk et al 2013) and a growing number of disease states including; rheumatoid arthritis (Bicker et al 2013, Lundberg et al 2005), COPD (Dicker et al 2018), psoriasis (Hu et al 2016) and cancer (Mohanan et al 2012, van der Windt DJ et al 2018). Because of this, interest in the potential therapeutic benefit of inhibiting these processes has greatly increased in previous years. The majority of PAD inhibitory research focuses upon the use of Cl-amidine, an irreversible non-specific inhibitor of PAD enzymes first synthesised in 2006 as an analogue to the previous (and less potent) synthesised PAD inhibitor F-amidine (Luo et al 2006).

Due to the limited information regarding the safety of Cl-amidine for use in humans much of the *in vivo* research looking at the impact of Cl-amidine on NETosis and disease severity is currently based on murine models. However, the results of these studies suggest that the molecule may offer potential therapeutic benefits as an anti-inflammatory treatment and may reduce the severity of several diseases. Examples of this include results showing Cl-amidine promotes wound healing in diabetic ulcers (Fadini et al 2016) as well as reduced severity of ulcerative colitis and arthritis (Chumanevich et al 2011, Willis et al 2011).

Prior studies suggest that in addition to inhibiting PAD activity, Cl-amidine also inhibits NETosis (Lewis et al 2015), however there are relatively little data evaluating the molecules impact on other aspects of neutrophil biology (e.g. phagocytosis, superoxide production, cytokine secretion). The published data suggests Cl-amidine has anti-inflammatory properties in mouse models (with potential for therapeutic use) but may have an underlying impact on several key neutrophilic functions. It is possible Cl-amidine may alter processes that play an important role in the infection-inflammatory response. I therefore obtained data on the effect of Cl-amidine on these aspects of neutrophil

function to guide future decision making regarding the potential of Cl-amidine as a therapy

6.1.2 *Hypothesis*

PAD inhibitor (Cl-amidine) pre-treatment of healthy volunteer derived peripheral blood neutrophils *in vitro* will significantly decrease PAD activity and NETosis rates.

Other aspects of neutrophil function (i.e. phagocytosis, superoxide production, longevity and CXCL8 secretion) will be significantly impacted by Cl-amidine treatment.

6.2 Results

6.2.1 *Establishing a non-toxic concentration of Cl-amidine*

As several of the assays used in the thesis may be impacted by apoptosis/necrosis rates, it was first important to establish a concentration of Cl-amidine that would not promote neutrophil death within the timeframe that experiments were taking place. Other *in vitro* studies examining the effect of Cl-amidine on NETosis reported using Cl-amidine at a concentration of 200µM (Kraaij et al 2018, Knight et al 2013, Li et al 2017), however there appears to be no published information on any impact of this concentration of Cl-amidine on neutrophil viability.

Three concentrations of Cl-amidine (100µM, 200µM and 300µM) were selected and assessed for their impact on neutrophil viability after 5-hour incubation. As shown in *figure 6.1*, of the assessed concentrations only 300µM Cl-amidine was observed to result in a statistically significant decrease of 9% in neutrophil viability at 5 hours compared to untreated neutrophils. Using these results, 200µM was the selected concentration used in all experiments involving Cl-amidine inhibition of neutrophils.

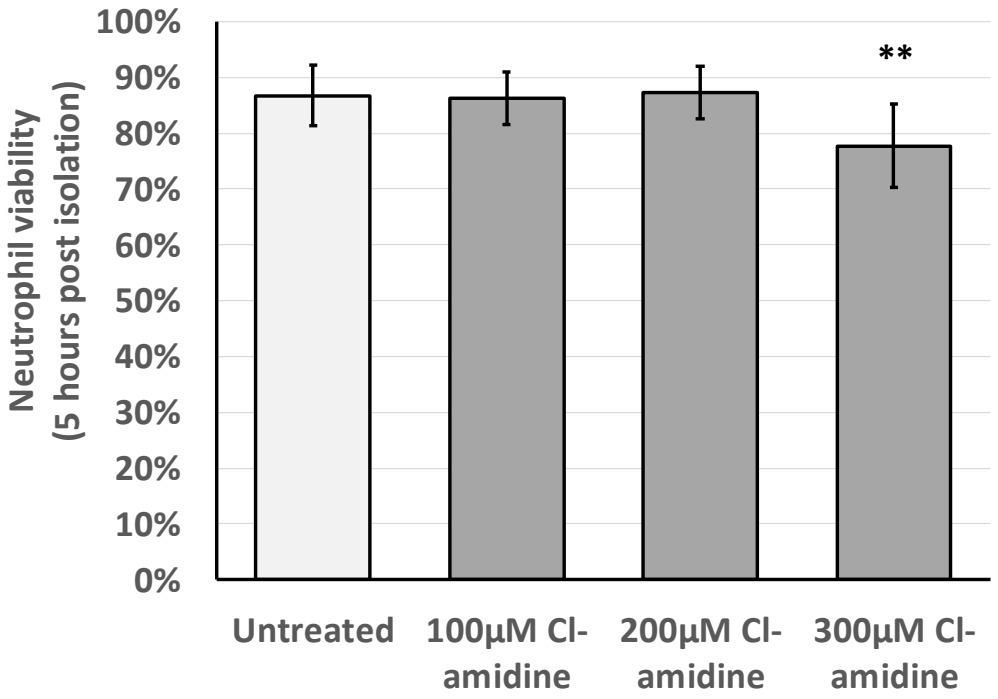

Figure 6.1 Cl-amidine concentration on 5-hour viability rates of neutrophils in vitro. Neutrophil viability showed no change with 100µM and 200µM Cl-amidine. Incubation with 300µM Cl-amidine significantly decreased 5-hour viability (n=5 95% CI, ** $p < 0.01$).

6.2.2 *Cl-amidine decreases extracellular citrullination activity*

Supernatants from neutrophils incubated with/without Cl-amidine then stimulated with PMA, LPS and TNF-α were assessed for PAD activity (*Figure 6.2*). Stimulation with PMA, LPS and TNF-α resulted in a 3.3-fold, 1.8-fold and 1.9-fold increase in supernatant PAD activity (measured by commercial Modiquest Research PAD activity assay) compared to unstimulated neutrophils. Pre-treatment and incubation of neutrophils with Cl-amidine resulted in an observed decrease in PAD activity for all conditions with large decreases seen for neutrophils stimulated with PMA (2.02mU PAD activity without Cl-amidine, 0.68mU PAD activity with Cl-amidine), LPS (1.10mU PAD activity without Cl-amidine, 0.60mU PAD activity with Cl-amidine) and TNF-α (1.12mU PAD activity without Cl-amidine, 0.52mU PAD activity with Cl-amidine). The decrease between uninhibited and Cl-amidine treated neutrophils was found to be statistically significant for PMA and TNF-α stimulated neutrophils. This suggests that Cl-amidine significantly inhibits any increase in extracellular PAD activity following stimulation.

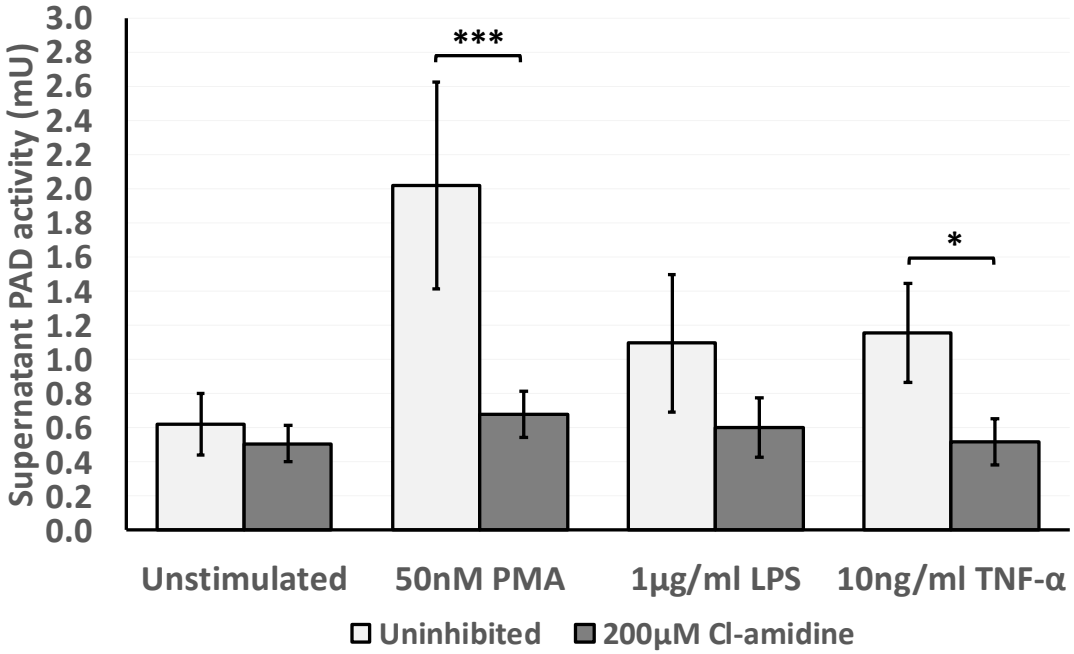

Figure 6.2 Supernatant PAD activity from stimulated neutrophils incubated with Cl-amidine. Cl-amidine resulted in decreases in PAD activity for all conditions. PAD activity was significantly lower in supernatants from Cl-amidine treated neutrophils stimulated with PMA and TNF-α. Despite the large decrease in PAD activity with Cl-amidine seen for LPS stimulated neutrophil no significant difference was shown, perhaps explained by the larger variation in spread for these results highlighted by the overlapping error bars (n=8 95% CI, * $p < 0.05$, *** $p < 0.001$)

6.2.3 *Cl-amidine decreases in vitro NETosis rates in healthy peripheral blood neutrophils*

Immunofluorescent (IF) counts (adherent NETs), NET ELISAs (non-adherent NETs) and SYTOX extracellular DNA staining were used to assess NETosis rates in samples incubated with and without 200µM Cl-amidine. IF counts of NETs for neutrophils stimulated with PMA and LPS showed a statistically significant decrease in the number of NETs observed when incubated with Cl-amidine (*Figure 6.3*). Interestingly, treatment with Cl-amidine did not result in a complete reduction of adherent NETs to the levels seen in unstimulated neutrophils, instead a decrease in NETosis rates of 14% and 11% was observed for PMA and LPS respectively.

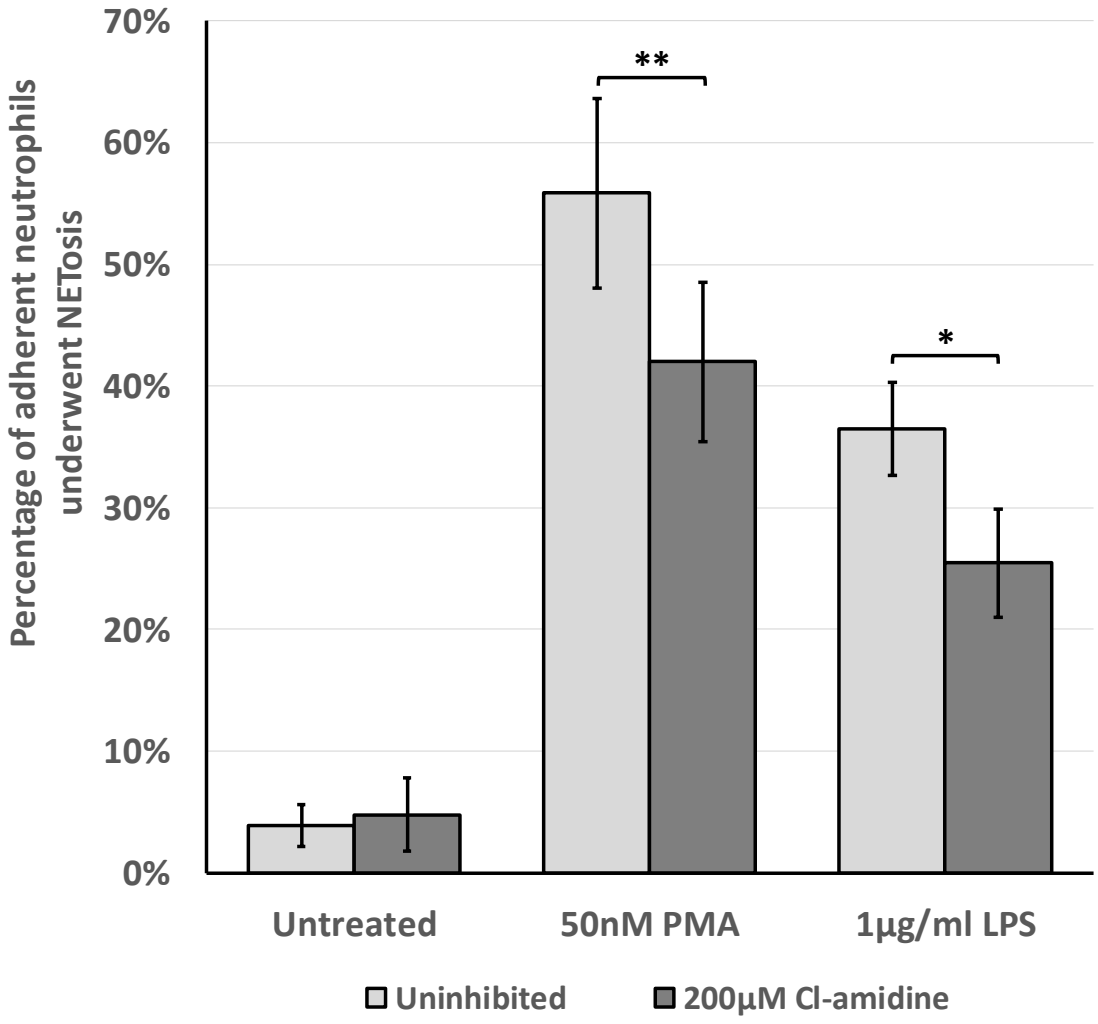

Figure 6.3 Cl-amidine on NETosis rates in adherent neutrophils in vitro. Incubation with Cl-amidine resulted in a decrease in NETosis rates for PMA and LPS stimulated adherent neutrophils. Although NETosis was not completely inhibited the decrease was shown to be statistically significant (n=8 95% CI, * $p < 0.05$, ** $p < 0.01$).

NET ELISAs of supernatants obtained from neutrophils stimulated with PMA, LPS, fMLP and TNF-α showed a much larger relative decrease in NETosis (*Figure 6.4*). For all conditions (including unstimulated neutrophils) there was a highly significant decrease in the arbitrary units (AU) measurements with Cl-amidine, to a nearly undetectable level with several results falling below the lower limit of detection (LLD = 0.01AU). This suggests there are significantly fewer NETs present in supernatants from unstimulated/stimulated neutrophils incubated with Cl-amidine.

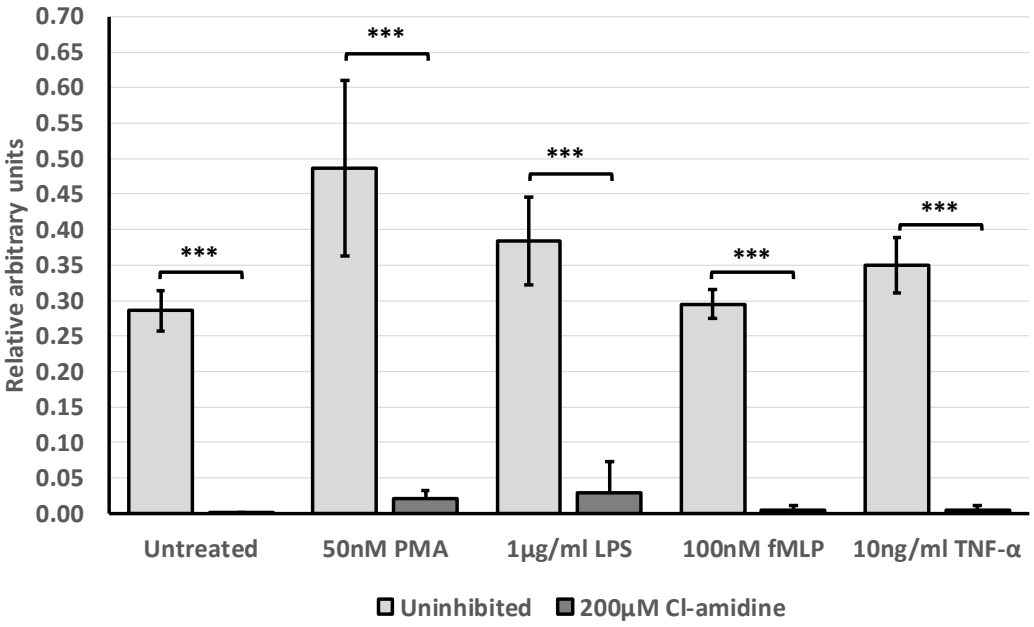

Figure 6.4 NET ELISAs of supernatants from stimulated neutrophils treated with Cl-amidine. The mean AU measurements for each condition decreased significantly when the neutrophils were incubated in the presence of Cl-amidine. As AU is determined using a NET ELISA, with a biological standard curve known to contain high number of NETs, this suggests the levels of non-adherent NETs in each supernatant has decreased significantly following incubation with Cl-amidine (n=8 95% CI, *** $p < 0.001$).

Finally, SYTOX staining was used to assess extracellular DNA release as a measure of total NETosis (adherent and non-adherent) within given conditions *in vitro* (*Figure 6.5*). Stimulation with PMA, as expected, resulted in a significant increase in relative fluorescence (i.e. fluorescent relative to unstimulated neutrophils), from 100% to 191.7% (95% CI: 172.3%, 211.1%). Incubation with Cl-amidine alone had no significant impact on extracellular DNA release of unstimulated neutrophils. However, incubation of PMA stimulated neutrophils with Cl-amidine resulted in significant decrease of 53% in comparison to PMA stimulated neutrophil in the absence of Cl-amidine. These results suggest that Cl-amidine did result in a significant decrease in extracellular DNA release in response to PMA stimulation *in vitro*.

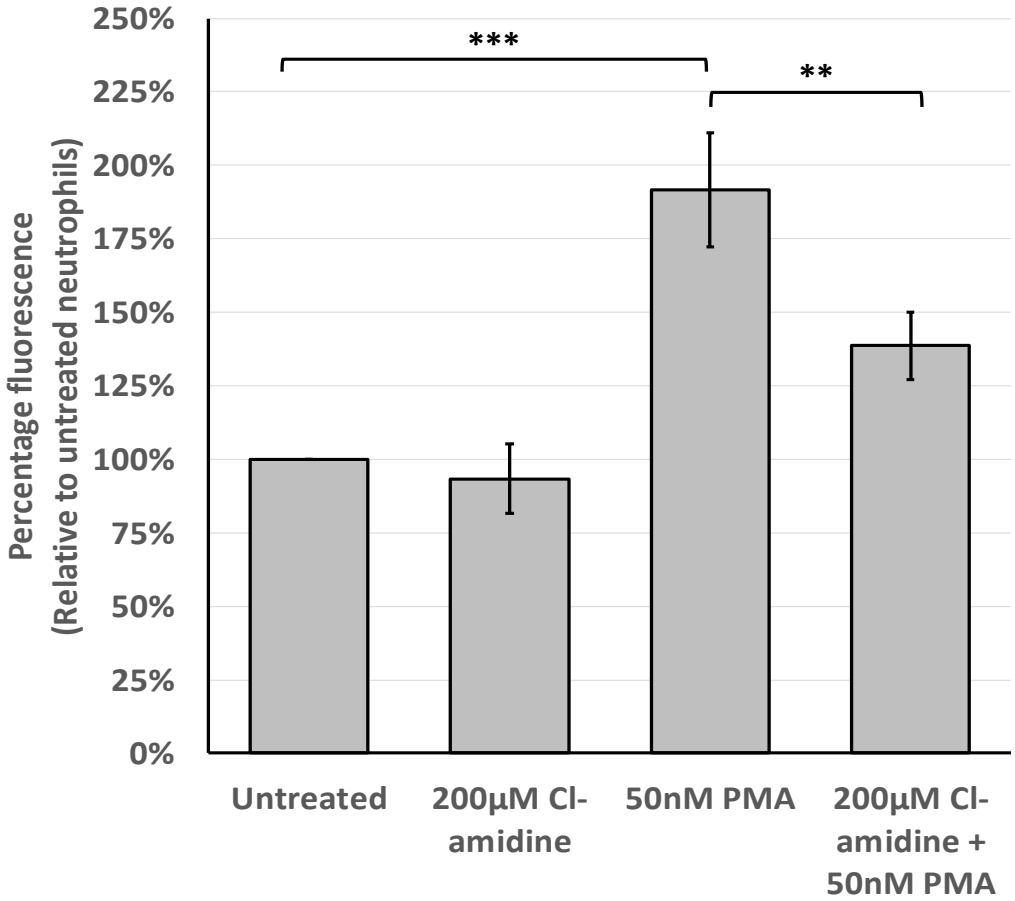

Figure 6.5 Cl-amidine on extracellular DNA release by PMA stimulated neutrophils in vitro. PMA treatment caused a significant increase in SYTOX depended relative fluorescence compared to untreated neutrophils. When incubated with Cl-amidine the increase in relative fluorescence is less than neutrophil stimulated with PMA alone. The decrease in fluorescence between PMA stimulated neutrophils incubated with/without Cl-amidine was found to be statistically significant (n=7 95% CI, ** $p < 0.01$, *** $p < 0.001$).

