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THE INTERPLAY BETWEEN *PSEUDOMONAS AERUGINOSA* AND HUMAN MACROPHAGES AND NEUTROPHILS

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DECLARATION

I hereby declare that the work presented in this thesis is my own except otherwise stated and that this thesis has not been submitted elsewhere for the award of a higher degree.



……

SIRINA MUNTAKA

DEDICATION

This thesis is dedicated to my beloved family for their splendid support and unflinching understanding always.

ABSTRACT

Pseudomonas aeruginosa (PA) is an opportunistic pathogen that can cause infections in patients with compromised immunity including patients with neutropenia, HIV infection, burns, cancer, organ transplant surgery or in intensive care as well as Cystic Fibrosis (CF) patients. CF patients may have a T-helper (Th)-2 and Th-17-biased immune response which suggest that, either the absence of Th-1 and/or enhanced Th-17 responses may contribute to chronic PA infection with a deterioration of lung function in CF. A previous study in our laboratory showed that interferon (IFN)-γ production by peripheral blood mononuclear cells (PBMCs) from CF patients positively correlates with lung function, particularly in patients chronically infected with PA. In contrast, IL-17A levels correlated negatively with lung function especially in patients chronically infected with PA. These results agreed with the notion that IFN-γ and IL-17A play protective and detrimental roles, respectively, in CF disease. To explore the protective effect of IFN-γ in CF, the effect of IFN-γ alone or in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF), on the ability of human macrophages to control PA growth, resist the cytotoxicity induced by PA or promote inflammation was investigated previously in our laboratory. Treatment of macrophages with IFN-γ, in the presence and absence of GM-CSF did not alter bacterial growth or macrophage survival upon PA infection, but made the macrophages more pro-inflammatory. As neutrophils have been reported to be the key effector cells for the clearance of PA, there was therefore the need to include neutrophils in the macrophage cultures so as to determine how inflammatory conditions affect PA clearance. Although neutrophils are essential for protection against extracellular bacteria, neutrophil-dominated inflammation can compromise organ function by causing tissue damage. Optimal anti-bacterial immunity should harness the microbicidal activity of neutrophils while minimising their potential for causing injury. However, neutrophils do not act in isolation, they respond to cues provided by other cells such as macrophages, which influence their activation and life span. *In vivo* models have dominated the study of anti-bacterial immunity and there is a need of human models for the dissection of macrophage/neutrophil communication during bacterial infection. This work describes the development of a human macrophage/neutrophil infection assay and its use to model Th-1 (IFN-γ) - and Th-17 (IL-17A)-driven microbicidal activity and inflammatory potential upon infection. Results show that IFN-γ and IL-17A have opposite effects on the killing ability of macrophages and neutrophils co-cultures; bacterial killing was reduced by IFN-γ and promoted by IL-17A. In addition, macrophages and neutrophils specifically collaborated for the production of IL-1β and IL-1α and IFN-γ-treated co-cultures generated significantly less IL-1β and IL-1α compared to those treated with IL-17A. This effect was not observed with other cytokines such as TNF-α, IL-6, MIP-1α and MCP-1. Also, there was increased

production of IL-1β upon addition of neutrophils to infected macrophage cultures and this depended on caspase-1 activity. Thus phagocyte co-cultures provide a suitable model of human anti-microbial immunity and unveil an unappreciated collaboration between macrophages and neutrophils in the promotion of IL-1-mediated inflammation. The reduction of inflammation in the presence of IFN-γ highlights the potential of IFN-γ as a therapeutic strategy in extracellular bacterial infections such as infections with PA.

LIST OF PUBLICATIONS

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- **S. Muntaka**, Y. Almuhanna, A. Asante, M. Camara and L. Martinez-Pomares (2017). IL-17A and IFN-γ establish the balance between inflammatory potential and bacteria killing by human phagocytes during *Pseudomonas aeruginosa* infection. Journal of Leucocytes Biology (In review)

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AECs	Airway epithelial cells
AHL	N-acyl homoserine lactones
AIM2	Absent in melanoma 2
ALRs	AIM2–like receptors
AP	Activated protein
APCs	Antigen-presenting cells
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guerin
BSA	Bovine serum albumin
C4-HSL	N-butyryl-L-homoserine lactone
CAP	Community associated pneumonia
CARD	Caspase activation and recruitment domain
CBS	Centre for Biomolecular Science
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFU	Colony forming units

CGD	chronic granulomatous disease
CM	Complete medium
CNS	Central Nervous System
CRISP3	Cysteine-Rich Secretory Protein 3
CSF	Colony stimulating factor
CSF1R	Colony stimulating factor 1 receptor
DAMPs	Damage-associated molecular patterns
DCs	Dendritic Cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco’s phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
EIII	Neutrophil Elastase Inhibitor 3
ELISA	Enzyme-Linked Immunosorbent Assay
EPS	Extracellular Polymeric Substance
FBS	Foetal bovine serum
FC	Fragment crystallizable
FIIND	Function-to-find domain

FSC	Forward scatter
G-CSF	granulocyte colony stimulating factor
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte Macrophage- Colony Stimulating Factor
GSH	Glutathione
h	Hour or Hours
HBECs	Human Bronchoepithelial Cells
HBSS	Hanks balanced salt solution
Hcp	Hemolysin-coregulated protein
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HI-NHS	Heat Inactivated Normal Human Serum
HMGB1	High mobility group box 1
HNP-1	Human Neutrophil Peptide-1
hpi	Hours post infection
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
HSA	Human Serum Albumin
IFI16	IFN-γ-inducible protein 16
IFN	Interferon

IFNs	Interferons
IKK	IκB kinase
IL	Interleukin
IPAF	IL1-β converting enzyme protease activating factor
IRAK	IL-1 receptor-associated kinases
IRF	Interferon regulatory factors
KC	Neutrophil Chemokine CXCL-1
LB	Lysogeny broth
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharides
LRR	Leucine-rich repeats
MAC	Membrane Attack Complex
MAMPs	Microbe-associated molecular patterns
MAP	Mitogen-Activated Protein
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage- Colony Stimulating Factor
MFG-8E	Milk fat globule epidermal growth factor 8
MHC	Major Histocompatibility Complex
min	Minute or minutes

MIP	Monocyte inflammatory proten
MMP9	Matrix metalloproteinase 9
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary response gene 88
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor-kappa B
NHS	Normal Human Serum
NK	Natural Killer
NLR	Nod-like receptor
NLRP	Nod-like receptor protein
NOD	nucleotide oligomerization domain
NS	Not significant
OD	Optical density
PA	*Pseudomonas aeruginosa*
PAMPs	Pathogen-associated molecular patterns

PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	phycoerythrin
PI	Propidium Iodide
PMNs	Polymorphonuclear neutrophils
PQS	Pseudomonas quinolone signal
PRR	pattern recognition receptors
PS	phosphatidylserine
QS	Quorum sensing
RBC	Red blood cells
rhIFN-γ	Recombinant human interferon gamma
RIG	Retinoic acid-inducible gene
RLRs	Retinoic acid-inducible gene (RIG) - like receptors
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
RP	Rhodamine phalloidin
rpm	Revolutions per minute
SLB	Society for Leukocyte Biology
SOCS	Suppressors of cytokine signalling

SP-A	Surfactant proteins A
SP-D	Surfactant proteins D
SSC	Side scatter
T2SS	Type 2 secretion systems
T3SS	Type 3 secretion systems
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
TICAM-1	Toll like receptor adaptor molecule-1
TIR	Toll/IL-1 receptor
TIRAP	TIR-associated protein
TLR	Toll like receptor
TMB	Tetramethylbenzidine
TNF	Tumor Necrosis Factor
tps	Two-partner secretions
VAP	Ventilator-associated-pneumonia
VgrG	Valine–glycine repeat

Chapter 1

General introduction

Chapter 1: General introduction

The introductory section will focus on the characteristics of PA and diseases it causes, particularly the main diseases of the airways caused by PA, how mutations in CFTR predispose to PA infections, virulence factors of PA and PA adaptations to the Cystic Fibrosis (CF) lung, the immune response to PA and altered immune response in CF.