6.2.4 *200μM Cl-amidine has no impact on neutrophil longevity in vitro*

As neutrophils an early responder to inflammation/infection they play a critical role in host defence. However, a key feature of neutrophil biology is their lack of proliferation capabilities and limited lifespan, which was reflected chapter 4 where only 5% of neutrophils remained viable after 24 hours incubated at 37°C in culture medium *in vitro*. This limited lifespan in some manner is a protective mechanism, as it ensures prolonged/ excessive neutrophil accumulation in tissues with resolved infection does not occur.

The work in this chapter has shown Cl-amidine did not impact viability at 5 hours, however there is some question if it may impact viability at later timepoints. This may be of significance for *in vivo* use of Cl-amidine as any change in neutrophil longevity could have severe implications for the health/survival of the organism receiving the molecule (e.g. neutropenia and infection susceptibility or excessive neutrophilic inflammation).

Neutrophil viability in the presence/absence of Cl-amidine was assessed over a 24-hour period with measurements taken every 4 hours. As seen in *figure 6.6,* there was no clear difference in the viability rates at the various timepoints, with no statistically significant differences being shown.

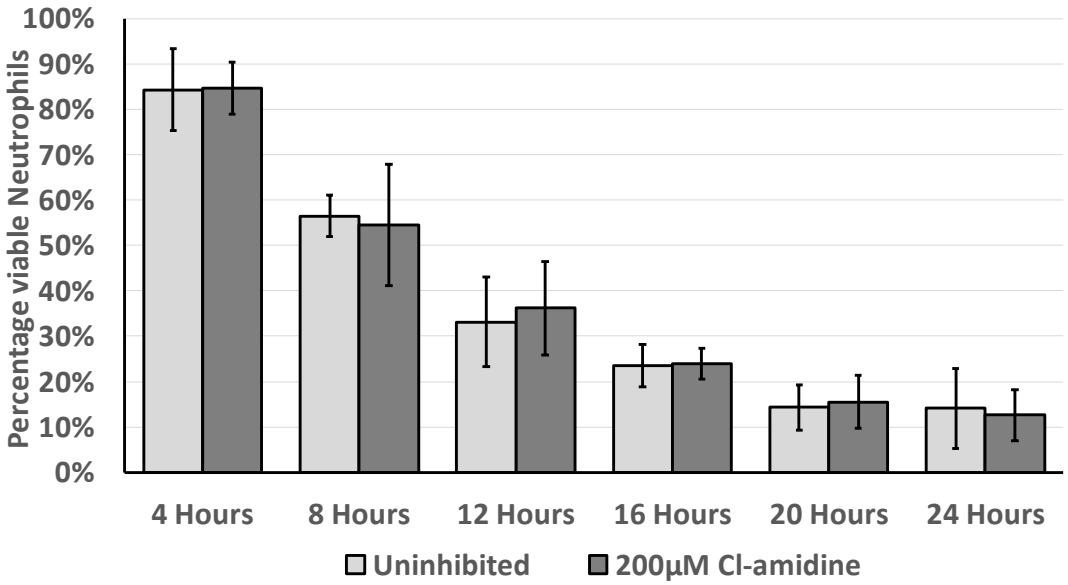

Figure 6.6 Neutrophil viability over 24-hour period when incubated with Cl-amidine. There was no significant difference in neutrophil viability over a 24-hour period (post-isolation) when incubated with Cl-amidine (n=3).

6.2.5 *Superoxide production and phagocytosis decreased following inhibition by Cl-amidine*

Two key features of broad microbial defence carried out by neutrophils are the processes of oxidative burst and phagocytosis. Decreases in function of one or both processes can have severe implications in neutrophil microbial responses. To assess the oxidative bust response of neutrophils treated with Cl-amidine, superoxide anion release was measured by recording absorbance changes due to superoxide dependent reduction of cytochrome c *in vitro* (using Beet-Lambert law to determine the molar concentration of superoxide anions released by the neutrophils).

Neutrophils stimulated with PMA and LPS (both of which activate NADPH oxidase and increase superoxide production) had mean superoxide anion concentrations higher than unstimulated neutrophils (*Figure 6.7*), with the increase in response to PMA and LPS being statistically significant ($p<0.001$ and $p<0.01$ respectively). Treatment with Cl-amidine resulted in a significant decrease in superoxide anion release for both PMA and LPS stimulated neutrophils. This suggests that Cl-amidine does have an inhibitory effect on the oxidative burst response in neutrophils.

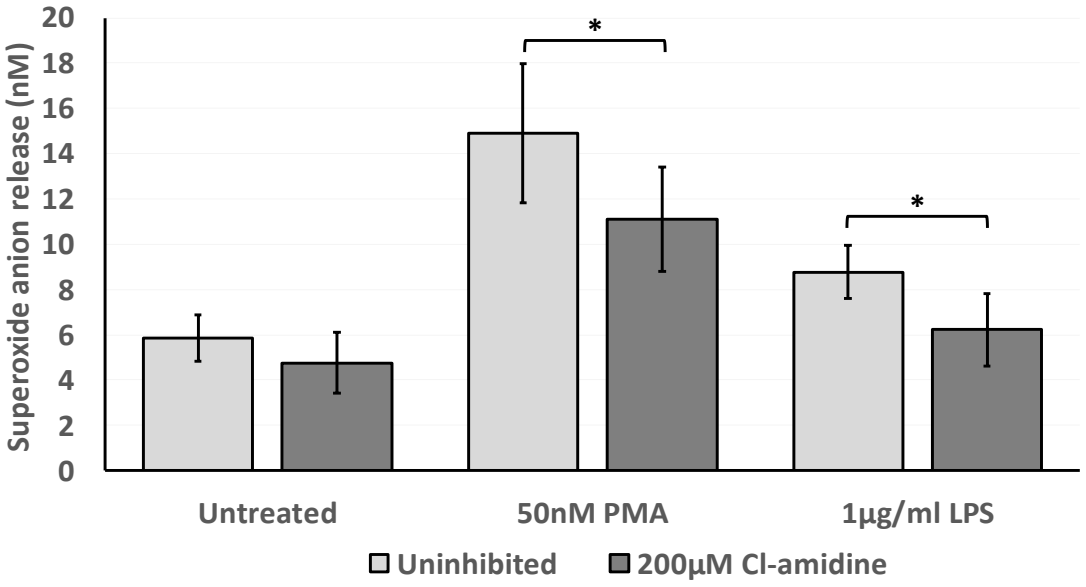

Figure 6.7 Superoxide anion release by stimulated neutrophil incubated with Cl-amidine. Stimulation with PMA and LPS resulted in the release of significantly higher quantities of superoxide anions ($p<0.001$ and $p<0.01$). Significant decreases in superoxide anion were recorded in PMA and LPS stimulated neutrophils incubated with Cl-amidine (n=7, * $p<0.05$).

Microscopy counts of neutrophils (fixed and Giemsa stained) that had been incubate with and engulfed 2 or more zymosan particles (similar to as done previously by Morris et al 2009) allowed analysis of phagocytosis rates following incubation with Cl-amidine (*figure 6.8*). Mean phagocytosis rates in uninhibited neutrophils were 69% (95% CI: 62.4%, 75.6%) compared to the significantly lower paired results for Cl-amidine treated neutrophils which had a mean phagocytosis rate of 58% (95% CI: 50.5%, 65.5%). These results suggest that Cl-amidine has some inhibitory effect on phagocytosis rates in neutrophils.

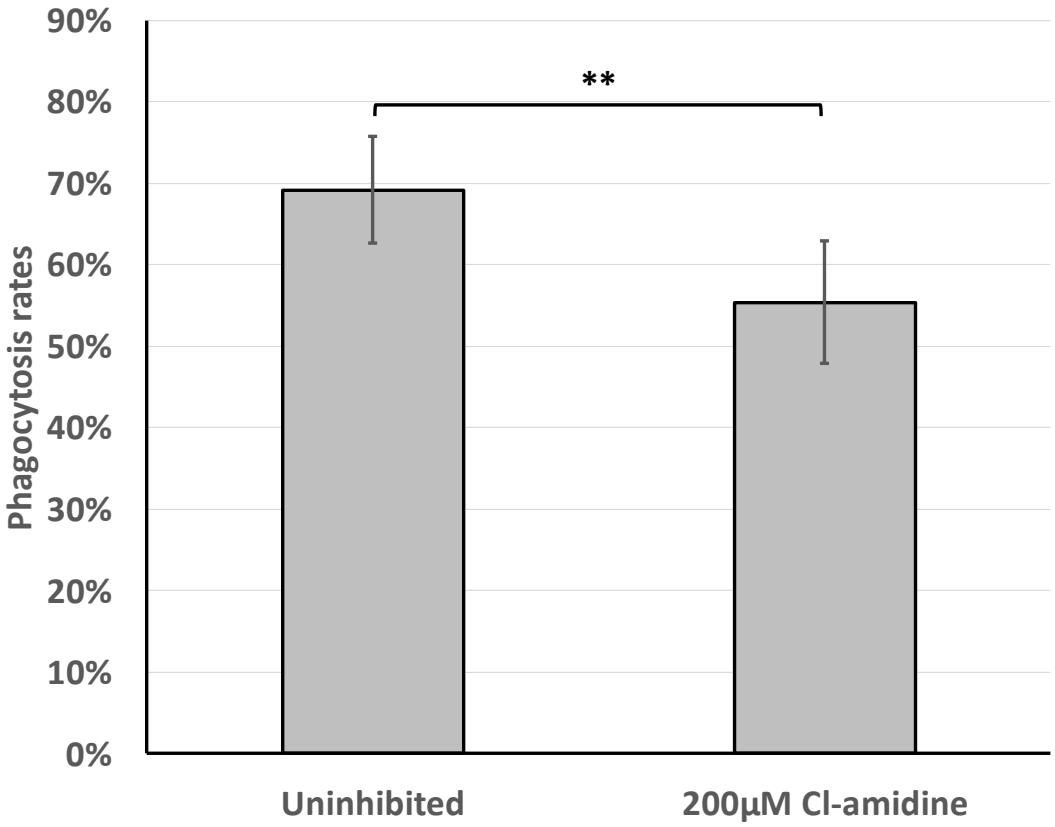

Figure 6.8 Phagocytosis rates in uninhibited and Cl-amidine treated neutrophils.
Phagocytosis rates were assessed by microscopy recording of the number of neutrophils that had phagocytosed 2 or more zymosan particles. A significant decrease in mean phagocytosis rate of 11% was seen when neutrophils were incubated with 200µM Cl-amidine (n=10, ** $p < 0.01$).

6.2.6 CXCL8 *secretion inhibited by Cl-amidine*

CXCL8 has long been established as an activator and potent chemoattractant for recruiting neutrophils to sites of infection/inflammation. As neutrophils are also a source of CXCL8 when activated, CXCL8 secretion by neutrophils is likely an important factor in essential neutrophil responses to infections. CXCL8 concentrations in supernatants from stimulated neutrophils were confirmed using ELISA and compared to paired values obtained from neutrophils incubated with Cl-amidine.

Figure 6.9 shows the mean CXCL8 concentrations in collected neutrophil supernatants. Stimulation with PMA, LPS and fMLP resulted in a highly significant ($p < 0.001$ for PMA, LPS and fMLP) increase in CXCL8 secretion compared to unstimulated neutrophils (similar to as shown in chapter 4.2.3). The mean supernatant CXCL8 concentrations for Cl-amidine inhibited neutrophils were lower than uninhibited neutrophils in all conditions, however only PMA stimulated neutrophils showed a significant decrease in supernatant CXCL8 concentration when inhibited by Cl-amidine. This suggests Cl-amidine has an impact on CXCL8 secretion by neutrophils following stimulation.

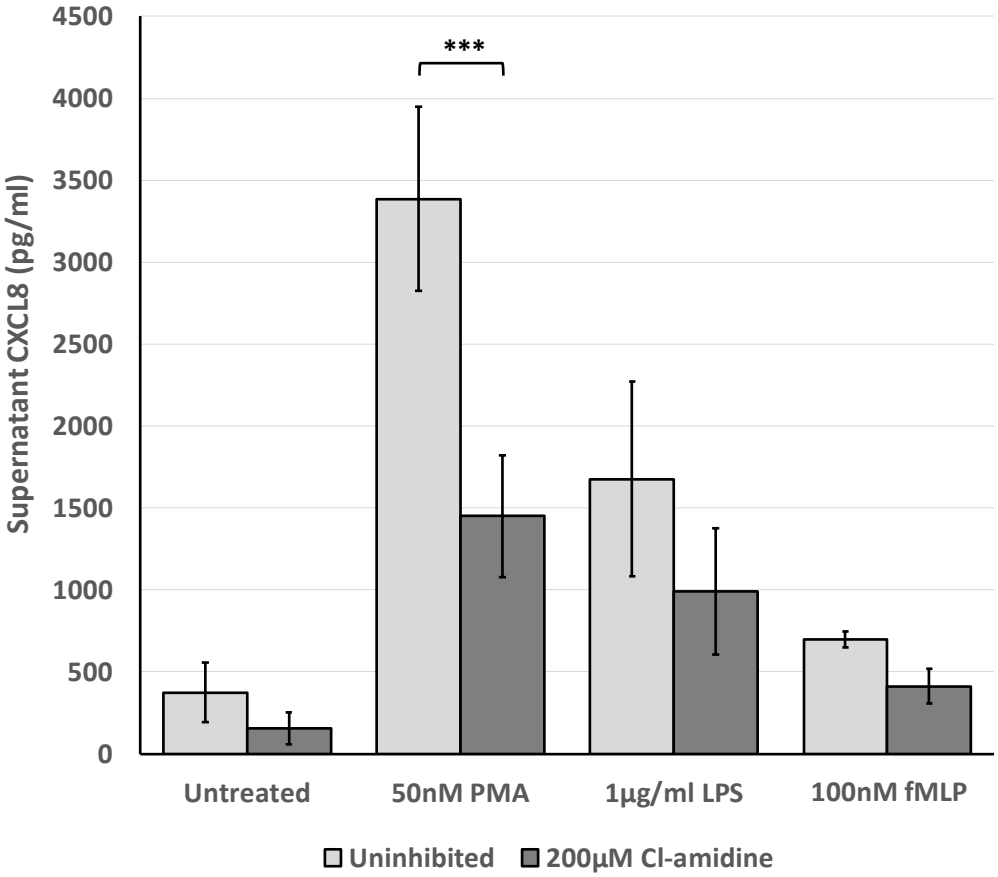

Figure 6.9 CXCL8 secretion by stimulated neutrophils inhibited by Cl-amidine. CXCL8secretion was significantly higher when neutrophils were stimulated by PMA, LPS and fMLP ($p < 0.001$ for all stimuli). Cl-amidine resulted in a decrease in CXCL8 secretion by neutrophils for all condition, however only PMA stimulated neutrophils showed a significant decrease in CXCL8 secretion in response to Cl-amidine (n=4, *** $p < 0.001$).

6.2.7 *Summary*

Cl-amidine treatment significantly reduced extracellular PAD activity in response to all stimuli. NETosis assays all suggested a decrease in NET formation when neutrophils were incubated with Cl-amidine, which is expected given the suggested role of citrullination in NETosis. Assays of neutrophil function effected by Cl-amidine showed no difference in long term (24-hour) viability *in vitro* for neutrophils, however there was some evidence that superoxide anion release, phagocytosis rates and CXCL8 secretion were inhibited by Cl-amidine.

6.3 Discussion

6.3.1 *PAD inhibition on extracellular citrullination*

Prior studies examining Cl-amidine and biological citrullination have used measurements such as IF staining for citrullinated histone to measure intracellular citrullination activity (Li et al 2017, Kan et al 2012), this thesis have included assessing PAD activity in the extracellular environment. This approach was selected as the results from a previous chapter (Chapter 4) had shown that stimulated neutrophil supernatants had higher levels of PAD activity, with an interpretation of this result being that activated neutrophils in vivo may create a pro-citrullination microenvironment over chronic periods of disease. Whilst methods such as IF staining for citrullinated histones or proteomic analysis would provide insight into the intracellular environment of Cl-amidine treated neutrophils it was deemed more important to evaluate the impact of Cl-amidine on neutrophils ability to create a promote extracellular environment, which is arguably likely to be a greater contributor to the development of neo-antigens and the onset of autoimmunity.

PAD activity was observed to significantly decrease in two conditions (PMA and TNF-α) when neutrophils were incubated with Cl-amidine. All conditions treated with Cl-amidine appeared to show a supernatant PAD activity close to that of unstimulated neutrophils (i.e. 0.5mU). Why PAD activity was not completely inhibited is unclear. One potential explanation is that Cl-amidine has different selectivity for the PAD enzymes. Data provided by the manufacturer suggest an IC_{50} value of 0.8, 6.2 and 5.9μM for PAD1,3 and 4 (no data available on value for PAD2), meaning the remaining PAD activity in supernatants detected in Cl-amidine treated neutrophils may be a result of a PAD enzyme that Cl-amidine has lower potency towards (compared to the one responsible for the increase in citrullination seen upon stimulation). Despite this, overall the data herein suggests that Cl-amidine can successfully inhibit PAD enzyme activity in an extracellular environment.

6.3.2 *PAD inhibition on NETosis*

A significant decrease in extracellular DNA release (SYTOX assay) in response to PMA was observed when neutrophils were inhibited by Cl-amidine, suggesting that Cl-amidine was downregulating NETosis. This matches the results of Fadini et al (2016) who

showed a significant decrease in extracellular DNA release in murine neutrophils treated with Cl-amidine. Although there were some differences between the results of this chapter and Fadini et al, which showed a larger decrease in PMA induced extracellular DNA release following pre-treatment with Cl-amidine, this is easily explainable by differences in experiment format (e.g. concentration of SYTOX used) and the use of human neutrophils in comparison to mouse derived neutrophils. Whilst the results may be interpreted as demonstration that Cl-amidine effects NETosis rates *in vitro* the low specificity of the assay (i.e. inability to establish NETosis from other cell death processes) must be considered.

Results from adherent NET counts (IF staining) and semi-quantification of free NETs in supernatant (NET ELISA) both supported the conclusion that NETosis rates significantly decrease in response to Cl-amidine. NET IF staining showed a significant decrease in the number of adherent NETs detected for PMA and LPS stimulated neutrophils when incubated with Cl-amidine. Other published work quantifying NETosis rates report a much larger decrease, to levels indicating almost no NETs are present (Kusonoki et al 2016, Braster et al 2016). However, it is important to note key differences between the research that may explain this difference. For example, Braster et al used a concentration of Cl-amidine 5-fold higher than that used in this work, whilst the methodology of Kusonoki et al featured several distinct differences including; stimulation with 20nM concentration of PMA (as opposed to 50nM), using only citrullinated histone H3 as a staining target for IF microscopy and using the total number of NETs counted for analysis (rather than proportions).

The lack of complete inhibition of NETosis seen in the IF staining counts could be explained by dosing or that Cl-amidine only partially inhibited intracellular histone citrullination, therefore resulting in partial inhibition of NETosis. The results for extracellular PAD activity following Cl-amidine treatment (i.e. significant decrease but not complete inhibition) appear to support this explanation for incomplete inhibition of NETosis. It can be expected that intracellular PAD activity would be of greater difficulty for Cl-amidine to effectively inhibit than extracellular PAD activity. Therefore, if extracellular PAD activity also showed incomplete inhibition following PAD activity it is

arguable that intracellular histone citrullination would also be expected to show partial inhibition by Cl-amidine.