1.1 *Pseudomonas aeruginosa* (PA)

Pseudomonas aeruginosa (PA) is a Gram-negative bacterium ubiquitous in nature. PA is found in the soil and moist surfaces but can also survive in numerous environments both natural and artificial (Gellatly *et al.*, 2013). PA is characteristically an opportunistic pathogen that causes infections in patients with tissue damage or compromised immunity (Gellatly *et al.*, 2013). These include patients suffering from neutropenia, CF, thermal injury, cancer and those undergoing organ transplant surgery or in intensive care units (Lyczak *et al.*, 2000). Infections caused by PA are difficult to treat because of PA's ability to form biofilms, modulate the immune response and resist antimicrobials (Willmann *et al.*, 2013).

The antimicrobial resistance of PA has been attributed to several mechanisms including multidrug efflux systems, production of enzymes that inactivate antibiotics and targeted mutational changes (Hirsch *et al.*, 2010). PA is equipped with a number of efflux pumps to expel drugs and other substances out of the cells (Livermore, 2002). Similarly, PA produces enzymes that inactivate several antibiotics for instance the AmpC β-lactamase that hydrolyses most β-lactam drugs (Hirsch *et al.*, 2010). Furthermore, PA can undergo mutational changes leading to resistance or acquire resistance through horizontal gene transfer (Hirsch *et al.*, 2010).

The types of infections caused by PA in susceptible individuals include infections of the skin (such as ecthyma), eyes (such as keratitis) and ears (such as otitis medium) (Lutz *et al.*, 2011). PA can also cause urinary tract infections and infections in the heart (such as endocarditis) and the central nervous system (CNS, such as brain abscess and meningitis) (Pollack, 2000),

as well as infections in the gastrointestinal tract and the bloodstream, bones and joints (Pollack, 2000). One of the most common and detrimental infections caused by PA in vulnerable persons are infections of the airways (Williams *et al.*, 2010).

1.2 Airway diseases caused by PA

Infections of the respiratory tract are some of the most deleterious and include acute infections such as community associated pneumonia (CAP) and nosocomial infections (e.g. ventilator-associated-pneumonia: VAP). A typical case of chronic infections caused by PA are observed in CF (Williams *et al.*, 2010). CF is a genetic disorder resulting from mutations in the CF transmembrane conductance regulator (CFTR) gene on chromosome 7, which show a high incidence among Caucasians (Tan *et al.*, 2011b). The CFTR protein channel in healthy epithelial cells functions as a regulator of chloride ions across membranes of secretory cells producing mucus, saliva, sweat, tears, and digestive enzymes (Nichols *et al.*, 2008). Movement of chloride ions subsequently regulates water movement in cells which aids in controlling mucus viscosity (Munkholm *et al.*, 2014). The CFTR channel also indirectly regulates the transport of sodium and bicarbonate across cell membranes (Coakley *et al.*, 2003; Cohen *et al.*, 2012). In the airways of healthy individuals, the cilia beats in a synchronised manner which helps to propel mucus with or without trapped bacteria out of the lungs for cleansing (Munkholm *et al.*, 2014). However in the CF airways, this function of the cilia is impaired by the highly viscous mucus which makes the environment highly favourable to infections (Munkholm *et al.*, 2014).

The CF lung is colonised by a number of pathogens ranging from bacteria (such as PA, *S.aureus*, *B. cepacia complex* and non-tuberculous mycobacteria), fungi (such as *C. albicans* and *A. fumigatus*) and viruses (such as Respiratory syncytial virus) (Saiman *et al.*, 2004; Harrison, 2007; Coutinho *et al.*, 2008; Filkins *et al.*, 2015). Meanwhile, several other emerging pathogens have been identified in CF (such as *Klebsiella species*, *Alcaligenes xylosoxidans* and *Stenotrophomonas maltophilia*) (Harrison, 2007; Filkins *et al.*, 2015). Although the common pathogens in CF infants are *S. aureus* and *H. influenzae* (Filkins *et al.*, 2015), a

longitudinal assessment of CF children within their first 3 years using bronchoalveolar lavage (BAL) found 72.5% of PA colonisation based on culture and 97% of PA colonisation based on combined serology and culture results (Burns *et al.*, 2001). Similarly, the common pathogens in CF adults are PA and *B. cepacia* (Filkins *et al.*, 2015); however, colonisation with PA still dominates in CF adults and it accounts for the majority of morbidity and mortality in CF (Lyczak *et al.*, 2002; Harrison, 2007).

1.3 Mutations in the CFTR gene

Since the initial identification of the CFTR gene in 1989 (Riordan *et al.*, 1989), over 1300 specific mutations in the CFTR gene have been described (MacDonald *et al.*, 2007). The most common mutation is the ΔF508 which accounts for more than 70% of CF patients (Roque *et al.*, 2001; Kreindler, 2010). CFTR gene mutations are classified into six classes based on how they affect CFTR protein (Kreindler, 2010). Class I mutations cause a shortened protein and are exemplified by the W1282X mutation [where a stop codon is introduced at the region for tryptophan (W) at position 1282 resulting in a truncated CFTR protein (Kreindler, 2010). Class II mutations cause a failure of the CFTR protein to reach cell membrane and this is exemplified by the ΔF508 mutation which results in deletion of phenylalanine (F) at position 508 (Kreindler, 2010). Class III mutations are caused by improper regulation of the CFTR channel and this is exemplified by G551D missense mutation [where there is a replacement of aspartate (D) by a glycine (G) at position 551] (Kreindler, 2010). Class IV mutations cause a reduction in the transport of chloride ions across the CFTR channel as illustrated by the R117H missense mutation [where an arginine (R) at position 117 is replaced by histidine] (Kreindler, 2010). The Class V mutations result in a reduction of normal CFTR protein synthesis as exemplified by the 3849+10KbC>T [where there is a splice-site mutation in intron 16] (Duguépéroux *et al.*, 2005; Kreindler, 2010). Class VI mutations are caused by an alteration in the residence time of CFTR at the apical membrane and this is exemplified by the Q1412X [where there is failure of insertion of glutamine (Q) at position 1412] (Kreindler, 2010).

Class I, II and III mutations are often associated with more severe disease while Class IV, V and VI mutations often lead to mild disease (Duguépéroux *et al.*, 2005; Kreindler, 2010).

Current therapy in CF involves management and the use of medications that target the specific mutations (Rowe *et al.*, 2013; McCallum K *et al.*, 2016). These include the use of pottentiators (such as Ivacaftor), correctors (such as lumacaftor) or a combination of Ivacaftor and lumacaftor (Rowe *et al.*, 2013; McCallum K *et al.*, 2016). See Chapter 6 for more details on therapy in CF and also these references (Kreindler, 2010; Rowe *et al.*, 2013; McCallum K *et al.*, 2016).