The NET ELISA results indicate that supernatants of neutrophils treated with Cl-amidine, in all stimulatory conditions (e.g. unstimulated, PMA, LPS), showed a significant decrease in the relative number of NETs detected by the assay. In contrast to the results obtained for IF staining counts of adherent NETs, the results from the NET ELISA suggest the number of supernatant NETs from stimulated neutrophils inhibited by Cl-amidine was even lower than that of unstimulated neutrophil supernatants. This could suggest that Cl-amidine is having a far greater inhibitory impact on NETosis rates following stimulation than predicted in the IF staining results, however as the NET ELISA is a semi-quantified measure (i.e. values relating to a biological standard known to contain high amounts of NETs) this decrease in NETosis rate cannot be used to establish the extent to which the number of free non-adherent NETs has decreased.

Overall the results obtained using the separate methodologies for assessing NETosis rates collectively suggest that NET formation *in vitro* is significantly inhibited by Cl-amidine treatment.

6.3.3 *Neutrophil function linked to citrullination*

As Cl-amidine has reported anti-inflammatory properties *in vivo*, the impact of Cl-amidine on selected aspects of neutrophil biology was assessed. There was no difference in viability between uninhibited and Cl-amidine treated neutrophils across a 24-hour period *in vitro*. A criticism of these results may be that neutrophil lifespan in circulation *in vivo* may be extremely different. The subject of *in vivo* half life itself is a debated matter with key pieces of the literature claiming a circulating half-life of 8-10 hours and some papers proposing a neutrophils life span of 5.4 days in circulation (Pillay et al 2010, Tofts et al 2011). The separation between *in vivo* and *in vitro* conditions does present many complications to result interpretations in neutrophil research. However, from the results obtained in this thesis we can currently conclude there is no clear difference in lifespan *in vitro* for neutrophil incubated with 200µM Cl-amidine

There was a significant increase in superoxide anion release by neutrophils stimulated with PMA and LPS. These results (in regard to unstimulated and LPS stimulated neutrophils) showed great similarity to the values reported in previously published work from our lab group that used the same methodology, supporting the accuracy of the results obtained (Ruchaud-Sparagano et al 2014). Treatment with Cl-amidine resulted in a significant decrease in the concentration of superoxide anion release by neutrophils when stimulated with PMA and LPS, suggesting that inhibition of PAD enzymes has some effect on the NADPH oxidase pathway. Publications on superoxide in relation to PAD function and/or NETosis are largely focused on reporting NADPH oxidase activation (and subsequent superoxide formation/activity) as being a requirement for the activation of PAD and/or the onset of NETosis (Remijsen et al 2011, Rohrbach et al 2012).

Currently the only similar published data is that detailed in an abstract presented at the 105th meeting of the American Association for Cancer Research (2014), which describes a finding that inflammatory cells produced less reactive oxygen species in response to Cl-amidine (Witalison et al 2014). Whilst the value of this information is largely limited (as no information is available on precise results or methodology), the notion that PAD inhibition has some impact on superoxide activity is an interesting one. This would have two implications; firstly, bacterial killing may be impaired by Cl-amidine (suggesting potential issues with therapeutic use) and secondly there may be some form of positive feedback control loop for PAD and superoxide, i.e. superoxide activity results in activation of PAD which in some way promotes further formation of superoxide. However, there is no current evidence demonstrating this is the case or how such a mechanism may potentially function.

Neutrophil phagocytosis also appeared to be inhibited by Cl-amidine. A relationship between PAD activity and phagocytosis has not been previously described. However, this could be explained by Cl-amidine disrupting “normal” PAD mediated citrullination of phagocytosis relevant proteins within the cell (e.g. cytoskeletal proteins) which impedes function. It has been shown that (in rheumatoid arthritis patients) PAD enzymes are capable of citrullinating cytoskeletal related proteins such as vimentin and β-actin (van Beers et al 2012) and that excessive citrullination of vimentin in macrophages is reported to disrupt vimentin polymerisation and have a severe impact on cytoskeletal

function (Hojo-Nakashima et al 2009). Furthermore, citrullination of intermediate filaments has been reported to be a physiological response to retinal trauma in mouse models (Wizeman et al 2016), showing PAD can be upregulated to alter cellular citrullination under certain circumstances. Whether citrullination plays any role in cytoskeletal regulation and phagocytosis in healthy functional neutrophils however is currently unknown,

Similar to superoxide, a decrease in phagocytic ability of neutrophils would have severe implications due to the critical importance of phagocytosis as an anti-microbial defence mechanism. In the current data the mean phagocytosis rate of Cl-amidine treated neutrophils was 55% (95% CI; 47.5%, 62.5%), 14% lower than uninhibited neutrophils. In their 2009 paper examining phagocytosis rates (using the same zymosan methodology) in patients with suspected ventilator associated pneumonia (VAP), Morris et al demonstrated that patient peripheral blood neutrophils had average phagocytosis rates lower than healthy (with the majority being <50%). Whilst direct comparisons between these results and the Morris results cannot be made (e.g. difference in age, health etc.), our results do suggest that 200µM Cl-amidine reduced peripheral blood phagocytosis rates in healthy volunteer derived neutrophils to values similar in severely ill VAP patients.

In addition to the possible inhibition of superoxide anion release and phagocytosis by Cl-amidine, CXCL8 secretion also appeared inhibited in response to Cl-amidine. Whilst decreases in mean CXCL8 supernatant concentration was observed for all conditions only PMA showed a significant decrease in CXCL8 secretion when treated with Cl-amidine. This is likely attributable to the relatively low sample size (n=4) when considering the spread of the data visible in *figure 6.9*. Regardless of this the results show that Cl-amidine is capable of inhibiting CXCL8 secretion from neutrophils upon stimulation, which could potentially *in vivo* reduce the ability of neutrophils to promote migration of other neutrophils to sites of infection/inflammation. Relatively little published information is available on the impact of PAD inhibition of cytokine secretion by neutrophils, however this result suggests that Cl-amidine (and by extension PAD enzymes) may have wide implications on cellular signalling.

The results for assessing neutrophil function in response to Cl-amidine inhibition suggest that inhibited neutrophils may have reduced superoxide anion release, phagocytic capacity and CXCL8 secretion (and potentially the secretion of other cytokines). Whilst several of these functions are central to neutrophil anti-microbial defence, future work would benefit from assessing bacterial killing rate by Cl-amidine treated neutrophils *in vitro.*

Some of these inhibitory effects of Cl-amidine on neutrophil function may in part explain results from *in vivo* animal studies showing improved survival in inflammatory disease models (e.g. diabetic limb ulcers and ulcerative colitis), however this raises concern over potential increased risk of severe/fatal microbial infections. This is somewhat refuted by a paper published in 2016 by Zhao et al, which demonstrated increased survival rates in mouse models of sepsis when administered Cl-amidine, suggesting no severe susceptibility risk. The increase in survival of mice in this paper was attributed to the reduction in pro-inflammatory cytokine secretion and a reported increase in circulating monocytes following Cl-amidine administration, which aided in resolving the systemic infection and promoting survival.

Collectively the available literature surrounding Cl-amidine and the work in this chapter highlight that Cl-amidine has a wide range of effects on neutrophil function, which may extend to other cell types within an organism. To address the questions surrounding the impact of Cl-amidine on individual cells and at a whole organism level, further research is required to guide potential future clinical trailing of Cl-amidine.

6.3.4 *Conclusion*

In conclusion Cl-amidine significantly inhibited extracellular citrullination and NETosis rates *in vitro* suggesting the molecule may prevent the formation of a pro-citrullination environment and the uncontrolled release of neutrophil cytoplasmic contents. Neutrophil functional assay results suggest that Cl-amidine had inhibitory effects on superoxide anion release, phagocytosis and CXCL8 secretion. Whilst this may explain the observed anti-inflammatory effects of Cl-amidine in published *in vivo* work further work

is needed to understand the broad effects of Cl-amidine (and PAD activity) on cell, tissue and whole organism function.

Chapter 7: Differences in citrullination, NETosis and neutrophil function in the bronchiectasis patient cohort

7.1 Introduction

7.1.1 *Overview*

The previous chapters have indicated increased neutrophil derived citrullination and NETosis occurs in response to bronchiectasis (BR) relevant stimuli, which may explain why BR may in some cases lead to an individual developing RA later in life (i.e. BROS patients). However, a question raised by these results is why only 3-5% of the BR patient patients' cohort develop rheumatoid arthritis? (Solanki et al 1992, Pasteur et al 2000)

It is possible that some BR patients have intrinsic differences which results in their neutrophils being more susceptible to undergoing NETosis and promoting citrullination in response to BR pathophysiology. Over time this would lead to the generation of higher quantities (and possible qualitative differences) of citrullinated self-peptide which may initiate an adaptive immune response and contribute to the onset of rheumatoid arthritis in some patients.

Given the lack of an appropriate animal model to study the impact of BR on NETosis and citrullination the best method to assess whether citrullination/NETosis is elevated in BROS patients would likely involve an appropriately powered pilot study. With interest in citrullination/NETosis increasing, owing partly to several studies identifying the phenomena as potential biomarkers and therapeutic targets for a range of diseases (Manzenreiter et al 2012, Obermayer et al 2014, György et al 2006), there may be potential soon for a pilot study of this kind to be carried out. However, due to a lack of existing data on NETosis, citrullination rates and general neutrophil function in the BR and BROS groups there is insufficient information available to power and inform the development of a methodology for such a pilot study.

The work presented in this chapter aimed to carry out a feasibility study to produce preliminary data on NETosis/citrullination responses by BR and BROS patient peripheral blood neutrophils in addition to assessing other aspects of neutrophil function (i.e. *in vitro* lifespan, CXCL8 secretion superoxide, phagocytosis rates) in these groups.

Whilst it would be arguably of greater relevance to study NETosis/citrullination responses in pulmonary neutrophils obtained from BR and BROS patients there are a number of clinical and technical difficulties in this approach. Therefore the responses of peripheral blood neutrophils were assessed (as a more pragmatic approach) before giving consideration to assessing these phenomena in airway sampled neutrophils.

7.1.2 *Hypothesis*

Peripheral blood neutrophils from BROS patients will show indications of higher levels of NETosis and citrullination than neutrophils from BR patients without comorbid rheumatoid arthritis.

7.2 Results

7.2.1 Patient *population demographics*

Initially the study was planned to involve age matched healthy volunteers and patients diagnosed with BR (including "BR only" diagnosis and BROS diagnosis) or RA. However, due to time constraints only a limited number of BR and BROS patients were recruited for use in the study. Although age matched healthy volunteers were not obtained for this study, samples/results obtained from healthy volunteers as part of a separate study (used in prior results chapters) were available to act as comparators to patient population results in this chapter. As these healthy volunteers were not age matched (ranging from 18-55 years of age) to the BR patients recruited, age differences between healthy volunteer results and BR patient results must be considered in interpretation of all results

A total of 15 bronchiectasis patients consented to take part in the study, with 2 patients being excluded from taking part due to failure to obtain peripheral blood. Samples were collected and processed blinded to disease category until all lab data relating to the samples was collected. Clinical data recorded by research staff was used to establish the demographics of the BR and BROS patient subgroups within the recruited cohort (*table 7.1*). The BR population had a mean age of 62.7 (SEM: 4.8) years of age at the time of recruitment, with the BROS having a similar mean age of 65.3 (SEM: 6.4). All other key clinical characteristics (including BSI) for the two patient groups showed a great deal of overlap, with the only distinct difference being in gender distribution.

Additional information was gathered on other variables which may impact patient results including patient comorbidities and colonisation status (described on an individual patient basis in table 7.2). As can be seen in the table the majority of the BR subgroup presented without any comorbidities (with only one patient presenting with COPD) and are classified as idiopathic bronchiectasis patients. In relation to colonisation status there also appears to be a difference between the BR and BROS subgroup, where none of the BROS patients were positive for colonisation status whereas there are two individual cases of colonisation by *E. coli* and *H. influenzae* in the BR patient subgroup.

In addition to the information on colonisation status, information on individual participant medication usage was also recorded to better characterise differences in treatments received between the groups (see *table 7.3*). This included current antibiotic therapies as well as current and recent (<18 months) treatment with RA targeting therapeutics and steroidal anti-inflammatory medication. No patient in either subgroup was reported as having received leflunomide, methotrexate and hydroxychloroquine, however some patients in both groups were reported as having received other medications including: nebulised antibiotics, long term macrolide therapy, rituximab and prednisolone.

In summary, the two groups appear to be matched on several key demographics however some differences were observed in gender distribution, colonisation status and medication usage within the subgroups. As this work was relatively small in scale (i.e. aiming to establish preliminary date) these differences are not currently of great concern in the context of the work. However future work building on this research should take note of potential differences in these individual variables between groups.

Table 7.1 Demographic information for bronchiectasis and BROS patients recruited for study. Information on a variety of clinical characteristics were recorded by research staff during recruitment of patients to take part in the study (± = SEM, [A] n=8 , [B] n=2)

Characteristics	Bronchiectasis patients (n=10)	BROS patients (n=3)
Age at consent	62.7 ± 4.8	65.3 ± 6.4
Percentage male	56%	0%
BMI	27.1 ± 1.2	23.2 ± 0.1
BSI Scores	7.4 ± 1.2[A]	5.5 ± 0.4[B]
Number of exacerbations in the last 12 months	2.9 ± 0.6	2 ± 0.00
FEV1	2.1 ± 0.2	2.2 ± 0.2
FEV1 % Predicted	75.7 ± 6.7	96.1 ± 18.6
SGRQ - Symptoms	58.2 ± 8.5	34.0 ± 13.5
SGRQ - Activity	40.6 ± 10.5	60.5 ± 15.6
SGRQ - Impacts	33.4 ± 8.6	27.5 ± 6.1
SGRQ - Total	39.7 ± 8.6	39.1 ± 8.8

Table 7.2 Demographic information and colonisation status for individual patients within the bronchiectasis and BROS subgroups. The demographic info (e.g. age, BMI, gender) for each individual participant in the two disease subgroup was documented in addition to the followingred for each individual including: BSI scores, number of exacerbations in the past year, whether participants are diagnosed with osteoporosis and the colonisation status of participants (in cases where the patient was able to produce sputum for analysis).

Bronchiectasis subgroup

Study Number	BMI	Comorbidities	Age	Male or Female	BSI	Known osteoporosis?	No. exacerbations in last 12 months	Colonisation status
01/003	20.8	None	71	M	9	No	4	*E. coli*
01/005	29.7	None	80	F	10	Yes	2	Negative
01/006	32.2	None	50	F	5	No	3	Negative
01/009	22.1	None	71	M	8	No	2	*H. influenzae*
01/010	28.5	None	79	M	8	Yes	0	Negative
01/011	22.8	COPD	63	F	15	No	4	Negative
01/012	29.6	None	60	F	5	No	3	N/A (Difficulty expectorating)
01/013	27.1	None	66	M	NA	Yes	7	N/A (Difficulty expectorating)
01/014	26.1	None	38	M	4	No	3	Negative
01/015	27.6	None	49	F	3	No	2	Negative

BROS subgroup

Study Number	BMI	Comorbidities	Age	Male or Female	BSI	Known osteoporosis?	No. exacerbations in last 12 months	Colonisation status
01/001	23.4	RA	64	F	NA	No	2	N/A (Difficulty expectorating)
01/004	22.9	RA	55	F	6	No	2	Negative
01/008	23.2	RA	77	F	5	Yes	2	Negative

Table 7.3 Reported medication usage by patients in the Bronchiectasis and BROS subgroups. Information was gathered for each participant on their current and/or recent (within past 18 months of enrolment) prescribed medications. These covered a range of standard of care medications used in the treatment of bronchiectasis and rheumatoid arthritis (i.e. antibiotics, RA therapies, steroidal anti-inflammatories). As expected there are some differences in medications used between the groups, most notable for rituximab which can be expected given its use as an RA therapeutic.

Bronchiectasis subgroup

Study Number	Current therapies				Current or Recent (past 18 months) therapies		
	Nebulised antibiotic?	Long term macrolide?	Leflunomide?	Methotrexate?	Rituximab?	Hydroxychloroquine?	Long term prednisolone?
01/003	No	Yes	No	No	No	No	No
01/005	No	No	No	No	No	No	No
01/006	No	No	No	No	No	No	No
01/009	No	No	No	No	No	No	No
01/010	No	No	No	No	No	No	No
01/011	No	Yes	No	No	No	No	No
01/012	No	No	No	No	No	No	No
01/013	Yes	Yes	No	No	No	No	No
01/014	No	No	No	No	No	No	No
01/015	No	No	No	No	No	No	No

BROS subgroup

Study Number	Current therapies				Current or Recent (past 18 months) therapies		
	Nebulised antibiotic?	Long term macrolide?	Leflunomide?	Methotrexate?	Rituximab?	Hydroxychloroquine?	Long term prednisolone?
01/001	No	No	No	No	Yes	No	No
01/004	No	Yes	No	No	Yes	No	No
01/008	No	No	No	No	No	No	Yes

7.2.2 *Confirming purity of patient neutrophil isolations*

Isolation of peripheral blood neutrophils from BR patient whole blood samples was carried out using the same methodology used to isolate neutrophils from healthy volunteer whole blood by percoll gradient separation. Purity of neutrophils isolations of BR patient samples was assessed alongside a healthy parallel sample isolated on the same day by nuclear morphology counts (*figure 7.1*). Healthy volunteer blood preps were shown to have a mean neutrophil purity of 94.9% (95% CI: 93.8%, 96%), similarly BR patient blood preps had a mean purity of 93.8% (95% CI: 92.9%, 94.7%). There was no significant difference in purity values.

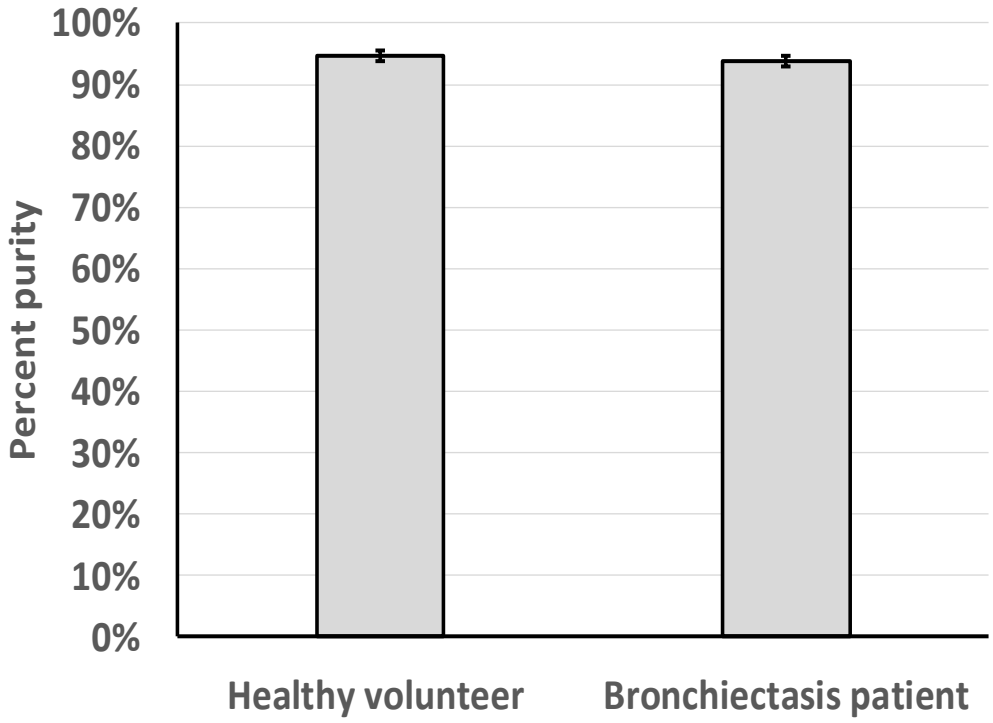

Figure 7.1 Purity of neutrophil isolations from whole blood of healthy volunteer and bronchiectasis patient samples. Purity of neutrophil isolations were seen to be similar in both healthy volunteer and bronchiectasis patient samples. With the mean purity of both results being above the arbitrary cut off point for work involving the study of neutrophils (i.e. 85% purity).

7.2.3 *PAD activity in Bronchiectasis patients*

Similar to results obtained from healthy volunteers, stimulation of BR derived peripheral blood neutrophils resulted in a statistically significant increase in supernatant PAD activity for PMA ($p<0.001$), LPS ($p<0.001$) and *P. aeruginosa* WCLs ($p<0.01$), however BR patient neutrophils were also observed to have a significant increase in supernatant PAD activity for *H. influenzae* WCLs stimulated neutrophils ($p<0.05$). In comparison to supernatant PAD activity for stimulated healthy volunteer neutrophils (previously presented in chapter 4), mean neutrophils supernatant PAD activity in bronchiectasis patient derived samples was observed to be higher following stimulation with PMA, LPS, *P. aeruginosa* WCLs and *H. influenzae* (*figure 7.*2). However only *H. influenzae* stimulated BR neutrophils were suggested to have significantly higher supernatant PAD activity than healthy volunteer neutrophils.

Comparison of BR and BROS patient groups showed no significant difference in PAD activity in any of the assayed conditions, with a large amount of overlap seen in the error bars for the results obtained from the two patient groups (*figure 7.3*). Collectively these results suggest extracellular PAD activity increases in BR patient peripheral blood neutrophils following stimulation with relevant stimuli (as was shown in healthy neutrophil responses to the same stimuli). However, increased citrullination in response to *H. influenzae* WCLs was only seen in BR patient neutrophils. Differences in neutrophils extracellular PAD activity between patients diagnosed with BR and BROS patients was minimal with no significant difference being shown between the two groups in response to any of the tested stimuli.

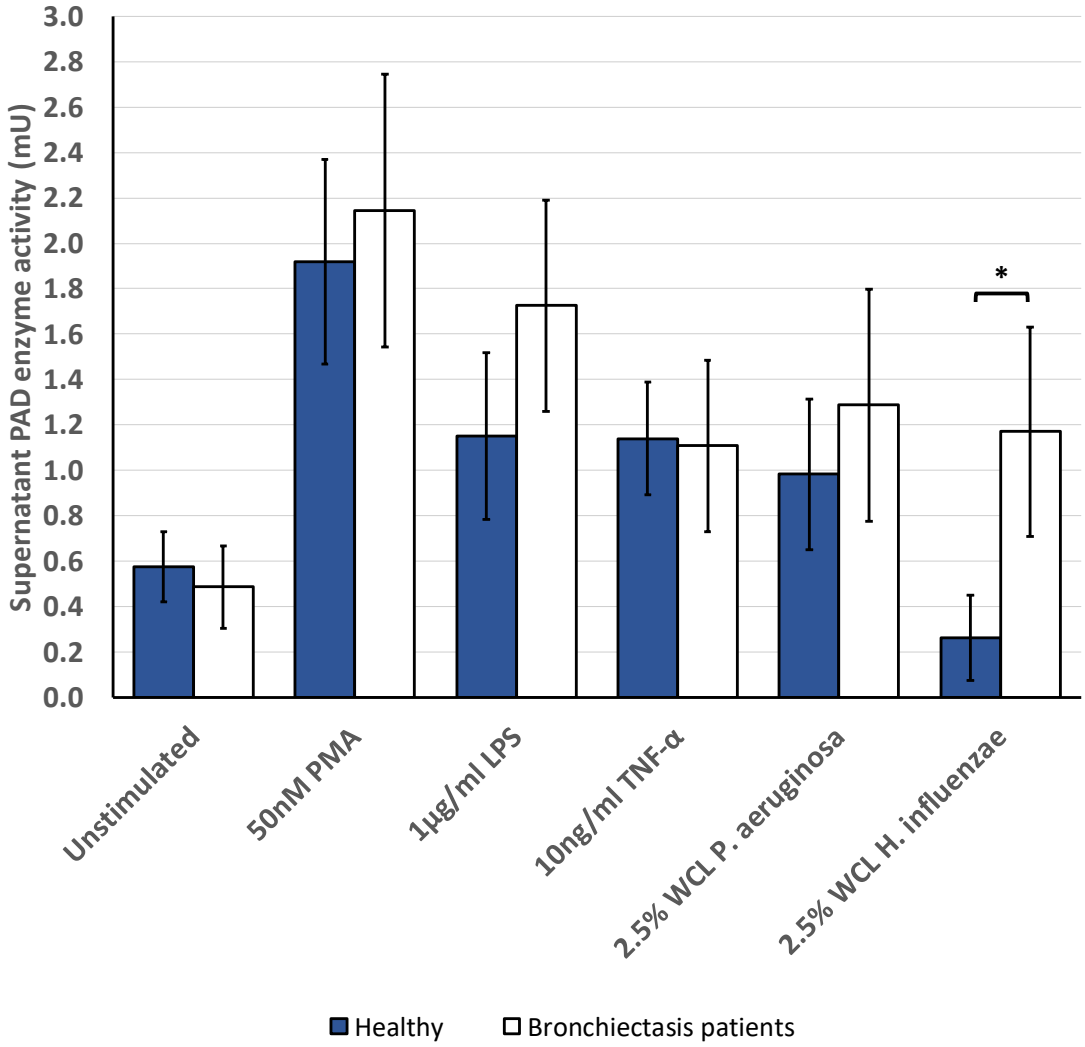

Figure 7.2 Mean supernatant PAD activity in neutrophils from healthy volunteers and bronchiectasis patients. Supernatant PAD activity was assessed in healthy neutrophils either unstimulated (n=16) or incubated with PMA (n=16), LPS (n=16), TNF-α (n=10), WCL P. aeruginosa (n=16) and WCL H. influenzae (n=6). Supernatant PAD activity in response to the same stimuli was assessed for bronchiectasis patient neutrophils (all conditions n=13). Comparing supernatant PAD activity in healthy and bronchiectasis derived neutrophils suggested only H. influenzae resulted in a significant difference between the two groups (* $p < 0.05$, +/- 95% CI).

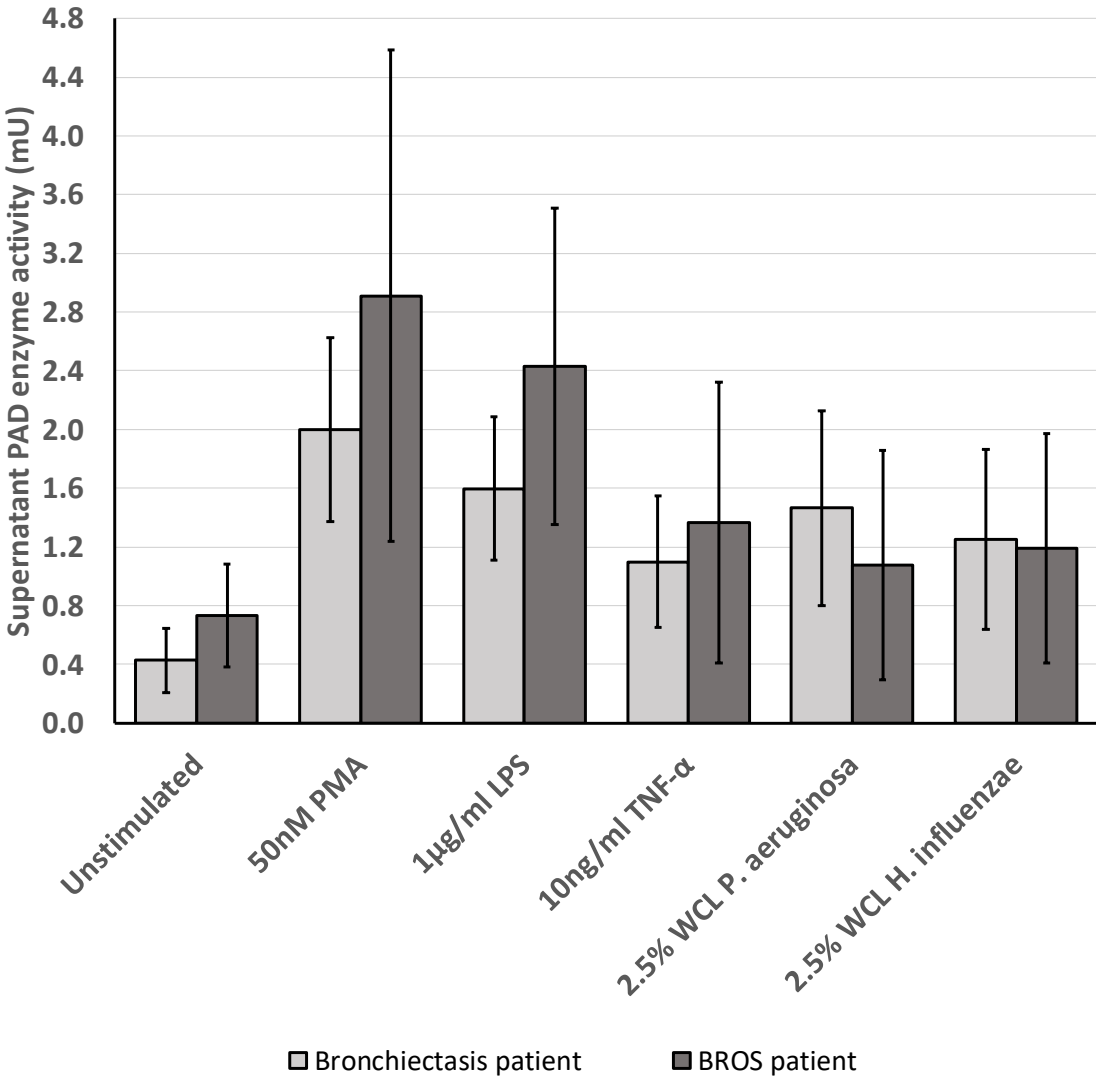

Figure 7.3 Mean supernatant PAD activity in neutrophils from bronchiectasis and BROS patients. Mean supernatant PAD activity was reportedly higher in BROS patient neutrophils (n=3) in comparison to bronchiectasis patient neutrophils (n=10), however none of these differences were shown to be statistically significant (+/- 95% CI).

7.2.4 *Extracellular DNA release in Bronchiectasis patients*

Extracellular DNA release (determined by SYTOX dependent fluorescence) in patient samples significantly increased following stimulation with PMA and LPS ($p<0.001$). No significant increase in DNA release (in comparison to unstimulated neutrophils) was observed following stimulation with fMLP and TNF-α. Mean DNA release in all stimulatory conditions for patient derived neutrophils showed similar levels of relative extracellular DNA release in comparison to healthy volunteer derived neutrophils (*figure 7.4*), suggesting no difference in NETosis rates between healthy volunteers and patient peripheral blood neutrophils. Comparison of extracellular DNA release in BR patients and the BROS patient subgroup showed no difference in DNA release in all conditions, with a large degree of overlap being seen in the 95% confidence intervals for the two groups (*figure 7.5*).

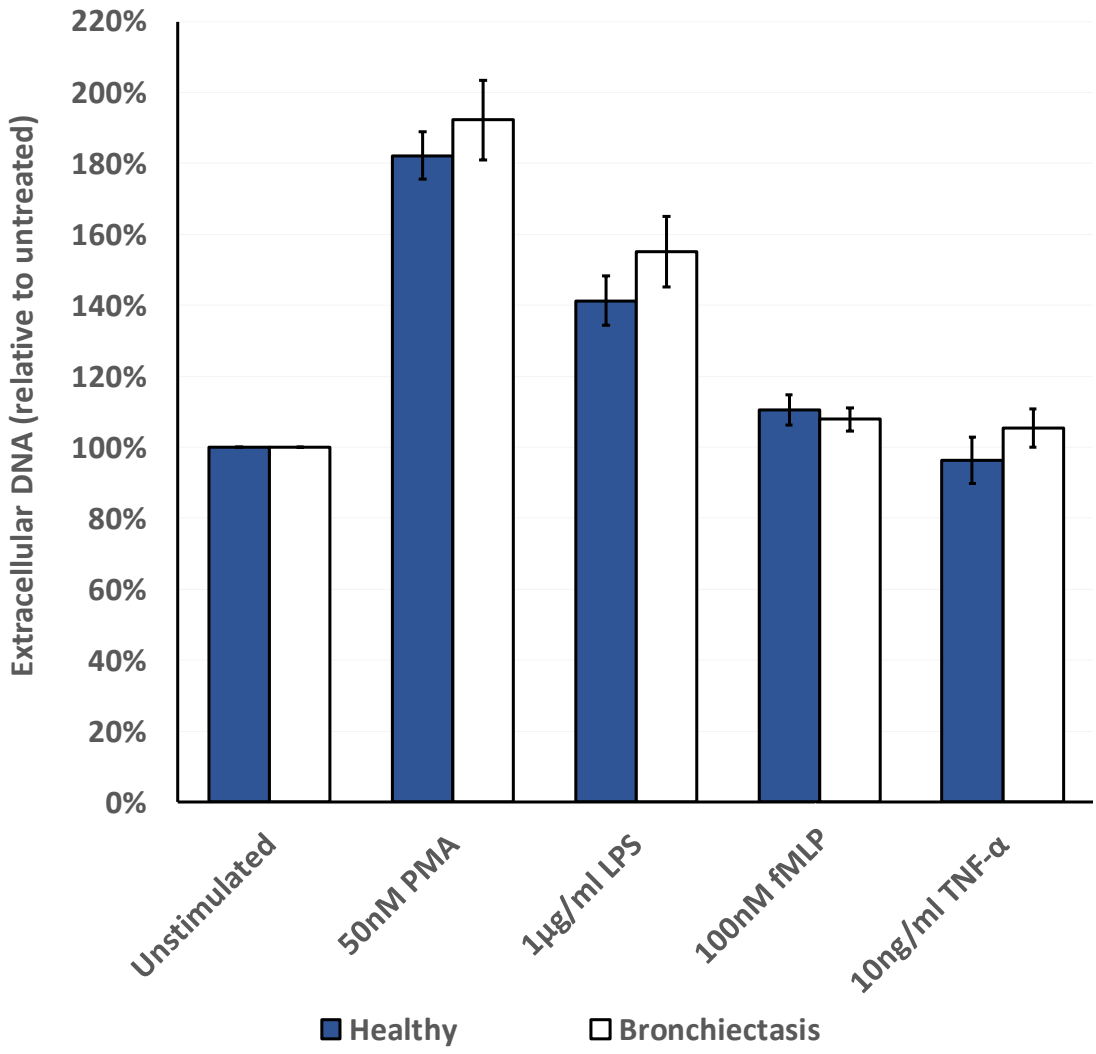

Figure 7.4 Extracellular DNA release in healthy volunteer and bronchiectasis patient peripheral blood neutrophil samples. Bronchiectasis patient peripheral blood neutrophils (n=13) showed significant increases in extracellular DNA release following stimulation with PMA and LPS. No significant differences were found between patient and healthy volunteer (n=6) neutrophil DNA release for any assayed conditions (+/- 95% CI).

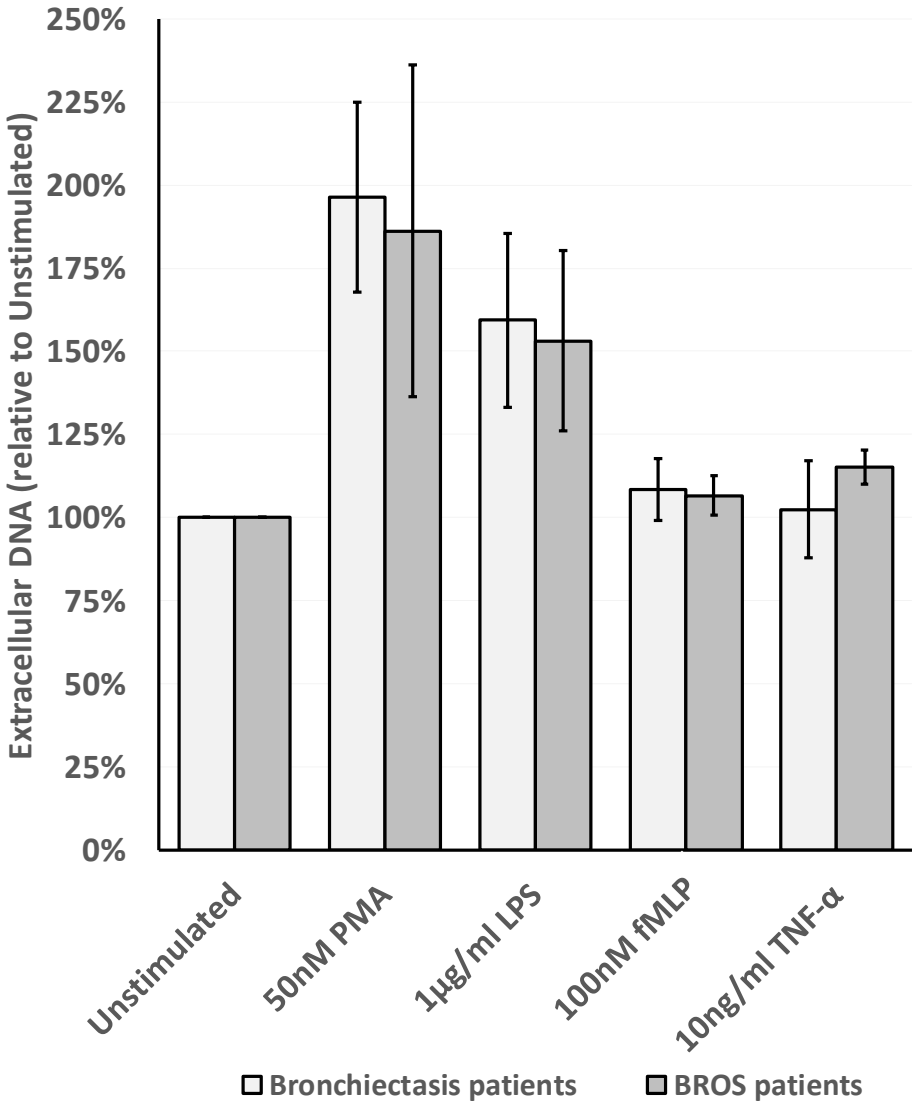

Figure 7.5 Extracellular DNA release in neutrophils obtained from Bronchiectasis and BROS patients. Bronchiectasis (n=10) and BROS (n=3) patient neutrophils showed similar levels of extracellular DNA release in response to stimulation with no significant differences being detected (+/- 95% CI).

7.2.5 *Supernatant NET concentration in bronchiectasis samples*

Relative NET concentration in culture supernatants was assessed for all collected bronchiectasis patient samples and healthy volunteer comparators. Patient neutrophil supernatants were shown to have significantly higher levels of NETs in supernatant following stimulation with; PMA ($p<0.001$), LPS ($p<0.001$), TNF-α ($p<0.05$), *P. aeruginosa* WCLs ($p<0.001$) and *H. influenzae* WCLs ($p<0.001$). In comparison to healthy volunteer results, patient supernatant samples were shown to have a significantly lower number of NETs when unstimulated ($p<0.001$) or stimulated with fMLP ($p<0.001$) and TNF-α ($p<0.01$) (***figure 7.6***).

BR and BROS subgroups showed similar levels of NETs present in supernatant samples for the majority of stimulatory conditions, however BROS patients were observed to have significantly higher amounts of supernatant NETs following stimulation with fMLP ($p<0.05$) and *H. influenzae* WCLs ($p<0.05$) (***figure 7.7***). Which suggests that NET formation may be elevated in BROS patient neutrophils in response to certain stimuli.