1.4 Mutative CFTR predispose CF patients to PA infections

The high susceptibility of CF patients to PA infections depend in part on the class of mutation in the CFTR gene as well as adaptations of the bacteria to the CF lung. Studies have shown that CFTR itself can function as a receptor on epithelial cells to internalise both PA (Pier *et al.*, 1996; Pier *et al.*, 1997) and *S. typhi* (Pier et al., 1998). In particular, the complete outer core polysaccharide of PA LPS (lipopolysaccharides) served as the ligand for CFTR. It has been suggested that internalisation of PA by the epithelial cells is part of the immune defence mechanism. Thus, a mutated CFTR, reducing PA internalisation, would be associated to reduced PA clearance by epithelial cells (Zaidi et al., 1999).

1.5 Virulence factors of PA

PA has a large genome of about 6.3MB which provides the organism with a remarkable amount of metabolic flexibility. This enables PA to grow well in challenging environments which usually do not support many organisms (Stover *et al.*, 2000; Diggle *et al.*, 2002). The pathogenesis of PA depends on both the virulence factors of the bacteria (Figure 1.1) and the immune response mounted by the host. Each of PA virulence factors contribute to its pathogenicity (Collier *et al.*, 2002) by playing key roles in the ability of PA to establish a

successful infection in its host. The contribution by the various virulence factors of PA are described below:

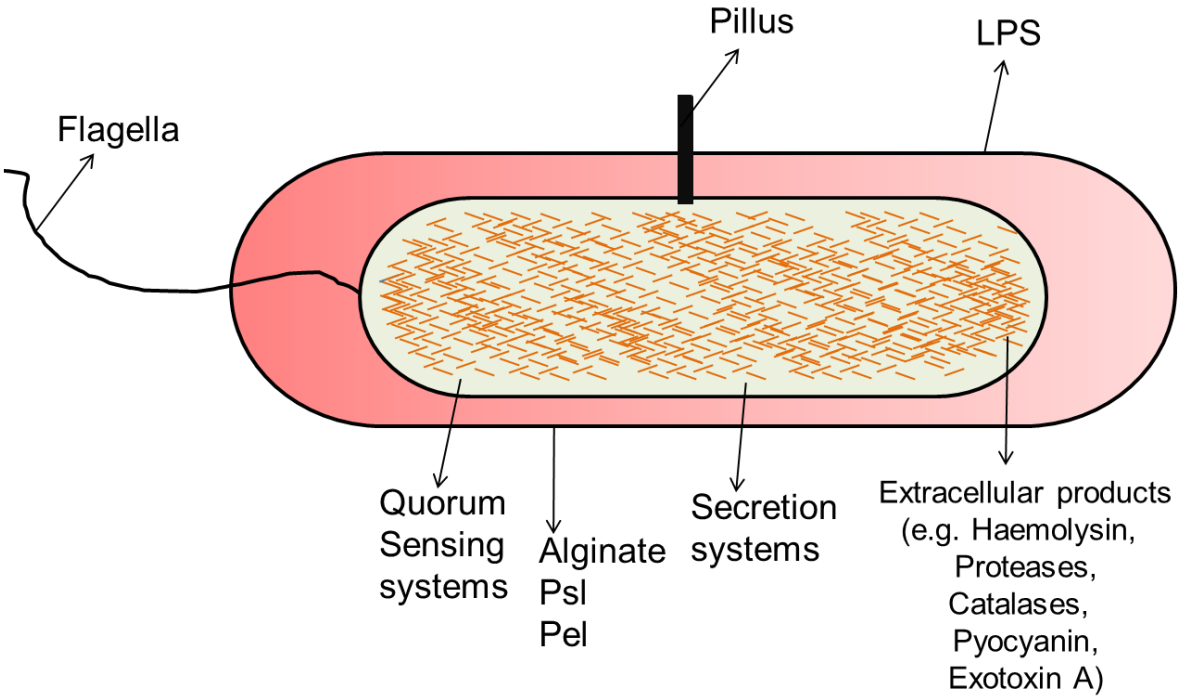


Figure 1.1: Virulence factors of PA.
PA has many virulence factors including surface structures (flagella, pili and LPS), Quorum sensing systems, extracellular polysaccharides (alginate, Psl and Pel), secretion systems and their secretory proteins and extracellular products. Figure adapted from (Sadikot *et al.*, 2005).

1.5.1 Surface structures

PA expresses polar pili at the cell surface which are used for attachment to host cells (Sadikot *et al.*, 2005) and for signalling through Toll-like receptor (TLR) 2 (Soong *et al.*, 2004). Another surface structure of PA is polar flagella which is essential for motility, flagella are also important during airway infections as they bind to TLR 5 on the airway epithelial cells (AECs) stimulating the immune response against the bacteria (Adamo *et al.*, 2004). However, just after PA colonises the lung, it switches off flagella expression and this coincides with the expression of genes for biofilm formation (Sadikot *et al.*, 2005). Also, the LPS of PA signals through TLR4 (Sadikot *et al.*, 2005) for an effective immune response against the bacteria, but PA can modify its LPS by the addition of palmitate and aminoarabinose (Sadikot *et al.*, 2005) as discussed below during PA adaptation to the CF lung. This modification in the LPS allows PA to evade immune recognition by escaping TLR4 recognition (Lafferty *et al.*, 2010). The addition of palmitate and aminoarabinose to the LPS of PA also make the LPS less negatively charged and thus conferring resistance to cationic antimicrobial peptides (Delcour, 2009).

1.5.2 PA Secretion systems

Secretion systems are mechanisms by which Gram-negative organisms such as PA introduce effector proteins, including enzymes or toxins into host cells (Filloux, 2011). Six types of secretion systems are well characterized so far (namely, Type I to Type VI often symbolized as T1SS through to T6SS) (Filloux, 2011).

PA has two of the T1SS (Bleves *et al.*, 2010). These include, the Apr system (consisting of AprD, AprE and AprF), which is involved in the secretion of alkaline protease (Duong *et al.*, 2001). The other T1SS utilizes the *has* genes (HasD, HasE and HasF) and is involved in iron depletion from host cells (Wandersman *et al.*, 2004). This involves the secretion of a protein called HasAP which binds haem from haemoglobin with a subsequent internalization by downstream mechanisms (Wandersman et al., 2004).

There are two T2SS in PA (Xcp and Hxc) (Bleves et al., 2010). The T2SS Xcp is involved in the secretion of virulence factors such as lipases, proteases and toxins in vivo (Lavoie *et al.*, 2011b) while Hxc mediates the secretion of alkaline phosphatase (Ball et al., 2002). An extensive review of the secretion systems in PA can be found in these references (Bleves *et al.*, 2010; Filloux, 2011).

The T3SS in PA enables the injection of toxins by the bacteria into host cells (Gauthier *et al.*, 2003). T3SS has been linked to acute invasive infections which necessitates contact between bacteria pili and the epithelium (Feldman *et al.*, 1998; Hauser *et al.*, 1998). The T3SS has three main components including the secretion component, the targeting or translocation component and the secreted toxins/effector proteins and associated chaperones (Gauthier *et al.*, 2003). PA secrete four main effector proteins through the T3SS which include, ExoU, ExoT, ExoS and ExoY (Sadikot *et al.*, 2005). The ExoU is a powerful cytotoxin while ExoT is bifunctional with an N-terminal domain with GTPase activity and a C-terminal domain with adenosine diphosphate (ADP) ribosyltransferase activity, ExoS is an ADP ribosyltransferase of ExoT and ExoY is an adenylate cyclase (Finck-Barbancon *et al.*, 1997; Sawa *et al.*, 1999; Epelman *et al.*, 2004).

ExoS induces the production of tumour necrosis factor alpha (TNF-α) through the activation of TLR2 and TLR4 in a MyD88-dependent manner. The dual ability of ExoS to activate TLR2 and a complex of TLR4/MD-2 was ascribed to the C- and N- terminals domains respectively in an experimental model where Alexa-flour 488-labelled recombinant ExoS (either the full length N-terminal or full length C-terminal) were used for stimulation of monocytes (Epelman *et al.*, 2004). The mechanism of action of ExoU and its cellular targets are not clear (Finck-Barbancon *et al.*, 1997; Sawa *et al.*, 1999; Epelman *et al.*, 2004); ExoU belongs to the phospholipase A enzyme family and has phospholipase A2 activity (Sadikot *et al.*, 2005). Sadikot and colleagues (Sadikot *et al.*, 2005) has shown irreversible cellular membrane damage as well as rapid necrotic death when ExoU was injected directly into mammalian cells

(Sato *et al.*, 2004). Meanwhile, ExoY has not yet been shown to have a direct toxic effect on eukaryotic cells (Sadikot *et al.*, 2005).

Various studies using animal models have demonstrated the contribution of PA T3SS to virulence. For instance, Shime and colleagues showed that neutralising T3SS proteins prevent septic shock as well as improve the rate of survival in mouse models of PA pneumonia (Shime *et al.*, 2001). Shime and colleagues (Shime *et al.*, 2001) administered polyclonal antibodies against *Pseudomonas* PcrV (a protein secreted by the T3SS itself which is responsible for translocating T3SS secreted toxin) and this treatment increased animal survival. Similarly, in a study by Hauser and colleagues (Hauser *et al.*, 2002) using patients with ventilator-associated pneumonia, showed worse clinical outcome in patients infected with T3SS-expressing PA.

PA was initially shown to have five out of the six secretion systems (i.e. all except T4SS) (Filloux, 2011). The T4SS is involved in horizontal gene transfer of various genomic islands of pathogens (Juhas et al., 2008). T4SS is sub divided into Type F and Type P, which together are termed as Type IVA and Type I which is termed as Type IVB (Juhas et al., 2008). Further studies have identified another lineage of T4SS on ICEHin1056 genomic island (Juhas et al., 2008). ICEHin1056 is a Haemophilus influenzae antibiotic resistance vector, interestingly however, this lineage of T4SS is shown to be present also in PA, *Pseudomonas fluorescens* and other pathogens (Juhas et al., 2008). Meanwhile, this T4SS is found to be associated with genomic islands only and hence termed as GI type (Juhas et al., 2007).

T5SS are divided into T5aSS, T5bSS and T5cSS (Bleves et al., 2010). T5aSS and T5cSS are autotransporters while T5bSS is a two-partner secretion system (Bleves et al., 2010). PA has an EstA autotransporter which has an esterase activity (Bleves et al., 2010). EstA is as well shown to be involved in rhamnolipids production by PA (Wilhelm et al., 2007). EstA mutants were defective in PA motility (including swimming, swarming and twitching) (Wilhelm et al., 2007). PA3535 and PA0328 autotransporters have also been predicted, with a serine protease

and an aminopeptidase activity, respectively (Bleves et al., 2010). Six of the two-partner secretions (i. e. tps 1 to 6) have been identified in PA, with some (such as tps1, 2, 3 and 5) yet to be well characterised (Bleves et al., 2010). LepA is a protein secreted downstream of the tps4 which has a protease activity (Bleves et al., 2010). LepA is also involved in activating the NF-κB pathway by digesting human receptors (Kida et al., 2008).

PA has 3 types of T6SS, namely, H1-T6SS, H2-T6SS and H3-T6SS (Hachani *et al.*, 2011; Sana *et al.*, 2012). H1-T6SS secretes lytic proteins such as Tse1, Tse2 and Tse3 (Chen et al., 2015), which destroy other co-existing pathogens and provide a survival advantage to PA (Chen et al., 2015). H2-T6SS contributes to PA virulence (Sana et al., 2012; Chen et al., 2015) and is controlled by quorum sensing (*las* and *rhl* systems) and negatively controlled by iron (Sana et al., 2012). There are two type 6 secretory proteins including, valine–glycine repeat (VgrG) and hemolysin-coregulated protein (Hcp) (Bleves et al., 2010; Hachani et al., 2011). The Hcp1 is an H1-T6SS secretory protein and was shown to be present in CF patient's sputum (Mougous, 2006). In a study by Hachani and colleagues (Hachani et al., 2011), they showed ten VgrG genes in the genome of PAO1. Of these ten, three (i.e. vgrG1a, vgrG1b and vgrG1c) were shown to be controlled along with the H1-T6SS. They also showed that either vgrG1a or vgrG1c was necessary for the secretion of Hcp1. Also, Hcp was shown to be involved in PA biofilm formation and antibiotic resistance caused by biofilms (Chen et al., 2015).

There is a tentative proposal for a T7SS in PA which is yet to be well characterized (unpublished, project topic: A novel secretion system in Gram-negative bacteria. Dr Chrystala Constantinidou Laboratory, University of Warwick).

1.5.3 Quorum sensing in PA

PA also has a well-developed mechanism of cell to cell communication called quorum sensing (QS) (Camara *et al.*, 2002; Diggle *et al.*, 2002; Cámara, 2006). QS is a complex regulatory system of cell-to-cell signalling, essential for PA to coordinate gene expression and to adapt to different environments (Passador *et al.*, 1993; Davies *et al.*, 1998). For a detailed review of QS in PA, consult Venturi (Venturi, 2006) and Lee and colleague (Lee *et al.*, 2015). The concentration of QS signal molecules gives an indication of bacteria cell numbers with a certain threshold signifying a “quorated” population (Camara *et al.*, 2002; Cámara, 2006). A number of elements are vital to the QS process including, genes for signal synthesis, signal transduction and the QS signal molecule (Diggle *et al.*, 2002). Initially, two entangled QS systems were identified in PA, these are the *las* system [consisting of the transcriptional regulator: LasR and the synthase protein: LasI (Smith *et al.*, 2003)] and the *rhl* systems [consisting of the RhlI and RhlR proteins (Smith *et al.*, 2003)]. Both systems are essential for PA virulence and biofilm formation (Diggle *et al.*, 2002). Each of these systems produce and respond to the QS signal molecules: N-acylhomoserine lactones (AHL) (Pearson *et al.*, 1994). Two AHL have been identified in PA: the *N*-3-oxododecanoyl homoserine lactone (3-O-C12-HSL) which is part of the *las* system and *N*-butyryl-L-homoserine lactone (C4-HSL) which is part of the *rhl* system (Fuqua *et al.*, 2002; Smith *et al.*, 2003). The *las* system activates rhlR transcription and some studies show that AHL regulates about 10 percent of genes in PA (Schuster *et al.*, 2003). Once the concentrations of the freely inducible AHL (also known as autoinducer) reach a particular threshold, the QS molecule binds to the LasR / RhlR transcriptional activators leading to the expression of several genes. PA also releases a 4-quinolone signal molecule whose activity has been shown to be facilitated by the *las* and *rhl* systems. This molecule has been termed PQS which stands for 2-heptyl-3-hydroxy-4(1H)-quinolone (Schuster *et al.*, 2003). QS molecules have a direct effect on modulating the immune system (Smith *et al.*, 2002; Tateda *et al.*, 2003). Tateda and colleagues (Tateda *et al.*, 2003) showed the ability of AHL to cause apoptosis of neutrophils and macrophages but

not epithelial cells. There is also evidence of QS molecules inducing the production of inflammatory cytokines in both airway epithelial cells (Burns *et al.*, 2001; Smith *et al.*, 2002) and macrophages (Telford *et al.*, 1998). Thus QS activation contributes to virulence of PA to it host.