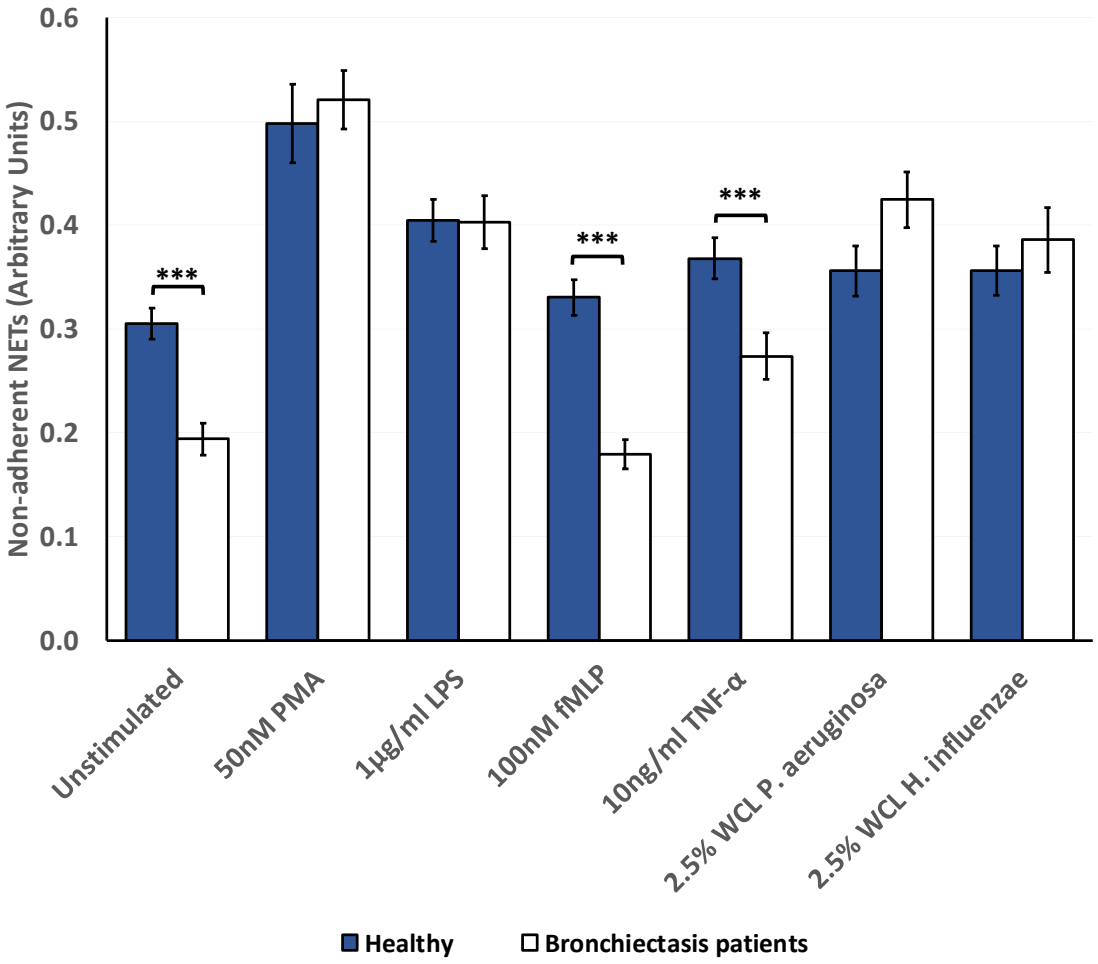

Figure 7.6 Supernatant NET ELISA results for healthy volunteer and bronchiectasis patient neutrophils. Healthy volunteer neutrophil supernatants (n=16) had similar relative amounts of non-adherent NETs to samples from recruited bronchiectasis samples (n=13) in several conditions. However significant differences were seen in the two groups for samples obtained from unstimulated, fMLP stimulated and TNF-α stimulated neutrophils ($p < 0.001$) (+/- 95% CI).

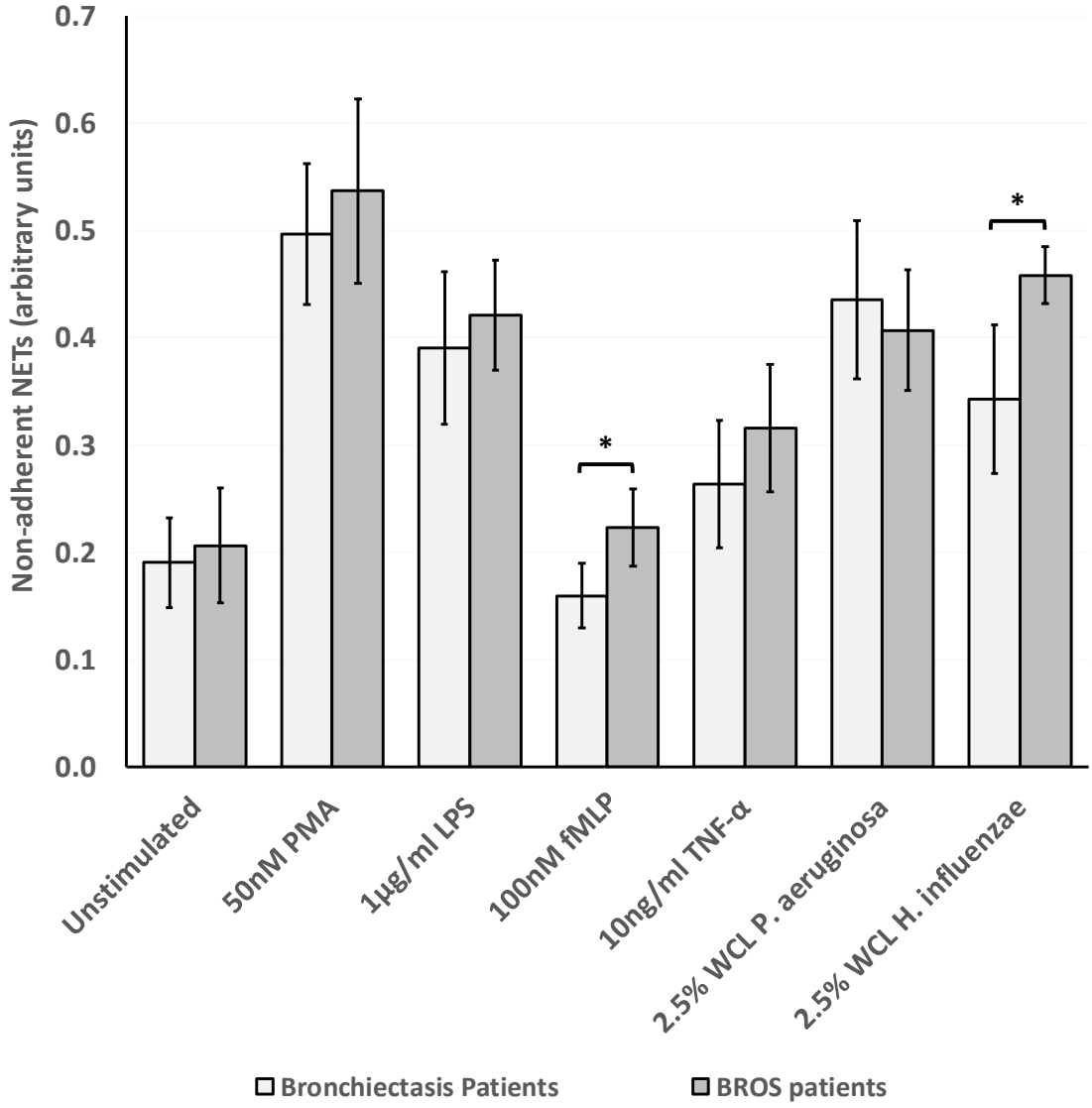

Figure 7.7 Supernatant NET ELISA results for Bronchiectasis and BROS patient neutrophils. BROS patient neutrophil supernatants (n=3) had a significantly higher amount of NETs than BR patient samples (n=10) following stimulation with fMLP and WCLs of H. influenzae ($p < 0.05$) (+/- 95% CI).

7.2.6 *Adherent NETosis rates in bronchiectasis patient samples*

IF counts were used to determine the proportion of adherent neutrophils that had fully underwent NETosis. Patient neutrophils showed similar results to NET counts for healthy volunteer neutrophils, with all stimulatory conditions (excluding fMLP) resulting in a highly significant increase ($p < 0.001$) in the number of adherent NETs formed in comparison to unstimulated neutrophils. No significant difference was found in the NETosis rates between healthy and patient neutrophils for all stimulatory conditions (*figure 7.8*).

Adherent NET formation rate results for BR and BROS patient subgroups showed no significant differences between the two groups (*figure 7.9*). This is reflected by the similar mean values determined for the two groups and large overlapping 95% confidence intervals in all conditions. Contradictory to the supernatant NET results for patient samples, this suggests no difference in NET formation between BR and BROS patient subgroups.

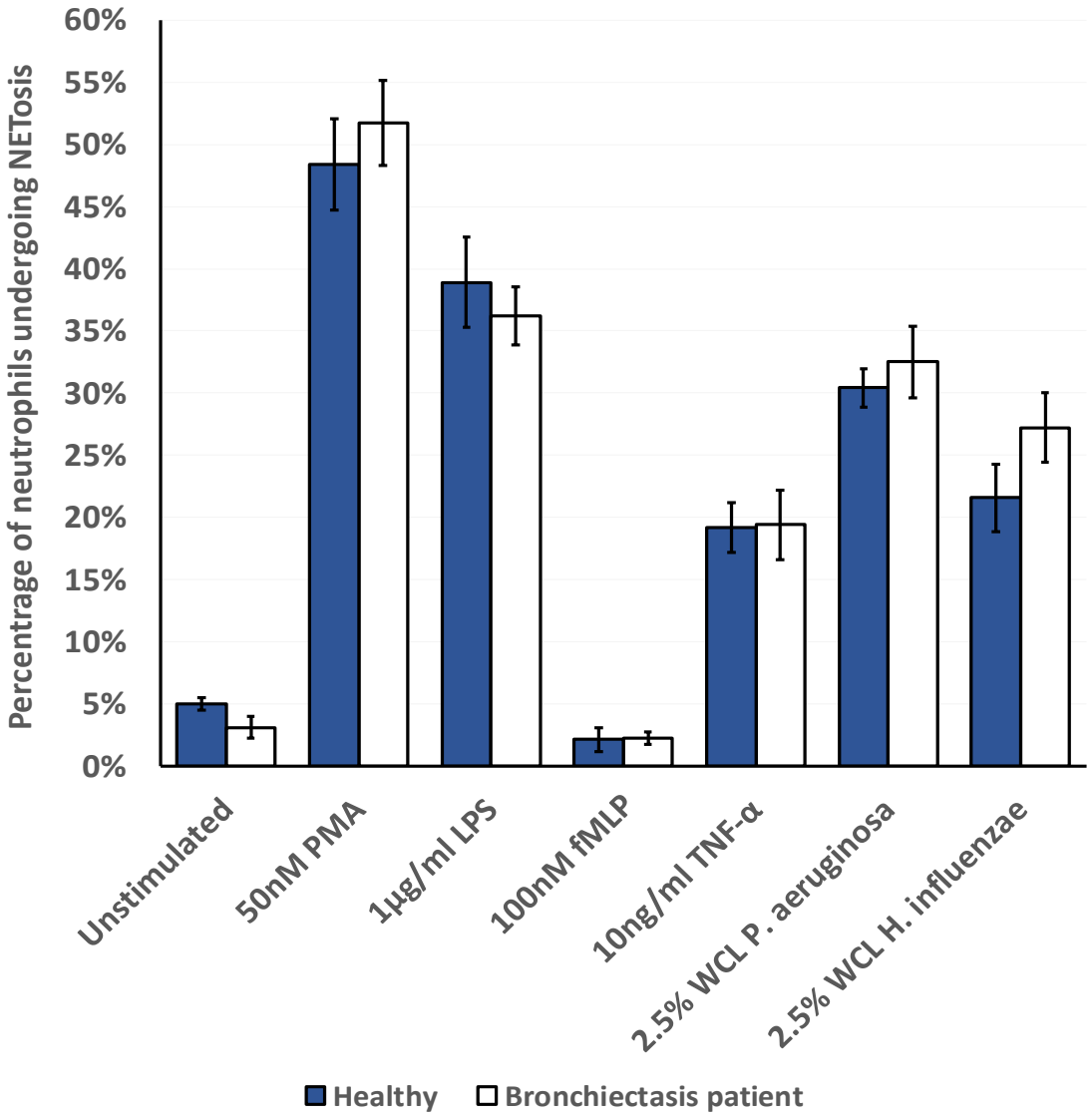

Figure 7.8 Adherent NET formation in healthy volunteer and Bronchiectasis patient samples. NETosis rates were significantly ($p<0.001$) higher in BR patient samples (n=13) following stimulation with PMA, LPS, TNF-α, P. aeruginosa and H. influenzae WCLs. No difference was observed in NETosis rates for healthy (n=6) and bronchiectasis peripheral blood neutrophil samples (+/- 95% CI).

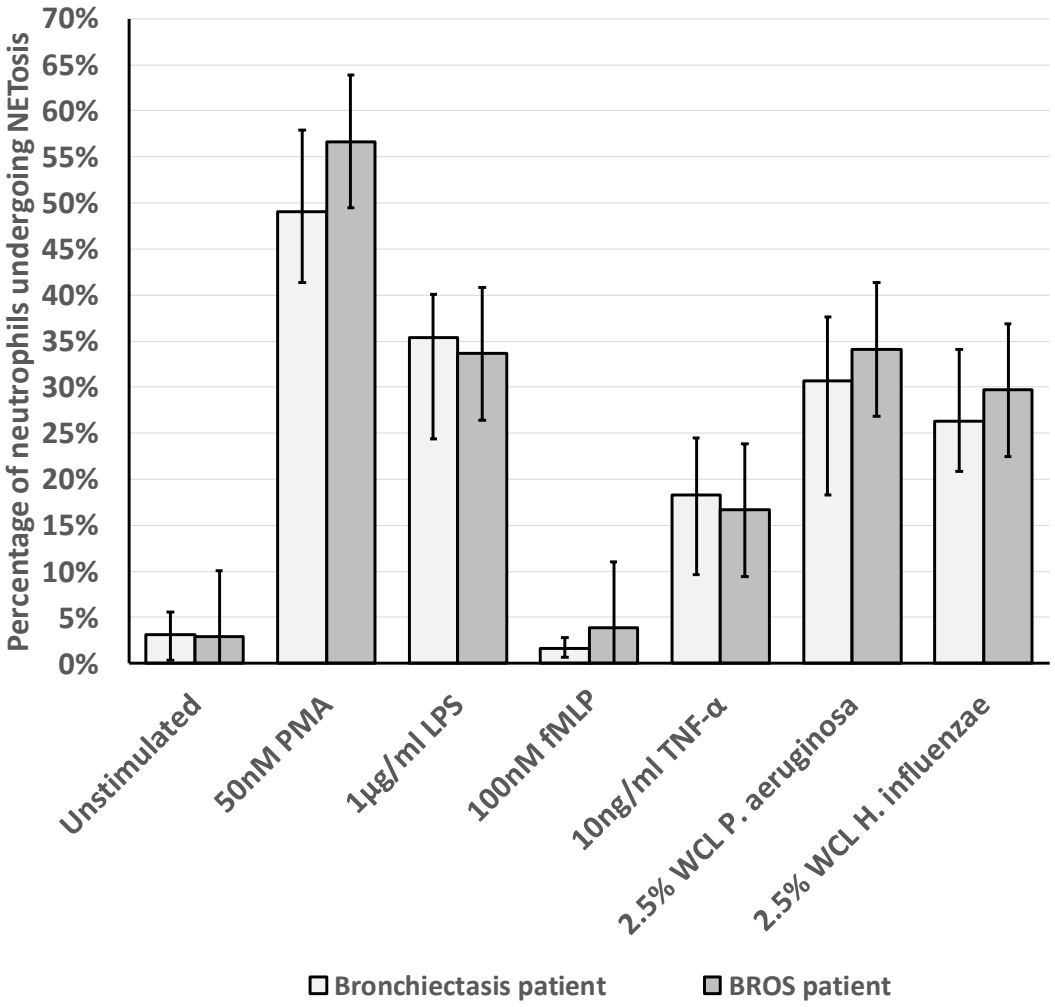

Figure 7.9 Adherent NET formation in Bronchiectasis and BROS patient subgroups. No significant difference was observed in NETosis rates for BR (n=10) and BROS patient (n=3) derived peripheral blood neutrophils (+/- 95% CI).

7.2.7 *In vitro lifespan differences between healthy and bronchiectasis patient neutrophils*

To investigate neutrophil function in patient peripheral blood neutrophils (in comparison to healthy volunteer peripheral blood neutrophils), viability of neutrophils was observed at 4-hour timepoints across a 24-hour period during *in vitro* incubation (as done in chapter 5). Although some slight differences were observed between the two groups (e.g. 4 and 8-hour BR viability being slightly lower, 16 and 20-hour viability being slightly higher), no significant difference in viability was shown between healthy and bronchiectasis patient neutrophils at any of the measured timepoints (*figure 7.10*). Which suggests there is no difference in *in vitro* viability between the two groups.

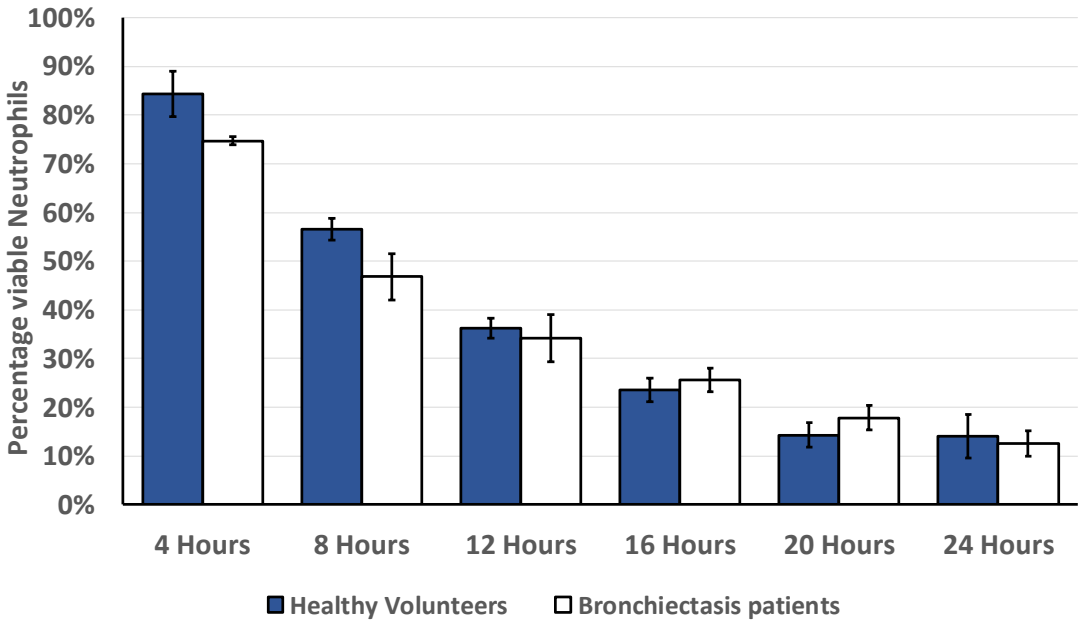

Figure 7.10 Viability of healthy and bronchiectasis peripheral blood neutrophils across a 24-hour period of in vitro incubation. As expected viability decreased over the 24-hour in vitro incubation period for both healthy (n=3) and bronchiectasis patients (n=3). No significant difference was observed in viability between the two groups during the 24-hours assessment period (+/- 95% CI).

7.2.8 CXCL8 *secretion by bronchiectasis patient peripheral blood neutrophils*

CXCL8 secretion by bronchiectasis patient neutrophils was significantly higher when stimulated by PMA ($p<0.001$), LPS ($p<0.05$), TNF-α ($p<0.01$), *P. aeruginosa* WCLs ($p<0.05$) and *H. influenzae* ($p<0.05$), similar to the trend in the results seen in healthy peripheral blood neutrophils. Mean CXCL8 neutrophil supernatant concentration was observed to be higher in all conditions for patient derived samples in comparison to healthy comparator results (*figure 7.11*). However, of all the assayed conditions significant increases in CXCL8 concentration was only observed in patient neutrophils when left unstimulated ($p<0.001$) or stimulated with TNF-α ($p<0.01$), *P. aeruginosa* WCLs ($p<0.01$) and *H. influenzae* WCLs ($p<0.01$). This suggests that suggests that CXCL8 secretion is elevated in bronchiectasis patient derived peripheral blood neutrophils in comparison to healthy peripheral blood neutrophils.

Comparison of CXCL8 neutrophil supernatant concentrations for BR and BROS patient derived samples showed the mean CXCL8 concentration of BROS samples was in most conditions lower than that seen in BR patient samples (excluding LPS) (*figure 7.12*). However, none of these differences were shown to be statistically significant. As the confidence intervals for all results (excluding unstimulated) were extremely large and overlapping (owing to the limited sample size available for the two groups) this cannot be used to form accurate predictions on potential differences in CXCL8 secretions between the patient subgroups.