1.5.4 Iron Scavenging

The survival of an organism depends on its capability to compete for ferric iron with host cells (Xiao *et al.*, 1997). PA initially was thought to expresses two siderophores for this purpose: pyochelin and pyoverdin (Meyer *et al.*, 1996; Vasil *et al.*, 1999; Takase *et al.*, 2000). Both molecules tightly bind to iron to be taken up by the bacteria through special receptors on the cell surface. Interestingly, the PA quorum sensing signal molecule PQS also has iron chelating activity (Bredenbruch *et al.*, 2006) which helps PA to deprive host cells of iron.

1.5.5 Additional factors

Additional factors such as exotoxin A and pyocianin are expressed by PA. Exotoxin A (recognised by the receptors CD91 on several cells including monocytes, macrophages, dendritic cells (DCs) and T cells (Binder *et al.*, 2004)) can lead to host cell death by inhibiting elongation factor 2 of the host cell with the consequent inhibition of protein synthesis (Gellatly *et al.*, 2013). Phospholipids and surfactant lipids of host cells can also be disrupted by lipases and phospholipases released by PA (Kipnis *et al.*, 2006). Other virulence factors produced by PA are proteases such as serine protease and alkaline proteases. The serine protease (protease IV) has been reported to degrade both host surfactant proteins A and D (Malloy *et al.*, 2005), protease IV can also damage antibodies, complement components and fibrinogen (Malloy *et al.*, 2005). Alkaline proteases disrupt epithelial cell tight junctions and damage antibodies and fibrin, hence their reputable roles in sepsis and ocular infections caused by PA (Engel *et al.*, 1998). PA also produces a blue poisonous pigment called pyocyanin, which can induce stress in host cells, cause neutrophil apoptosis *in vitro* and disrupts catalase (which in healthy individuals acts as an antioxidant enzyme) in lung epithelial cells (O'Malley *et al.*,

2003). Pyocyanin may also be associated with protection of PA against reactive oxygen species (ROS) produced by phagocytes during the course of an infection (Lau *et al.*, 2004). Also, the ability of PA to phenotypically change in the CF lung from a non-mucoid to mucoid phenotype, by over expression of alginate can lead to the scavenging of ROS, which contributes to escape of host immune defence, disruption of complement activation, chemotaxis, and evasion of phagocytic killing by neutrophils and macrophages (Mishra *et al.*, 2012). PA elastase can cleave collagen, immunoglobulins (such as IgG and IgA) and complement components (Wilson *et al.*, 1987). Additionally PA expresses cytotoxins (e.g leukocidin) that are pore forming proteins, which enhance bacterial invasion and are cytotoxic to the host cells (Wilson *et al.*, 1987). Two haemolysins (e.g. phospholipase and lecithinase) function in concert to break down lipids and are also cytotoxic to host cells (Wilson *et al.*, 1987). The arsenal of virulence factors produced by PA enables its adaptation to new environments, competition with host cells for iron and other nutrients and also avoiding phagocytic and complement mediated killing, thus allowing continual survival in the host (Sadikot *et al.*, 2005).

1.6 Adaptation of PA to the CF lung

Major CF pathogens display genetic adaptability to the CF airway, such as antibiotic resistance, metabolic resourcefulness and the ability to elude the innate immunity, which explains why the CF lung is mainly colonized by rather distinct groups of organisms such as *S.aureus* and PA (Cohen *et al.*, 2012).

As mentioned above, PA isolated from chronically infected CF patients are phenotypically distinct from non CF patients and environmental strains (Sadikot *et al.*, 2005). These isolates have acquired changes in motility, multidrug resistance, LPS structure and the T3SS (Hoboth *et al.*, 2009; Lee *et al.*, 2011) as illustrated in Figure 1.2. The ability of PA to form biofilms is a major contributing factor for chronicity (Costerton *et al.*, 1999; Shirtliff *et al.*, 2002). Biofilms are structured microbial communities enclosed in an extracellular polymeric substance (EPS) (Wei *et al.*, 2013). Wei and Ma as well as Karatan and Watnick (Karatan *et al.*, 2009; Wei *et*

al., 2013) give an excellent review on biofilm formation and regulation in PA. Biofilm formation enables bacteria to withstand hostile environments and thus persist in their niches (Drenkard *et al.*, 2002; Lesprit *et al.*, 2003). Once bacteria form biofilms, they are more difficult to eradicate by the immune response or antibiotics (Kavanaugh *et al.*, 2012). Biofilms may serve as reservoirs where planktonic forms are released periodically to cause infections (Costerton *et al.*, 1999). However, a study by Bester and colleagues (Bester *et al.*, 2005) have shown that PA released from biofilms were characteristically different from attached biofilms and from planktonic PA (Bester *et al.*, 2005). Within the biofilm, PA loses its pili, flagella and T3SS, increases alginate production, releases DNA and several virulence factors (Cohen *et al.*, 2012).

PA inhibits CFTR recycling by releasing an inhibitory protein known as CFTR inhibitory factor (Cif) which is contained in outer membrane vesicles (Wade *et al.*, 2005). Cif redirects CFTR from the endosomes, where it is to be recycled, to the lysosomes where it is degraded (Bomberger *et al.*, 2011). In addition, MucA mutants are spontaneously selected within the population of PA in the biofilm leading to over expression of alginate, which facilitates evasion of phagocytic uptake (Martin *et al.*, 1993; Silo-Suh *et al.*, 2002). Indeed, the presence of alginate-producing PA isolates indicates that the bacteria has adapted well to the environment of its host which makes it more difficult to control PA infection by the innate immune system (Cohen *et al.*, 2012). Furthermore, lipid A modification by the addition of palmitate and aminoarabinose, as mentioned earlier, eventually leads to increased resistance to antimicrobial peptides and IL-8 production (Ernst *et al.*, 1999; Ernst *et al.*, 2007). The increase in IL-8 is attributed to recognition by human TLR4 (Hajjar *et al.*, 2002). Hajjar and colleagues (Hajjar *et al.*, 2002) showed that human TLR4 was able to distinguish the structures of CF Lipid A and that CF-derived LPS elicited much more robust pro-inflammatory response compared to non-CF LPS (Hajjar *et al.*, 2002). This observation was not true for mouse cells but only observed in human cells, implying that this distinctive recognition of LPS adaptation by TLR4 is species specific (Hajjar *et al.*, 2002).

Another factor that has been shown to aid PA adaptation to the CF lung is pyocyanin, a toxic phenazine pigment that is produced by PA especially within biofilms (Schaber *et al.*, 2004). Pyocyanin can inhibit the function of antioxidants, such as glutathione (GSH) and N-acetylcysteine (Rada *et al.*, 2008). Pyocyanin also has the ability to block chloride transport in human bronchoepithelial cells (HBECs), thereby exacerbating the defective CFTR function (Schwarzer *et al.*, 2008).

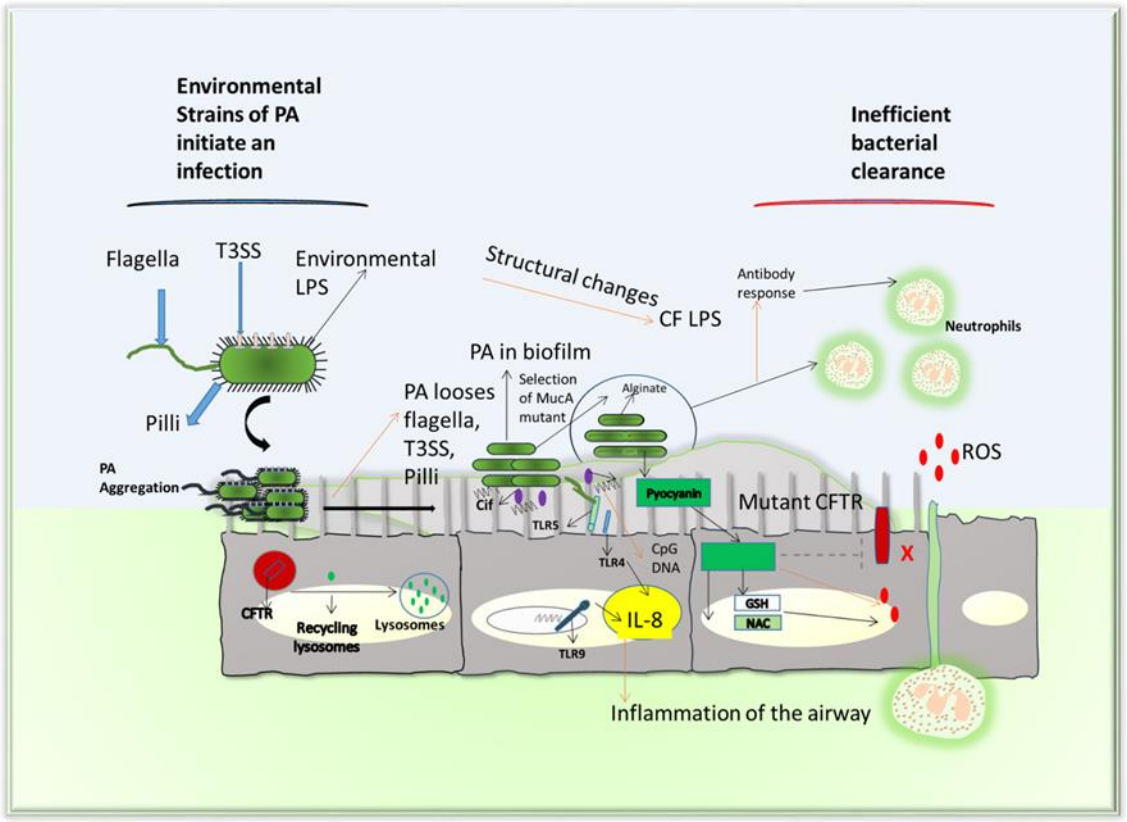


Figure 1.2: Adaptation of PA to the CF Lung.
As PA accumulates in the CF lung and reaches critical numbers, they form biofilms. Once in the biofilm, PA loses its pili, flagella and T3SS, increases alginate production, releases bacterial DNA and a variety of virulence factors which aid to evade the host immune response. PA also modifies the lipid A by the addition of palmitate and aminoarabinose which eventually leads to an increased resistance to antibiotics and IL-8 production. In addition, PA inhibits CFTR recycling by releasing an inhibitory protein known as Cif contained in its outer membrane vesicle which compounds the defective function of the mutated gene. Figure adapted from (Cohen *et al.*, 2012).

A current study by Huus and colleagues (Huus *et al.*, 2016) also showed that PA isolated from CF patients, both with stable disease and exacerbations, were unable to activate the inflammasome compared to environmental PA isolates and acute non-CF infection isolates (Huus *et al.*, 2016). Huus and colleagues (Huus *et al.*, 2016) ascribed the inability of these PA isolates from CF to activate the inflammasome to reduction in inflammasome ligands and reduced bacteria motility (Huus *et al.*, 2016). Thus PA adaptation to evade inflammasome activation might be another mechanism of PA adaptation to the CF lung.

These adaptations of PA, together with the defective function of the mutated CFTR gene, further promotes bacterial persistence and defective immune responses in CF (Cohen *et al.*, 2012).

1.7 The immune response to PA

Given the ubiquitous nature of PA, it is likely that a healthy immune system is well equipped to fight against this pathogen. The immune defences against PA encompasses various mechanical barriers which physically block entry of pathogens into the body (e.g. the skin) as well as other innate and the adaptive immune responses (Williams *et al.*, 2010). Innate immunity represents the first line of defence against invading pathogens and is therefore critical in the protection against PA infection (Lavoie *et al.*, 2011b).

1.7.1 The role of microbe associated molecular patterns (MAMPs) and pattern recognition receptors (PRRs) in the defence against PA

There are various preserved structures associated with microbes that allow their recognition by the host and the subsequent activation of the innate immune system. These structures are called microbe-associated molecular patterns (MAMPs). MAMPs in PA include flagella, alginate, lectins, the T3SS, effector exoenzymes (ExoS, ExoU, ExoT and ExoY) and exopolysaccharides (LPS, Psl and Pel) (Singh, 2012). These MAMPs are recognised by pattern recognition receptors (PRRs) (Parker *et al.*, 2011). PRRs are a set of germ-line encoded molecules comprising soluble proteins as well as cell surface, endosomal and cytosolic receptors (Parker *et al.*, 2011). Reference (Takeuchi *et al.*, 2010) gives a detailed review on the various PRRs. Four major classes of PRRs have been identified including transmembrane receptor proteins such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), cytoplasmic receptors such as the Retinoic acid-inducible gene (RIG) - like receptors (RLRs) and NOD-like receptors (NLRs) (Takeuchi *et al.*, 2010; Parker *et al.*, 2011). PRRs are expressed in multiple cell types including professional phagocytes (e.g. macrophages, DCs and neutrophils) and non-professional phagocytes [e.g. epithelial cells, fibroblasts and endothelial cells (Takeuchi *et al.*, 2010)]. Interaction between MAMPs and PRRs initiate several signalling cascades leading to effective immune responses against pathogens (Takeuchi *et al.*, 2010).