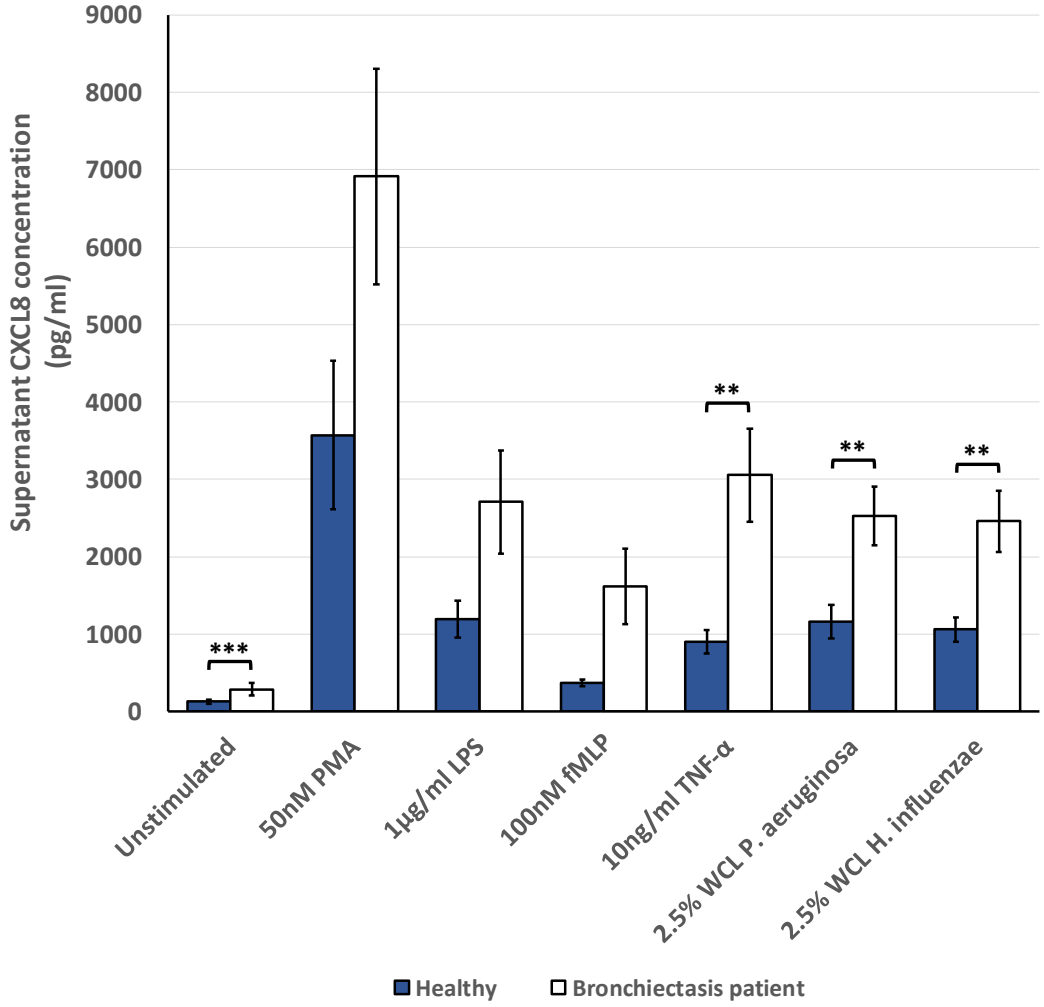

Figure 7.11 CXCL8 supernatant concertation in stimulated neutrophils obtained from healthy volunteers and bronchiectasis patients. BR patient (n=11) CXCL8 supernatant concentration was significantly higher neutrophils following stimulation with all conditions excluding fMLP (in comparison to unstimulated neutrophils). BR patient neutrophils were observed to have significantly higher CXCL8 supernatant concentrations than healthy neutrophil samples (n=12) in the following conditions; unstimulated, TNF-α, P. aeruginosa WCLs and H. influenzae WCLs (** $p < 0.01$, *** $p < 0.001$, +/- 95% CI).

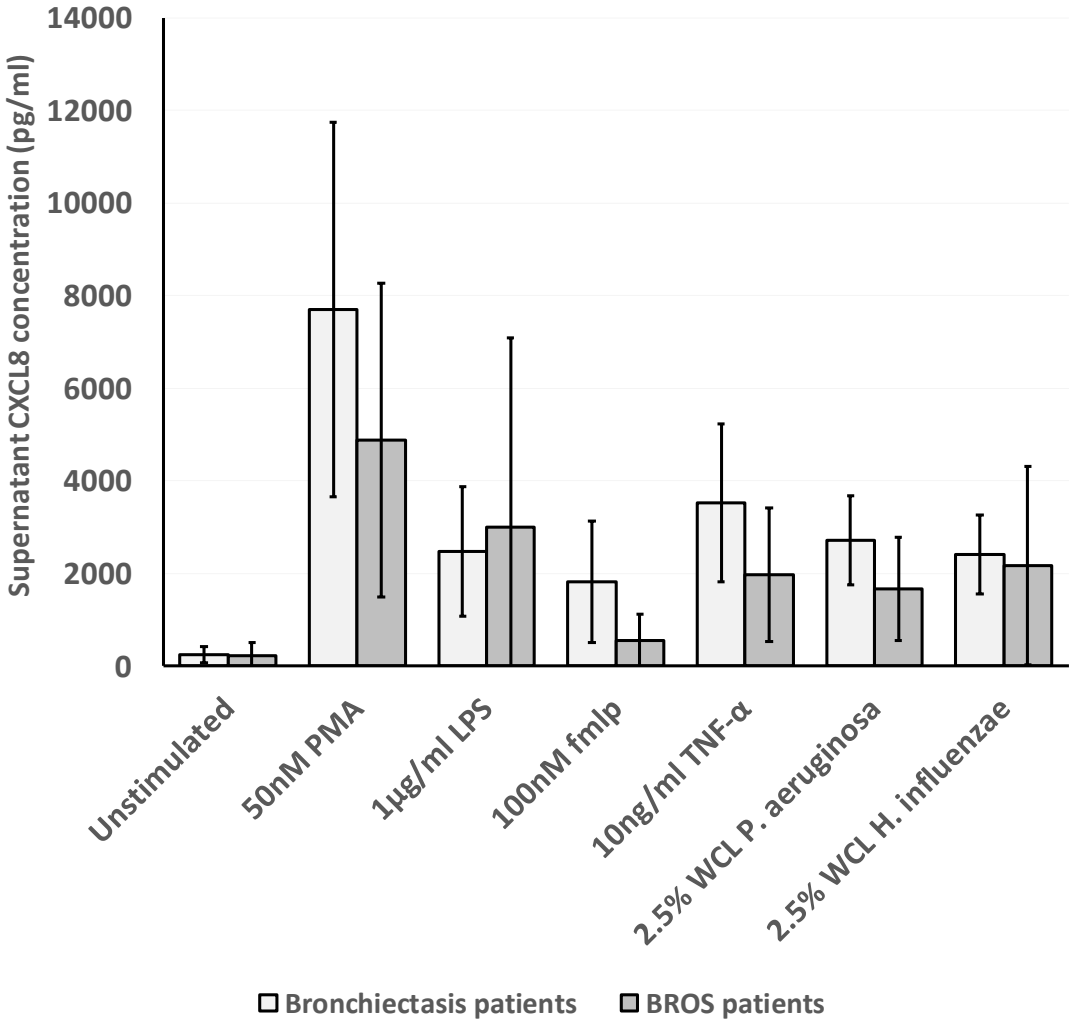

Figure 7.12 CXCL8 supernatant concertation in bronchiectasis and BROS patient samples. Differences in mean supernatant CXCL8 concentration were observed between bronchiectasis (n=8) and BROS (n=3) results, however none of these differences were shown to be statistically significant (*** $p < 0.001$, +/- 95% CI).

7.2.9 *Superoxide production by patient neutrophils*

As previously shown with healthy volunteer peripheral blood neutrophils (chapter 4), bronchiectasis neutrophils produced significantly ($p<0.001$) larger quantities of superoxide anions following stimulation with PMA (14.34nM, 95% CI: 12.67, 16.01), LPS (8.89nM, 95% CI: 7.31, 10.46) and fMLP (6.06nM, 95% CI: 4.53, 7.58) in comparison to unstimulated neutrophils (2.43nM, 95% CI: 1.53, 3.33). Mean superoxide anion release for healthy and bronchiectasis patients were similar for all conditions with no results showing statistical significance (*figure 7.13*). Comparison of BR and BROS subgroups results showed little difference in mean superoxide anion release across all stimulatory conditions with no significant difference being shown (*figure 7.14*).

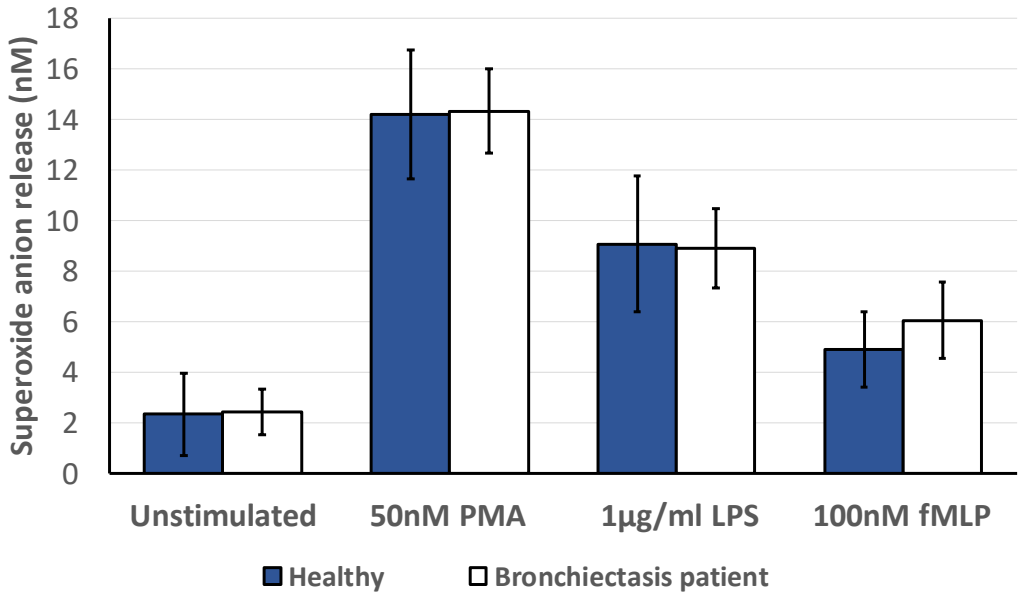

Figure 7.13 Superoxide production by healthy and bronchiectasis patient neutrophils. All stimulatory conditions caused a significant ($p<0.001$) increase in superoxide production in bronchiectasis patient (n=13). Healthy volunteer derived neutrophils (n=8) produced similar results with no significant differences between healthy and bronchiectasis samples being detected (+/- 95% CI).

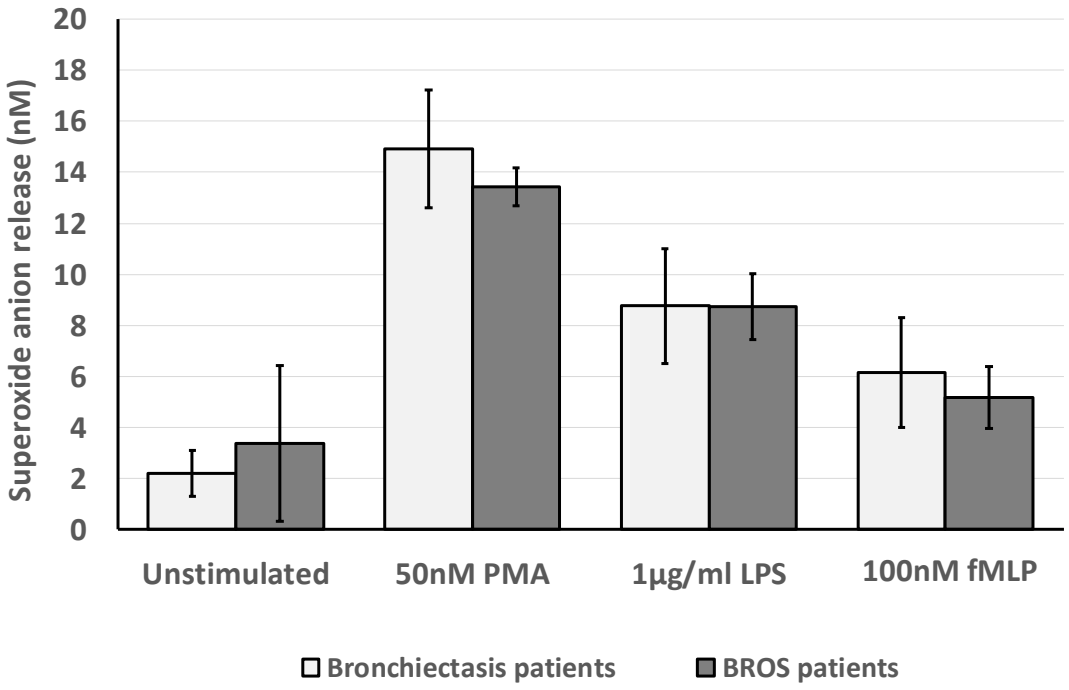

Figure 7.14 Superoxide production by bronchiectasis and BROS patient neutrophils. Superoxide production was similar across all conditions for neutrophils from bronchiectasis patients (n=10) and BROS patients (n=3) (+/- 95% CI).

7.2.10 *Phagocytosis rates in patient neutrophils*

Phagocytosis rates were assessed in neutrophils obtained from healthy volunteers and the recruited bronchiectasis patients. In addition, phagocytosis rates in healthy volunteers incubated with dexamethasone, a clinically used steroid argued to suppress phagocytosis of zymosan particles (Mlambo et al 2003), was also assessed as an additional comparator for patient results. Healthy neutrophils had a mean zymosan phagocytosis rate of 75.7% (95% CI: 71.5%, 79.9%), 30-minute incubation (pre-stimulation) with dexamethasone resulted in the mean phagocytosis rate in the same healthy volunteer samples decreasing to 61.6% (95% CI: 57.9%, 65.3%).

BR patient neutrophils has a mean phagocytosis rate of 66.4% (95% CI: 64.0%, 68.8%), which was significantly ($p<0.001$) lower than uninhibited neutrophils healthy neutrophils (*figure 7.15*). Comparison of BR and BROS phagocytosis rates showed similar mean values (63.6% and 64.6% respectively) with no statistically significant difference between the two being detected (*figure 7.16*).

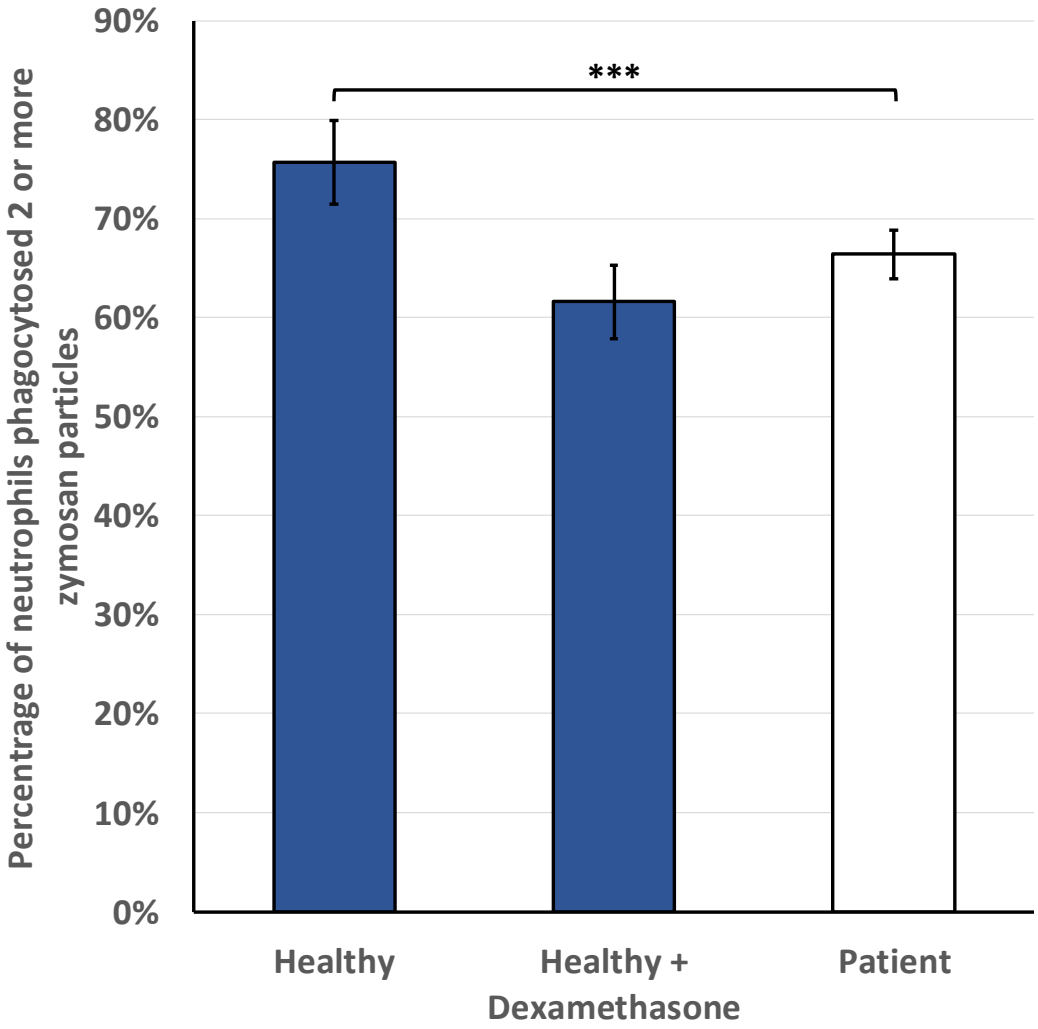

Figure 7.15 Phagocytosis rates for uninhibited and dexamethasone inhibited healthy volunteer neutrophils in comparison to bronchiectasis patient neutrophils. Phagocytosis rates in healthy volunteers (n=6) was shown to decrease following treatment with dexamethasone (n=6). Rates in patient neutrophils were significantly lower than that of uninhibited healthy neutrophils (n=13) (*** $p < 0.001$, +/- 95% CI).

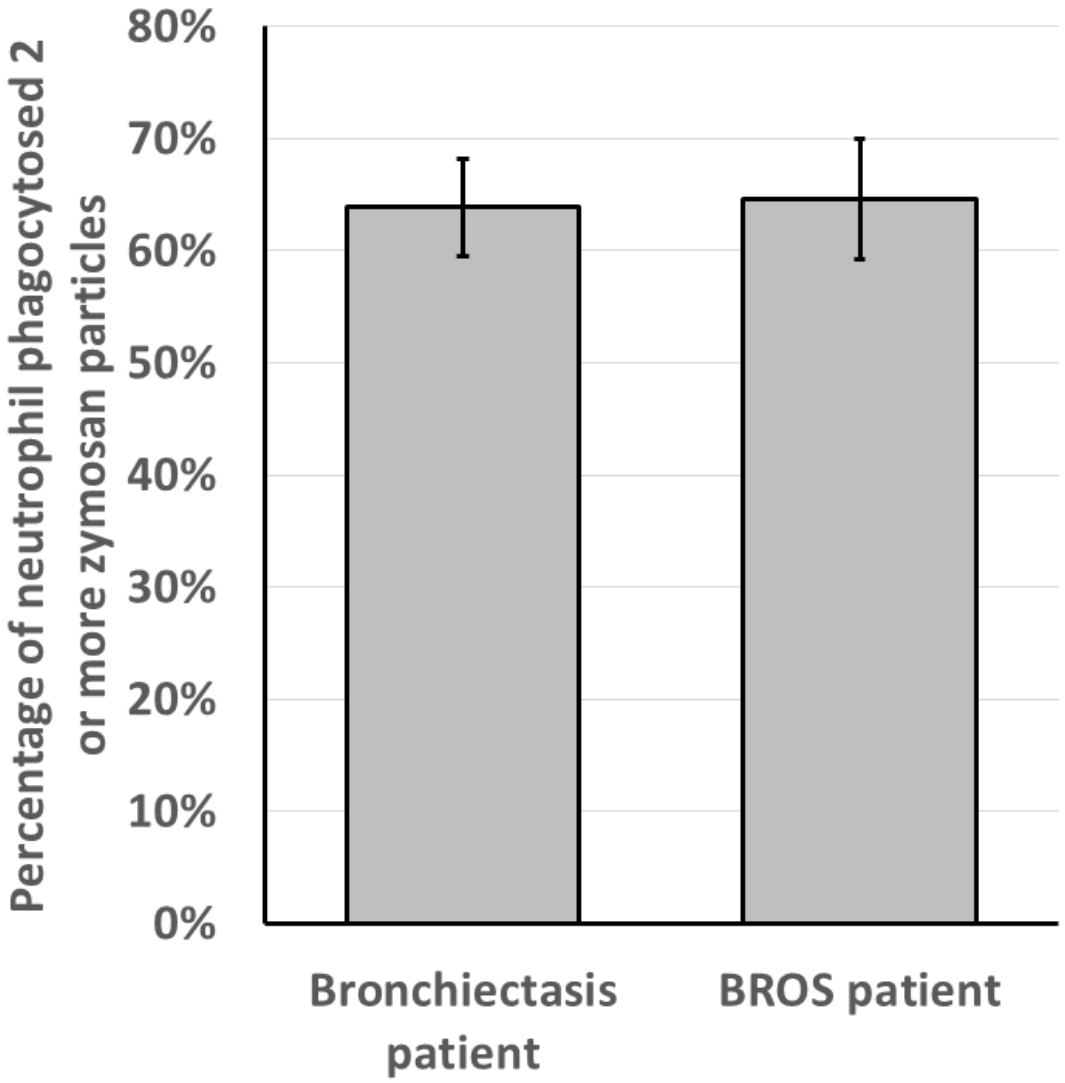

Figure 7.16 Phagocytosis rates for bronchiectasis and BROS patient derived neutrophils. Phagocytosis rates for BR (n=10) and BROS (n=3) patient subgroups were highly similar with no statistically significant difference between the groups being shown (+/- 95% CI).