1.7.2 The role of TLRs in protection against PA

TLRs are members of the PRR family whch upon exposure to microbes or their products, initiate intracellular signalling pathways (Beutler, 2004). There are eleven and twelve TLRs in humans and mice, respectively (Beutler, 2004). These TLRs are type I transmembrane proteins, which contain leucine-rich repeats (LRRs) at their extracellular domain and a Toll/IL-1 receptor (TIR) domain at their cytoplasmic tail (Kawasaki *et al.*, 2014). Most TLRs (i.e. TLR1, 2, 4, 5, 6 and 11) are located on the plasma membranes whilst TLR3, 7, 8, and 9 are located in the endosomal compartment (Nishiya *et al.*, 2004). TLR engagement triggers signalling cascades leading to activation of NF-κB, MAP kinases, and activated protein (AP)-1 pathways (Takeda *et al.*, 2003).

The function of almost all TLRs except TLR3 depends on the adaptor molecule MyD88 (Zhang *et al.*, 2007). When TLRs become activated by either microbes or microbial products, the TLRs form hetero or homodimers and induce the recruitment of adaptor proteins through their TIR domain (Kawasaki *et al.*, 2014). Examples of adaptor proteins include MyD88, MyD88 adaptor-like protein (Mal), TIR-associated protein (TIRAP), TIR domain-containing adaptor protein-inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) (Kawasaki *et al.*, 2014). Upon recruitment of MyD88 for instance, MyD88 in turn recruits IL-1 receptor-associated kinases 1 and 4 (i.e. IRAK1 and IRAK4) (Kawasaki *et al.*, 2014). IRAK4 subsequently activates IRAK1 by phosphorylation, then IRAK1 and IRAK4 become ubiquitinated by associating with TRAF6 (Kawasaki *et al.*, 2014). This association with TRAF6 activates the IκB kinase (KK) complex with a subsequent activation of NF-κB and of MAP kinases (Kawasaki *et al.*, 2014).

The different TLRs use different adaptor proteins, TLR4 and TLR2 require TIRAP (also known as Mal) for their signalling through MyD88 (Hemmi *et al.*, 2002). TLR3 upon stimulation by double-stranded RNA, uses the adaptor TRIF (also known as Toll like receptor adaptor molecule-1 (TICAM-1)) to induce the production of IFN-β in a MyD88-independent manner (Hayashi *et al.*, 2001). TRIF is also involved in TLR4 mediated signalling in a MyD88-

independent manner (Hu *et al.*, 2015). TLR3, TLR7, TLR8 and TLR9 become stimulated by recognition of nucleic acids from viruses and induces the production of type I IFNs by signalling through different interferon regulatory factors (IRFs) (Sato *et al.*, 2000) . Three IRFs (i.e. (IRF3, IRF5 and IRF7) are involved with signalling by these TLRs. IRF3 and IRF7 are activated by TLR3 and TLR4 (Sato *et al.*, 2000) while IRF5 and IRF7 are activated by TLR7 and TLR8 (Cao *et al.*, 1996).

The adaptor molecule MyD88 is essential for PA control during lung infection *in vivo* (Mijares *et al.*, 2011a). Various components of PA interact with different TLRs as shown in Figure 1.3. The most important TLRs promoting defence against PA are TLR4, TLR2 and TLR5. TLR4 recognises LPS while TLR5 recognises flagellin (Feuillet *et al.*, 2006). Faure and colleagues (Faure *et al.*, 2004) demonstrated the importance of TLR4 for protection against PA in an infection model using wild type PA103 and a mutant PA103 deficient in T3SS, by showing that infections with wild type PA103 caused acute lung injury and sepsis in all cases. These effects were not observed with the mutant PA103. TLR4 signalling was essential in clearing infection and avoiding lung injury (Faure *et al.*, 2004) . TLR2 senses PA pili and plays a role in activation of epithelial cells (Sadikot *et al.*, 2005). The DNA of PA can be recognised through TLR9. However, the role of TLR2 and TLR9 in the control of PA infections is not as well studied (Sadikot *et al.*, 2005).

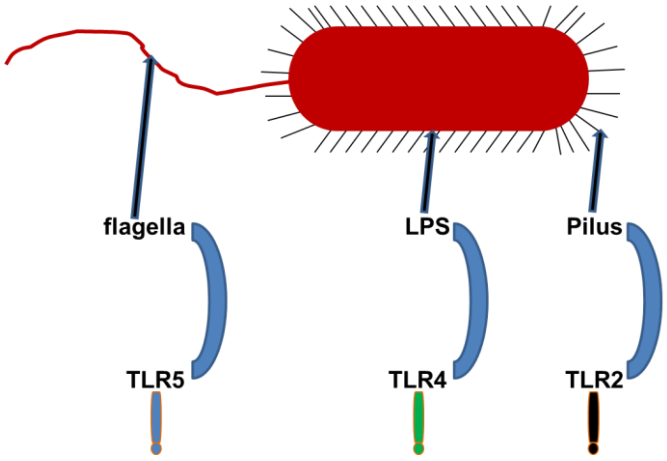


Figure 1.3: Interaction of various PA surface components with different TLRs.
Various surface components of PA interact with different TLRs. TLR 2 interacts with pili, TLR 4 interacts with LPS, TLR 5 interacts with the flagella

1.7.3 The role of the inflammasome in protection against PA

Another important process for the innate immune system ability to fight PA is the activation of the inflammasome. Inflammasomes are a group of scaffold and signalling proteins that result in signalling cascades involved in the detection of pathogens and stimuli, which results in the activation of the inactive cytokines pro-IL-1β and pro-IL-18 to their active forms and also sometimes, lead to the induction of an inflammatory form of cell death called pyroptosis (Latz *et al.*, 2013; Sharma *et al.*, 2016). More details on the inflammasomes is covered in Chapter 5.

Various PA components such as flagella, LPS and pilus (Figure 1.3), as well as an intact T3SS can activate the inflammasome in varying degrees (Cohen *et al.*, 2013). PA invasion of the airway activates caspase-1 through the NLR protein NLRC4. However, the T3SS effector proteins ExoS (Bals *et al.*, 1998) and ExoU (Costerton *et al.*, 1999) can cause an inhibition of caspase-1 activation through their activities as phospholipase A2 and ADP-ribosyltransferase, respectively (Sadikot *et al.*, 2005). Formation of the inflammasome complex recruits pro-caspase-1, which is activated into caspase-1.The activation of caspase-1 and the consequent IL-1 receptor (IL-1R)-dependent signalling is essential for the recruitment of neutrophil to infection sites (Mijares *et al.*, 2011a). Signalling of the IL-1R through MyD88 in epithelial cells of the airways is indispensable for a timely response by the host (Mijares *et al.*, 2011a). Likewise, Schultz and colleagues (Schultz *et al.*, 2002) reported that absence of IL-1R signalling significantly reduced the clearance of PA in the lung. It is as well reported that, MyD88 knockout mice failed to control the replication of PA in the lung, whereas knockout mice in individual MyD88-dependent pathways such as TLRs, IL-1R and Caspase-1 are able to control the replication of PA in the lung (Lavoie *et al.*, 2011b), thus highlighting the fact that various MyD88-dependent pathways collaborate to control PA infection (Lavoie *et al.*, 2011b).