7.2.11 *Summary*

The primary aim of this chapter was to utilise established methodologies and available patient samples to establish preliminary data in relation to extracellular citrullination, NETosis rates, *in vitro* lifespan, CXCL8 secretion, superoxide production and phagocytosis rates in the BR and BROS patient groups, which has been successful. The produced results suggest there may be some potential difference between healthy and bronchiectasis peripheral blood neutrophils in relation to citrullination, NETosis, CXCL8 production and phagocytosis. Data comparing the BR and BROS patient groups predicts relatively little differ (excluding one indication of increased NETosis in response to *H. influenzae* WCLs). Due to limitations of this study (i.e. sample size and demographics) interpretation of this work becomes difficult.

7.3 Discussion

7.3.1 *Comparison of healthy and bronchiectasis peripheral blood neutrophil responses*

Several results obtained suggested differences in neutrophil responses between healthy volunteer and BR patient samples. Citrullination rates were largely similar between the two groups, with one clear difference being PAD activity in response to *H. influenzae* WCL stimulation where patient neutrophils supernatant PAD activity was significantly higher than healthy neutrophil PAD activity rates.

Results for NETosis rates between the groups were conflicting. Extracellular DNA measurements and IF NET results showed no difference in NET formation between healthy and patient samples, however measurement of free NETs present in neutrophil supernatants (NET ELISA) suggested there were significantly fewer NETs in BR patient supernatant for several stimulatory conditions. As the NET ELISA assay is only semi-quantified and cannot provide an exact measurement of the decrease in the amount of NETs present in each sample, and given that two of the three methodologies consistently suggested there was no difference in NETosis, with one of these methods (IF microscopy) being one of the best-established methods for measuring NETosis, the data collectively supports the conclusion that there is no large difference in NET formation between healthy and BR patient peripheral blood neutrophils.

Neutrophil biology was similar in several aspects between the two population samples. No clear difference was shown in the 24-hour *in vitro* neutrophil viability rates, although limited sample size (n=3) may have some impact on this result. Similarly, no difference was seen in superoxide anion production between healthy and BR peripheral blood neutrophils, suggesting this aspect of neutrophil function is unchanged in peripheral blood neutrophils in bronchiectasis patients, which is to be expected given the lack of published data suggesting otherwise.

BR peripheral blood neutrophils may have had differences to healthy volunteers in two aspects; CXCL8 secretion and phagocytosis rates. BR peripheral blood neutrophil secreted significantly greater quantities of CXCL8 than healthy neutrophils following stimulation with disease relevant stimuli, with TNF-α induced CXCL8 secretion being 3-fold higher in BR neutrophils. Although all conditions showed higher levels of CXCL8

secretion in BR patient neutrophils, only four conditions (unstimulated, TNF-α, *P. aeruginosa* and *H. influenzae*) showed a significant difference. This lack of statistical significance in other stimulatory conditions may however be attributed to low sample size for patient samples which would need to be addressed in further study.

Phagocytosis rates in BR patient neutrophils was also shown to be significantly lower than those seen in healthy volunteers, with the mean phagocytosis rate being near that of healthy peripheral blood neutrophils inhibited by dexamethasone. Whilst these results present interesting differences between the healthy and BR derived neutrophils, there are several important considerations regarding the demographics of the two groups in the interpretation of these results.

The healthy volunteers whose blood was obtained for use in this study were not age-matched to bronchiectasis patients. As a result, the two groups likely differ greatly in mean age and age distribution. The recruited BR patient group has a mean age of 63.3 years old (95% CI: 56.4, 70.2), with the lowest age recorded being 38 years old. Healthy volunteers were recruited to take part in the study providing they were over that age 18 (with many volunteers coming from the research institute/university). Meaning there is likely great difference in the average age between the two groups. This becomes problematic in regard to the difference in phagocytosis results between the groups as published work has previously suggested neutrophil phagocytic ability decreases as age increases (Chiu et al 2011, Wei Li 2013). Therefore, it is possible that this difference may be a consequence of age differences between rather than disease state.

In addition, it is well established that the BR population is highly heterogenous in regard to several clinical aspects (ten Hacken 2007). Therefore, the limited sample size of BR patients recruited to take part in this work (n=13) means the results should be interpreted with caution. However, given this work was carried out as a feasibility study with the aim to establish preliminary data to guide further work, rather than conclusively establish differences between healthy volunteers and BR patient peripheral blood neutrophil responses, these issues of age matching and sample size do not undermine the value of the data acquired but instead serve as points for consideration in the design of future studies in this field.

7.3.2 *Bronchiectasis and BROS peripheral blood neutrophils responses*

Comparison of peripheral blood neutrophil responses showed no obvious differences in supernatant PAD activity, *in vitro* viability, CXCL8 secretion, superoxide and phagocytosis between the BROS and BR patient samples; the sample sizes however are limited meaning no firm conclusions can be made. Whilst NETosis results collected using extracellular DNA measurement and IF microscopy assessment methods also suggested no difference between the two patient sub-groups, NET ELISA suggested a greater number of NETs were present in BROS supernatants following incubation with fMLP or *H. influenzae* WCLs.

Although the difference in non-adherent supernatant NETs between the BR and BROS samples in these conditions was small (35% higher with fMLP, 26% higher with *H. influenzae* WCLs), the detection of a significant increase despite the limited sample size of each group (BR = 10, BROS = 3) implies that NET formation may be higher in BROS peripheral blood neutrophils. Whilst the extracellular DNA measurement and adherent NETs count assay results failed to show any significant difference in NET formation, several results from these assays showed some difference in the mean NETosis rates of BR and BROS patient samples (with the confidence intervals for these results overlapping greatly). Therefore, there may be potential for some difference in NETosis rates between the two patient subgroups which may be determined by further study using a larger sample size.

An additional point for consideration when interpreting the BR and BROS results focuses on the demographic differences between the two groups. As seen in table 7.1, the groups appeared largely similar in most recorded values differing only in gender distribution (BR = 56% male, BROS = 0% male), however the groups also differed in relation to medication taken by individual patient. BROS patients were reported to be currently taking several medications the majority of the BR cohort were not (rituximab, prednisolone and long-term macrolide therapy). As some of these medications have been suggested to impact neutrophil function (Wolach et al 2010, Trowald-Wigh et al 1998, Arai et al 2015) it is important to take these variables into consideration during interpretation of results (particularly in results relating to general neutrophil function e.g. phagocytosis, CXCL8 secretion).

7.3.3 *Conclusion*

There were several delays in getting ethical approval leading to a shortened recruitment period in the final year of the PhD. This led to a limited sample size, potentially making the BR/BROS sample population unrepresentative, which make addressing the hypothesis of validly establishing any differences in peripheral blood neutrophil citrullination, NETosis and neutrophil function between healthy volunteers, BR and BROS patients difficult. Therefore, the results of this chapter cannot be used to strongly support or dispute the established hypothesis for this chapter. However, given one of the central aims of this work was to establish preliminary data in order to guide the establishment of larger scale studies on the topic of neutrophil citrullination, NETosis and/or function, this chapter can be viewed as made significant progress towards this aim.

Chapter 8: Discussion & Conclusions

8.1 Bronchiectasis rheumatoid arthritis overlap syndrome, citrullination and NETosis overview

BR and RA have been long recognised to be comorbidities, with a significant minority of BR patients proceeding to develop RA (i.e. BROS patients). The comorbidity of two diseases is recognised as clinically important given BROS patients showing higher rates of mortality than patients with BR alone (De Soyza et al 2017). Despite this clinical importance our understanding of why recurring infection/inflammation of the airways may lead to the development of autoimmunity is lacking. Despite a large amount of research, the exact pathogenesis of RA remains elusive, with many factors (both genetic and environmental) likely playing a role in promoting the disease.

Amongst the many processes proposed to play a role in RA pathogenesis, citrullination has been identified as a common clinical feature in most RA patients (i.e. majority of patients testing positive for anti-citrullinated peptide antibodies) (Aletaha et al. 2010). It is believed that this uncommon modification of self-peptides may lead to a host's adaptive immune system misrecognising citrullinated peptides as foreign and therefore contribute to promoting an autoimmune response in RA patients as evidenced by ACPA. It is clear that this process is common but not universal in RA as ACPA negative cases arise.

As citrullination is carried out by members of the PAD enzyme family (which are expressed by a broad range of human cell types) it is plausible that cells known to express these PAD enzymes may play some role in initiating the onset of RA. In both RA and BR neutrophils are argued to play an important role in the progression of the disease (Lally et al. 2005, Schaaf et al. 2000, Chalmers et al 2017). Importantly neutrophils have also been identified as a cell type that expresses PAD enzymes, in particular PAD4 which plays a key role in the formation of NETs in response to infection and certain molecular triggers (e.g. PMA).

During the formation of NETs (NETosis), PAD4 migrates to the nucleus of the neutrophil where the enzyme citrullinates histones triggering chromatin decondensation. Following the breakdown of the nuclear envelope the contents of the neutrophil (including PAD4)

are released to the extracellular environment to restrict and possibly kill invading pathogens (Abdallah et al 2012).

Based on these features it is proposed that the chronic inflammatory and infectious airway environment in BR leads to the recruitment of neutrophils, which then undergo NETosis in response to the diseased airway environment. Over time this leads to the pulmonary microenvironment having rates of citrullination of self-peptides and/or the citrullination of peptides not typically targeted by PAD enzymes during normal healing and repair. Just as neutrophil elastase is thought to "spill over" from normal repair and cause damage it is possible that neutrophil derived PAD enzymes similarly cross a threshold to become harmful. In some genetically susceptible individuals the adaptive immune response may recognised these citrullinated peptides, which may promote the generation of an autoimmune response when combined with other factors (e.g. genetic predisposition) leading to the BR patient developing BROS.

The aims of this body of work largely focused on addressing several topics relating to BR, NETosis and BROS patient, including: whether BR relevant stimuli do promote neutrophil mediated citrullination and/or NETosis, the role of the two arms of the infection relevant LPS signalling pathway in citrullination and/or NETosis, the impact of PAD inhibition on citrullination, NETosis and neutrophil function and finally whether there are differences in neutrophil responses (citrullination, NETosis, phagocytosis, viability etc) between healthy, BR and BROS patients.

8.2 Summary of findings

8.2.1 NETosis and citrullination increase in response to bronchiectasis relevant stimuli

Chapter 4 described work investigating the ability of BR relevant stimuli to induce NETosis and neutrophil derived citrullination, the following two hypotheses were established for this work:

- Neutrophils stimulated *in vitro* with disease relevant molecules and bacterial cell lysates will show signs of elevated NETosis
- Neutrophil mediated citrullination will increase following stimulation with BR relevant stimuli in comparison to unstimulated neutrophils.

These hypotheses were tested by incubating healthy volunteer derived peripheral blood neutrophils *in vitro* with a BR relevant stimuli (e.g. PMA, LPS, WCLs of *P. aeruginosa* and *H. influenzae*), after which measurements were taken for changes in NETosis rates (using immunofluorescent staining of NETs, a NET ELISA assay and extracellular DNA measurements) and PAD activity (using an antibody based PAD activity assay).

The results suggest that *in vitro* NETosis rates and supernatant PAD activity increased following 4-hour incubation with 50nM PMA, 1µg/ml LPS, 10ng/ml TNF-α and 2.5%v/v *P. aeruginosa* WCLs, whereas no significant difference in NETosis or PAD activity was observed following stimulation with 100nM fMLP and 2.5%v/v *H. influenza* WCLs. As *P. aeruginosa,* LPS and TNF-α are common features within the infection prone pro-inflammatory airway environment in BR, or in the case of PMA activate signalling pathways central to neutrophil responses, these results support the hypotheses established for this chapter.

8.2.2 Inhibition of MyD88 and TRIF reduces in vitro NET formation

Chapter 5 described work addressing whether inhibition of the MyD88 dependent and the TRIF dependent arms of the LPS signalling pathway reduced LPS induced NETosis and PAD activation, with the hypothesis for this work being:

- Inhibition of MyD88 function in neutrophils will significantly reduce NETosis rates and/or PAD activity, whereas TRIF inhibition will have no impact on either feature.

To test the hypothesis healthy peripheral blood neutrophils were incubated with an inhibitor of TRIF or MyD88 (TRIF-pep and MyD88-pep respectively) for 3 hours prior to the addition of LPS (final concentration of 1µg/ml) for 4 hours, NETosis rates and PAD activity was then measured using the same methods described in chapter 4.

Although mean supernatant PAD activity was lower in LPS stimulated neutrophils pre-treated with TRIF-pep and MyD88-pep this decrease was not significant. Significant decreases were however observed in extracellular DNA measurements, NET ELISA results and immunofluorescent microscopy NET counts for LPS stimulated neutrophils pre-treated with TRIF-pep and MyD88-pep (in comparison to uninhibited LPS stimulated neutrophils), suggesting inhibition of either TRIF or MyD88 had inhibitory effects on LPS

induced NETosis. Therefore whilst TRIF and MyD88 inhibition had no impact on supernatant PAD activity, the results for NETosis rates suggest both the TRIF and MyD88 arm of the LPS pathway are implicated in NETosis, which disputes the hypothesis underpinning this body of work.

8.2.3 Cl-amidine treatment inhibits NETosis, PAD activity and additional neutrophil functions

Chapter 6 details work examining the impact of a PAD inhibitor (Cl-amidine) on healthy peripheral blood neutrophil NETosis rates, PAD activity and neutrophil function. Two hypotheses were created for this work to address:

- Pre-treatment of healthy volunteer derived peripheral blood neutrophils *in vitro* with the PAD inhibitor Cl-amidine will significantly decrease PAD activity and NETosis rates.
- Cl-amidine will significantly alter various aspects of neutrophil function (i.e. phagocytosis, superoxide production, lifespan and CXCL8 secretion).

Freshly isolated neutrophils were incubated for 30 minutes with 200μM Cl-amidine, after which bronchiectasis relevant stimuli were added to each condition and incubated for 4 hours. Following incubation NETosis rates and PAD activity were assessed as done for the previous chapters, in addition phagocytosis, superoxide, 24-hour viability and CXCL8 secretion were also assessed (methodologies outlined in chapter 3).

Results showed that pre-treatment with Cl-amidine significantly inhibited NET formation in response to all stimuli, with supernatant PAD activity (in response to PMA and TNF-α) also showing a significant decrease when incubated with Cl-amidine. Whilst viability over a 24-hour period appeared unchanged when peripheral blood neutrophil were incubated with Cl-amidine, significant decreases in superoxide release, phagocytosis rates and CXCL8 secretion in response to stimuli were observed for Cl-amidine treated neutrophils. Therefore the results produced from this work suggest Cl-amidine had inhibitory effects on NETosis, neutrophil PAD activity and general neutrophil immune function, which supports both hypotheses.

8.2.4 Differences in NETosis, PAD activity and neutrophil function between healthy, bronchiectasis and BROS neutrophils

Chapter 7 describes a study carried out which aimed to establish preliminary data on several biological responses/functions in peripheral blood neutrophils obtained from BR and BROS patients, with the following hypothesis established:

- Peripheral blood neutrophils from BROS patients will exhibit higher levels of NETosis and citrullination than BR patients without comorbid bronchiectasis results.

Peripheral blood neutrophils were obtained from healthy volunteers, BR patients and BROS patients. Following stimulation neutrophils were assessed for *in vitro* NETosis rates, extracellular PAD activity, viability, phagocytosis rates, superoxide production and CXCL8 secretion (using the methodologies described in the previous results chapters). The available results suggest there may be some differences between healthy and the whole BR patient cohort (i.e. BR and BROS) in regard to extracellular citrullination, phagocytosis rates, NETosis rates and CXCL8 secretion, however little difference was observed in neutrophil responses between BR and BROS patient samples.

Whilst these results oppose the initial hypothesis, issues with the patient population (i.e. limited size due to delays in approval and time constraints) make interpretation of these results difficult, therefore the results cannot be used to support or refute the established hypothesis. However as this body of work aimed to establish preliminary data to guide the powering of future larger studies, the results presented have arguably progressed towards achieving this purpose.

8.2.5 Summary of chapters results and conclusions

In conclusion the four bodies of work described have collectively attempted to establish: whether bronchiectasis stimuli can induce neutrophil PAD activation and NETosis (chapter 4), the roles of the two arms of the TLR4 signalling pathways in these processes (chapter 5) and the role of PAD in NETosis and other key neutrophil functions (chapter 6).

Whilst limitations to the population sample recruited for the work comparing healthy neutrophils to those from BR and BROS (chapter 7) prevent the work addressing the

underlying hypothesis the research has provided useful preliminary data on the responses of BR and BROS patient peripheral blood neutrophils in regards to citrullination, NETosis and other aspects of neutrophil function. *Figure 8.?* Summarises the questions raised for each results chapter along with the key findings and conclusions drawn.

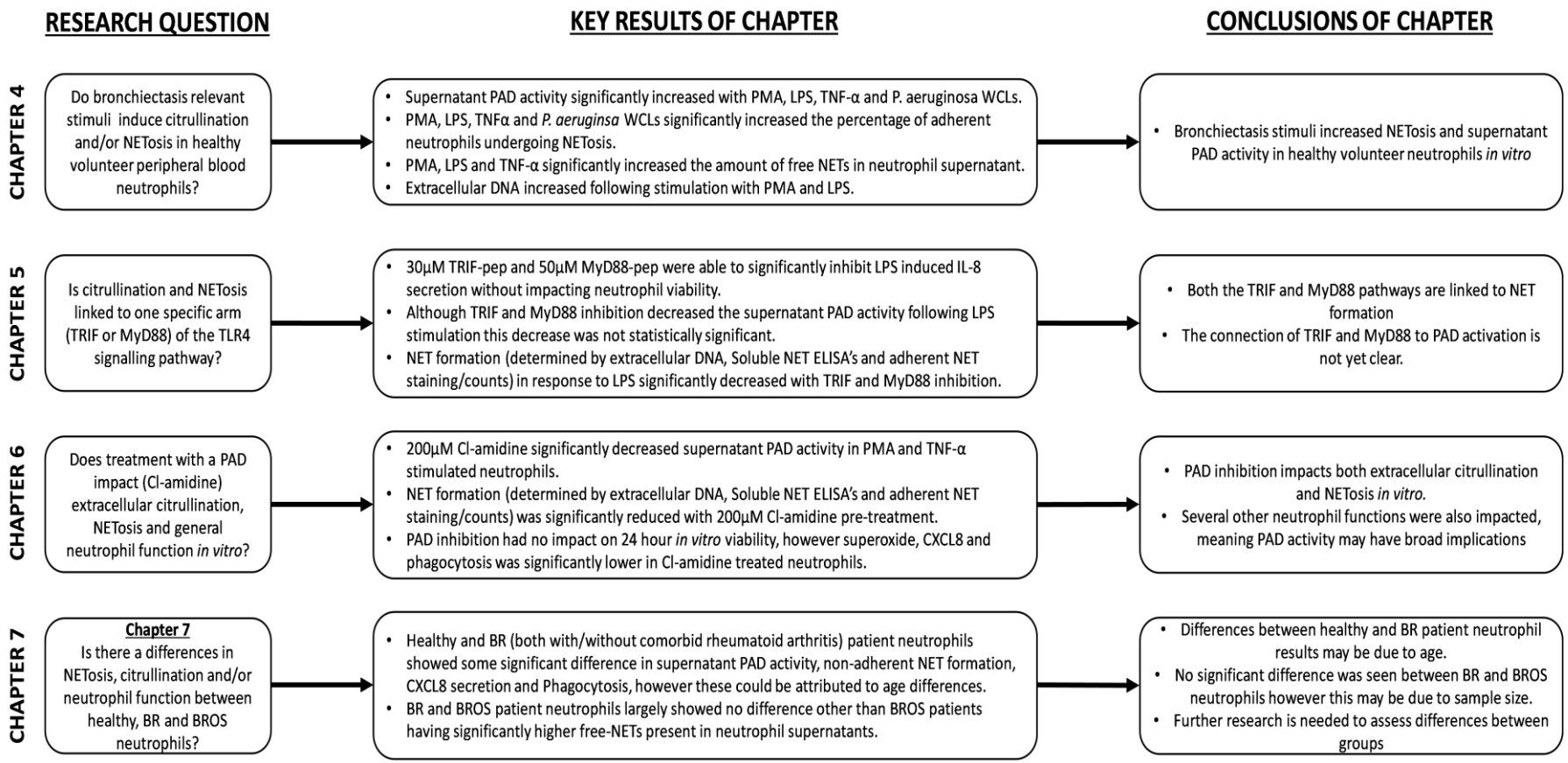

Figure 8.1 Summary of the work presented across the four results chapters. The underpinning research questions for each chapter of work along with the results for each of the chapters and the conclusions drawn from these results is shown above.

8.3 Strengths of work

8.3.1 Measurements of NETosis

A common criticism of work studying NETosis relates to the choice of methodologies. Although a variety of methodologies have been demonstrated within the literature, each have intrinsic flaws which make application of the method or valid interpretation of collected data difficult in most cases, because of this there is no established gold-standard methodology for the measurement of NETosis (Masuda et al 2016). Three well-established methodologies were used throughout this work to measure NETosis rates in isolated peripheral blood neutrophils; Immunofluorescent (IF) staining and microscopy counts of adherent NETs (Patel et al 2018, Hair et al 2018, Zou et al 2018), Histone-elastase complex (i.e. NET) ELISAs of supernatants (Nakazawa et al 2009, Söderberg et al 2015, Elaskalani et al 2018) and extracellular DNA measurements (Hakkim et al 2010, Zhang et al 2014, Hair et al 2018).

Each of these three methodologies have some limitation; extracellular DNA measurements having low specificity for NETosis, IF staining being subject to bias as well as potentially disregarding NETs which become detached prior to fixing (i.e. free NETs) and NET ELISAs disregard the adherent NETs left behind following extraction of supernatant whilst also only being semi-quantifiable. However, by using the three methodologies collectively we overcame several of these limitations (e.g. obtaining data on the presence of adherent and free NETs in samples) and are able to identify trends in the results (e.g. PMA elicited the highest NETosis rates in results collected using all three methodologies). Therefore the conclusions drawn from the three NET measuring assays results throughout this work (i.e. BR relevant stimuli promote NETosis, TRIF and MyD88 are both implicated in LPS induced NETosis, PAD inhibition downregulates NET formation) are given more validity than would be possible if only a single NET measuring methodology was used.

8.3.2 Supernatant PAD activity assessments

Another feature seen in all chapters of the thesis was the assessment of changes in citrullination (due to its central role in NETosis and rheumatoid arthritis pathogenesis). At the start of the research a central question arose regarding how to study this aspect of neutrophil biology. Two potential routes for investigating this phenomenon were

presented; investigating changes in intracellular PAD activity or extracellular PAD activity. After careful consideration it was decided to observe changes in extracellular PAD activity, this decision was made on the basis that the thesis was largely focused upon investigating whether neutrophils alter their extracellular environment (by NETosis dependent protein release and PAD release) to promote citrullination in BR. Therefore, whilst qualitative changes in intracellular PAD activity would be of great interest (although of some difficulty to study) it was deemed more relevant and vital to address each chapter in relation to how the extracellular environment is altered by neutrophils.

Several methods for studying PAD activity have been published, with the earliest dating back to 1939. However most of the known assays have several issues with sensitivity or their ability to be used with biological samples such as neutrophils. The ABAP assay (i.e. the commercial modiquest assay used in this work) however has been demonstrated to show high sensitivity for PAD dependent citrullination and function correctly with complex biological samples (Hensen et al 2014, Spengler et al 2015, Laugisch et al 2016), which made this methodology the best choice for assessing the large number of biological samples assessed throughout the thesis.

8.3.3 Neutrophil inhibition work

Whilst Cl-amidine has previously been used in several pieces of published neutrophil based *in vitro* work, with several papers using a concentration of 200μM (as done in the work presented in this thesis) (Wang et al 2009, Knight et al 2013, Li et al 2017), viability rates were assessed to confirm the inhibitor was not having a toxic effect upon neutrophils. Viability rates were also assessed in response to inhibition by MyD88-pep and TRIF-pep *in vitro* to establish a concentration of the inhibitors that was non-toxic but still successfully inhibited LPS induced activation of neutrophil function.

By confirming non-toxic but effective concentrations of the inhibitors for *in vitro* incubation, the possibility that inhibitor induced neutrophil death may be an explanation for any decrease in response is resolved. This allowed more valid conclusions to be drawn relating to the impact of these inhibitors on NETosis, PAD activity and general neutrophil function, allowing the hypotheses underpinning the bodies of work to be better addressed.

8.4 Limitations of work

8.4.1 Applicability of results

Both chapters 4 and 7 attempted to provide evidence to determine whether BR relevant stimuli promote NETosis/citrullination and whether BR (and BROS) patient derived neutrophils differ to healthy regarding NETosis/citrullination, which may explain why a proportion of BR patients proceed to develop RA. However there are several limitations in relation to the methodology which would prevent the results being extended to an in vivo disease environment; firstly, the work assesses neutrophil responses within a sterile in vitro environment with no other disease relevant cells/molecules present (e.g. endothelial cells, lymphocytes, epithelial cells, antibodies), secondly cells are given single stimuli (e.g. LPS) at a much higher concentration than likely seen in a physiological environment (as opposed to a range of inflammatory/infections stimuli at a low concentration) and finally the work involves the use of peripheral blood neutrophils which have not been exposed to the chronic inflammatory/infection pulmonary environment seen in bronchiectasis.

Whilst a true *in vivo* model of BR would involve a range of immune cell types and molecules, the purpose of this study was to assess neutrophil specific responses in relation to BR stimuli, therefore the inclusion of other disease relevant cells would over-complicate analysis and reduce the ability of the work to address its main focus of neutrophil biology in relation to BR. This was also the reason underpinning the decision to use single stimuli at concentrations likely far greater than physiologically relevant. As the work aimed to address whether specific stimuli could promote NETosis and extracellular citrullination, the main priority was not to replicate *in vivo* conditions as best as possible, but to instead achieve maximum stimulation of isolated neutrophils with each of the stimuli to determine if this had any impact on NETosis and PAD activity.

The use of pulmonary neutrophils would be of far greater relevance to assessing BR neutrophil responses (than the use of peripheral blood neutrophils as done in this work), however obtaining pulmonary neutrophils from human healthy volunteers is a difficult logistical obstacle (given the lack of inflammation and neutrophils present within the

healthy lung) and even obtaining pulmonary neutrophils samples from patients can present a difficult challenge for recruitment and sampling a sufficient number of patients to address research questions. Furthermore, as there is no well-established animal model for bronchiectasis there is no effective approach currently for assessing the *in vivo* environment of chronic pulmonary inflammation/infection seen in bronchiectasis.

8.4.2 Exclusion of vital NETosis

In the literature there have been two proposed types of NETosis; suicidal NETosis and vital NETosis. Suicidal NETosis is the mechanism of NETosis first described by Brinkmann et al (2004), known to involve hypercitrullination of histones and the death of the neutrophil within a roughly 4-hour period. Vital NETosis was first suggested by Clark et al (2007) and is described as a rapid (<30 minutes) alternative pathway of the process in which the neutrophil remains alive whilst excluding condensed chromatin with no evidence of hypercitrullination (Yipp et al 2013., Pilsczek et al 2010).

As NET measurements throughout the thesis were carried out on neutrophils incubated for at least 4 hours, and as the main methodology for studying NETosis responses (IF microscopic NET counts) used citrullinated histones as a marker for determining the presence of NETs, the results presented are likely a measure of changes in suicidal NETosis rates, potentially overlooking any changes or differences in vital NETosis. The decision to prioritise the study of suicidal NETosis over vital NETosis was made on two principles; firstly, that suicidal NETosis is more established within the literature with more validated methods for recording the process (whereas far less is known surrounding the molecular events associated with vital NETosis meaning specific assays are limited) and secondly that only suicidal NETosis is known to involve citrullination. Therefore, as the citrullination was a focus throughout the whole thesis, suicidal NETosis was decided to be the better choice to prioritise.

8.4.3 Choice of methodologies

Whilst the strengths and limitations of the methodologies used to assess NETosis rates throughout the thesis has previously been discussed in this chapter there are some potential limitations with regards to the the choice of other methods used in the research.

Neutrophil function was assessed using a variety of assays to measure viability rates (annexin V and propidium iodide stain), superoxide production (cytochrome C reduction), phagocytosis rates (zymosan phagocytosis) and CXCL8 secretion (commercial ELISA) *in vitro*. Whilst all these methods are well established in previous literature and address a variety of aspects of neutrophil function (Biswas et al 2015, Ruchaud-Sparagano et al 2014, Morris et al 2009), some aspects of neutrophil function were not assessed within the thesis, with two potentially important examples being chemotaxis and bacterial killing. Results collected using assays addressing these processes would be particularly useful in relation to chapter 6 (Cl-amidine), which showed decreased superoxide, phagocytosis and CXCL8 secretion in response to incubation with Cl-amidine (all relevant to anti-microbial activity and chemotaxis) and chapter 7, which would have provided more information on a broader range of healthy, BR and BROS patient neutrophils responses.

Another limitation with the methodology used in this work arises in regard to chapter 4 (BR relevant stimuli). The results from chapter 4 showed significant increases in healthy neutrophil supernatant PAD activity following stimulation, however no change in supernatant PAD4 concentration to match this (assessed by PAD4 ELISA). Inclusion of additional methods to confirm this was the case (i.e. PAD4 western blot) or assaying the same samples for the presence of other neutrophil derived PAD enzymes (i.e. PAD2) would have provided more information to establish whether the change in supernatant PAD activity was due to increased activation of PAD4 or release of PAD2, as suggested by other published research (Zhou et al 2017).

Furthermore, whilst the ABAP assay used to assess supernatant PAD activity is well validated and produced interesting results suggesting increased citrullination in response to BR stimuli there are questions raised by the results which were not addressed in the presented research. These included; was there any qualitative change in neutrophil derived protein citrullination following stimulation and whether the observed increase in PAD activity would have any substantial impact on the citrullination of host proteins? (such as those implicated in rheumatoid arthritis: α-enolase, fibrinogen). Both would be of interest if trying to place the produced results in context of BR and the formation of BROS.

8.4.4 Study of bronchiectasis and rheumatoid overlap patients

The limitations of using non-age matched healthy volunteers as a comparator and the limited sample size have already been discussed in chapter 7, however a further problem relating to studying the responses of BROS and BR patients is the ability to accurately distinguish the two. As described in the introduction of the thesis, there are likely two categories of BROS patients; those whom developed RA first (then due to extra-articular manifestations developed BR) and those whom developed BR first. As this thesis was focused upon "BR first" BROS patients, an important consideration when recruiting patients for this nature of research is establishing a clinical history of which condition was diagnosed first in BROS patient (and the difference in time between the diagnoses), which was not done for the study described in this thesis and should be considered in future study design and patient recruitment.

A further limitation to the work presented in chapter 7 relates to the lack of additional approaches in assessing BR and BROS patient responses. The approach selected focused upon identifying theorised differences in neutrophil mediated NETosis and PAD release in BROS patients (in comparison to healthy and BR patients) that may explain the onset of rheumatoid arthritis in these BR patients (i.e. via hypercitrullination). However, it is also possible that BROS patients do not differ to BR patients in neutrophil responses, but instead differ in adaptive immune responses (i.e. more likely present/recognise citrullinated self-peptides), which may be an explanation for why the patients developed RA (i.e. the adaptive immune response is pre-disposed to recognise citrullination). Whilst this question of differences in adaptive immune response was not assessed (owing to time constraints and sensitivity of neutrophil work) possessing unreported data on adaptive immune responses in healthy, BR and BROS would provide valuable insight into potential differences in the BROS cohort.

8.5 Future Work

8.5.1 Expanding on bronchiectasis relevant stimuli work

Future work for chapter 4 (BR stimuli) would first determine whether stimulated neutrophil supernatants showed any significant increase in PAD2 concentration (by

ELISA and/or western blot), which would establish which neutrophil expressed PAD enzyme was responsible for the observed increase in PAD activity. Initially neutrophil specific PAD enzyme production was going to be measured using real time PCR (rtPCR) in addition to ELISAs, however several challenges exist when attempting to apply this method in neutrophil based work (i.e. difficulty of complete neutrophil RNA isolation and the comparatively little mRNA expressed by neutrophils in comparison to other peripheral leukocytes) which make the potential for this technique to validly measure PAD production questionable.

Additional experiments would be included to compare the levels of citrullination of a protein relevant to rheumatoid arthritis citrullination (e.g. α-enolase) by supernatants from differing stimulatory conditions (e.g. unstimulated vs LPS stimulated). A proposed methodology for assessing this would involve incubation of the target protein with supernatants with various levels of PAD activity, with proteomic analysis (e.g. mass spectrometry) used to compare qualitative and quantitative differences in target protein citrullination between the various supernatants. Consultation with proteomics experts in Newcastle and Dundee university have suggested that whilst this approach is possible there are several issues in the current methodologies used to isolate, identify and quantify citrullination of peptides that make carrying out this work in complex biological samples difficult. These difficulties include: anti-citrulline antibodies lacking specificity, Phenylglyocal-rhodamine probes (another method of fluorescently identifying citrulline residues) having broad reactivities in biological complex samples, distinguishing between citrullinated and deamidated amino acid residues using mass spectrometry and detecting a specific citrullinated peptide at low abundance in a biological sample (Hensen et al 2014, Slade et al 2014, Lee et al 2018). Therefore future work of this nature will likely require a great deal of proteomic expertise, time and resources in order to validly assess any qualitative or quantitative changes in peptide specific citrullination by neutrophil derived PAD.

Other potential routes for further work leading on from the work in chapter 4 would include attempting to better replicate BR *in vivo* conditions to observe if any significant differences are seen in results obtained. Some improvements on the established *in vitro* model used in this study would include coating plastic well plates with a biologically

relevant matrix molecule such as fibrinogen to better replicate *in vivo* conditions. A further improvement would include using hypoxic conditions for the incubation and stimulation of isolated neutrophils. As neutrophils are arguably evolved to function optimally under hypoxia (i.e. due to the hypoxic environment within inflamed tissue) there is a possibility that neutrophil function may differ under these conditions (in comparison to responses in 21% normoxic atmospheric conditions). Therefore measuring neutrophil responses (as demonstrated in this thesis) under hypoxic conditions *in vitro* to would be of great interest and would likely be more representative of the conditions within the BR airway (Lodge et al 2016, Sarkar et al 2017).

8.5.2 Cell signalling and NETosis

As the results suggested that inhibition of TRIF and MyD88 (two upstream proteins in the LPS signalling pathway) impacted NETosis and citrullination, the next step in investigating signalling pathways implicated in these processes would involve inhibition of other downstream proteins in the LPS signalling pathway. The effect of inhibition of proteins within in the MAPK/ERK section of the LPS signalling pathway on NETosis/PAD activity would be the first assessed, as these proteins are believed to be a primary route by which NET inducing stimuli induce NETosis, inhibition of this pathway should theoretically prevent the initiation of NETosis. If NETosis persisted despite inhibition of MAPK/ERK, further work would involve examining other proteins associated with the LPS signalling pathway which are common to both TRIF and MyD88 signalling (e.g. NF-κB) to observe the impact of inhibition of these molecules on NETosis in order to establish specific molecules with a key role in TLR4 mediated pro-NETosis signalling.

8.5.3 Inhibition of PAD activity by Cl-amidine

From the results obtained in chapter 6, it was suggested that Cl-amidine downregulates both NETosis and citrullination (as expected), but also has negative effects on several aspects of neutrophil function. Two potential routes for future work building on the results of this chapter are proposed. Firstly, neutrophils pre-treated with Cl-amidine (unstimulated and stimulated) would be assessed in comparison to uninhibited neutrophils for qualitative differences in PAD activity. The results of this chapter showed a decrease in extracellular PAD activity, however changes in citrullination of neutrophil derived proteins were not assessed. To determine this, neutrophils (following inhibition

and stimulation) would be lysed with the resulting intracellular proteins analysed using a proteomic approach to determine how the neutrophil citrullination profile changes following inhibition of PAD. This would assist in addressing the question raised in the discussion of chapter 6 involving whether inhibition of NETosis by Cl-amidine only partial (compared to full inhibition of extracellular PAD activity) due to inability of the inhibitor to effectively inhibit citrullination within the neutrophil.

Secondly, the effect of Cl-amidine on other neutrophil functions would also be investigated. Cl-amidine was shown to inhibit superoxide, phagocytosis and CXCL8 secretion, all of which are important features in neutrophil function and may suggest Cl-amidine could have an immunosuppressive effect in an *in vivo* context. Additional methods would be used to assess the impact of the inhibitor on neutrophil mechanisms including; elastase secretion, chemotaxis ability and bacterial killing, all of which are also key aspects of physiological neutrophil function and likely of important clinical relevance.

8.5.4 Study of Bronchiectasis and BROS patient cohort

With the completion of the feasibility study and processing of results shown in chapter 7, there were some potential differences between the groups results relating to NETosis, PAD activity and neutrophil function. The results obtained will assist in the design and correct powering of future large-scale studies involving the BR and BROS patient groups, which will be better equipped to assess if any true significant differences between the groups exist.

Several changes should be applied to future study design to allow more valid comparisons between results obtained for the groups including; the use of a larger sample size, age matching the groups where possible, matching other aspects of key demographics (e.g. gender, bronchiectasis severity scores, colonisation status, medication usage) where possible and the inclusion of a group of RA only patients. Furthermore, as CXCL8 secretion showed the most potentially for difference between the groups (despite a limited sample size) it would be beneficial to assess differences in the secretion of other neutrophil derived molecules (e.g. elastase, IFN-γ, TNF-α) between the groups to better categorise differences in pro-inflammatory responses of peripheral blood neutrophils between patients.

8.6 Conclusions

By isolating peripheral blood neutrophils and using a variety of *in vitro* methodologies, this thesis has produced a large amount of data on NETosis and citrullination in relation to neutrophil biology and BR pathology. Using validated assays for measuring NETosis, several BR relevant stimuli have been shown to promote NETosis under *in vitro* conditions, with results also suggesting an increase in extracellular PAD activity following stimulation with these molecules. This may suggest BR relevant conditions promote NETosis and citrullination which may have impact on disease progression and the onset of autoimmunity, however this has not been established. Experiments exploring the under-investigated LPS signalling pathway in relation to NETosis/PAD activity suggests that the signalling pathways underpinning these processes may be more complex than initially thought, with further work required to gain a better understanding of the signalling events implicated in NETosis and neutrophil mediated citrullination.

Whilst Cl-amidine was shown to have an inhibitory effect on NETosis and citrullination, which may be of potential therapeutic use, the results obtained also suggest that Cl-amidine also impaired important immune functions carried out by neutrophils including superoxide generation, phagocytosis and CXCL8 secretion. This may imply that PAD activity may regulate aspects of neutrophil function other than NETosis and that the PAD inhibitor could have a negative impact on immune response generation in an *in vivo* context. Finally, preliminary data was obtained which suggested some potential differences between healthy volunteer peripheral blood neutrophil responses and BR patients, and whilst there are limitations which limit the interpretation of the results, the data obtained can be used to guide future larger scale studies to investigate true significant differences between healthy, BR and BROS peripheral blood neutrophil responses *in vitro*.

Chapter 9: Presentations and Publications

The work generated from this research has been presented at the following academic conferences: The World Bronchiectasis conference 2017 (poster presentation), the ERS congress 2017 (poster presentation) and the British thoracic society 2018 winter meeting (two spoken presentations), with the work also being printed in the Thorax supplementary publications for the 2018 winter meeting which can be located using the following two references

1. C Cole, J Scott, G Davies, G Jones, K Jiwa, J Chalmers, J Simpson, A De Soyza (2018) “Bronchiectasis relevant molecules promote NETosis and citrullination in human peripheral blood neutrophils” BTS Winter meeting *Thorax* 73: A54

2. C Cole, J Scott, G Davies, G Jones, K Jiwa, J Chalmers, J Simpson, A De Soyza (2018) “Interleukin 8 secretion but not superoxide anion production is different between healthy and bronchiectasis patients neutrophils” BTS winter meeting *Thorax* 73: A54

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