1.7.4 Role of immune cells in controlling PA infection

Various cell types contribute to the immune response against PA (Figure 1.4). These include, epithelial cells, neutrophils, macrophages, DCs and lymphocytes (Gellatly *et al.*, 2013). However, neutrophils are considered the most important cells for defence against PA infection (Williams *et al.*, 2010) . The contributions by the above individual immune cells in the context of airway infections with PA is described below:

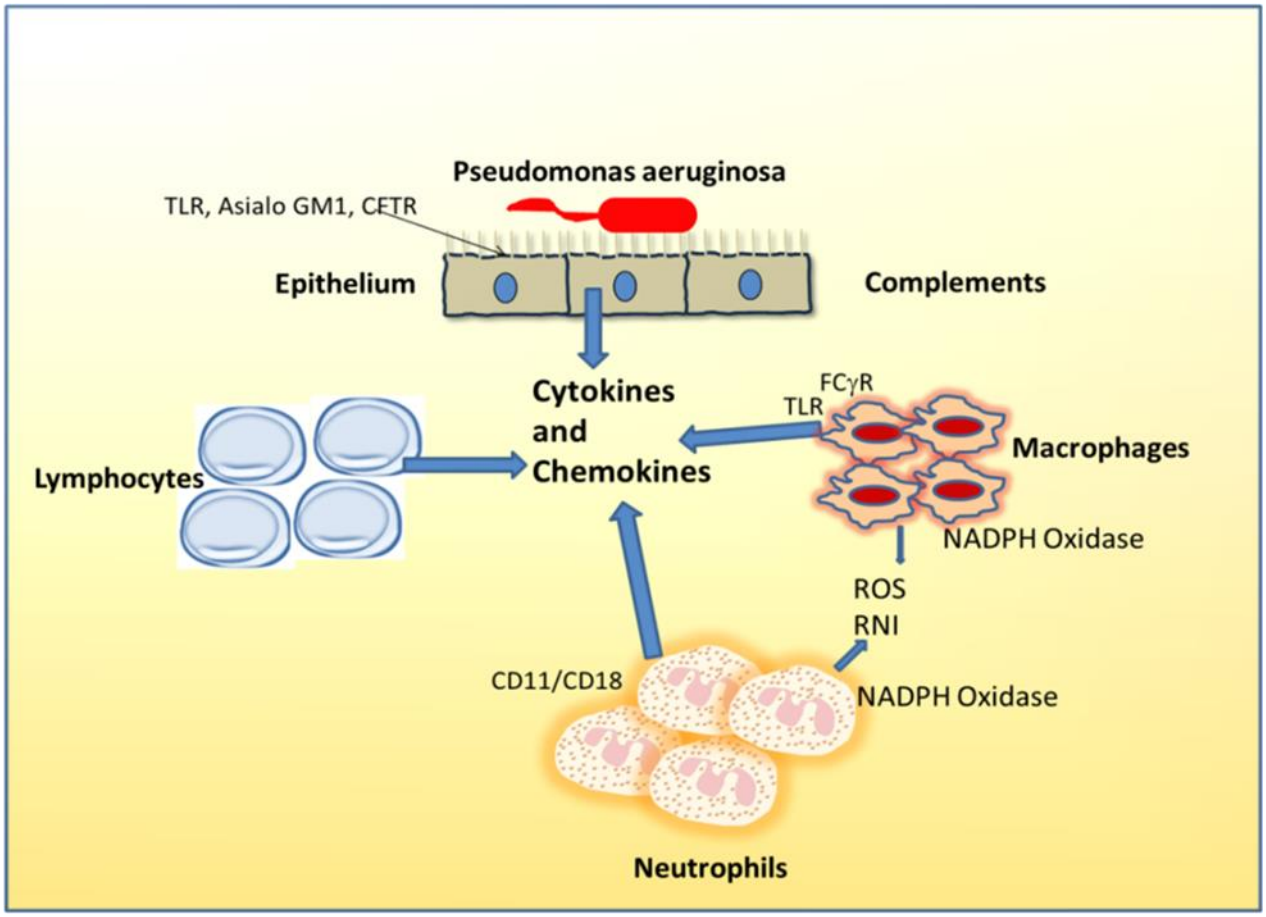


Figure 1.4: Role of different immune cells in response to PA.
Upon PA infection of the airways, several signalling cascades are activated and a successful elimination of the bacteria results from the concerted effort of various cell types. The contributions by the individual cell types such as epithelial cells, macrophages, neutrophils and lymphocytes are described below. TLR = Toll-like receptor, NADPH = reduced nicotinamide adenine dinucleotide phosphate, RNI = reactive nitrogen intermediates, ROS = reactive oxygen species. Figure adapted from (Sadikot *et al.*, 2005).

a. Epithelial cells

Epithelial cells are the first cells to encounter inhaled pathogens in the airways. The airway epithelium has about 8 morphologically distinct cell types which can be grouped into 3 categories (i.e. basal , ciliated and secretory) (Knight *et al.*, 2003), although immune cells (such as mast cells, macrophages, dendritic cells and intraepithelial lymphocytes) may also migrate to and reside in the epithelium (Knight *et al.*, 2003).

Over 50% of the airway epithelial cells (AECs) express cilia to physically remove foreign organisms trapped in mucus, by beating out organisms to body openings for elimination (Knight *et al.*, 2003). Other AECs specialise in producing the mucus that traps microorganisms to enhance their expulsion by cilia, while others produce surfactants containing antimicrobial opsonic proteins (for instance Surfactant protein A and D) (Knight *et al.*, 2003; Wright, 2005; Voynow *et al.*, 2009; Singh, 2012). Besides AECs function as a physical barrier (by their wealth of tight junctions, intermediate and gap junctions to maintain their structural integrity), they also express antimicrobial proteins, various cationic peptides, and a wide range of PRRs (Knight *et al.*, 2003), hence their important role in the innate immune system.

Signalling through PRRs as a result of stimulation by microbe associated molecular patterns (MAMPs) and damage associated molecular patterns (DAMPs), lead to activation of NF-κB pathway (Sadikot *et al.*, 2006b) and production of pro-inflammatory cytokines such as TNF-α and IL-8 (Sadikot *et al.*, 2006b). IL-8 eventually causes immune cell recruitment to infection sites (Sadikot *et al.*, 2006b). The production of surfactant proteins by AECs are also reported to be essential in defence against viral and bacterial infections including PA (Parker *et al.*, 2011).

b. Alveolar Macrophages

Macrophages could be tissue resident or derived from monocytes during inflammation (Martinez *et al.*, 2014). More details on macrophages and their function in the immune response is covered in Chapter 4.4. Macrophages adopt different phenotypes depending on the organ in which they are located, accordingly macrophages in different tissues have been named differently. For instance, Langerhans cells are the macrophages in the skin, Kupffer cells are the macrophages in the liver, Microglial cells are macrophages in the brain, Osteoclasts are macrophages in the bone and Alveolar macrophages are macrophages in the aveoli of the lung (Epelman et al., 2014). Alveolar macrophages are derived from foetal embryonic monocytes under GM-CSF dependent processes (Gordon et al., 2017) with their replication mostly independent of the contributions of monocytes (Gordon et al., 2017). These cells contribute about 20–30% of the metabolism of surfactant lipids and proteins (Gordon et al., 2017). These surfactant lipids and proteins reduce the surface tension in the alveoli by keeping them open in the course of breathing (Gordon et al., 2017).

Alveolar macrophages have unique characteristics that distinguish them from other macrophages (Guth *et al.*, 2009). For instance, studies have shown that alveolar macrophages have high expression levels of CD11c (Guth *et al.*, 2009). Meanwhile, CD11c is generally not expressed by other macrophages but expressed by DCs, some NK cells and activated T cell subsets (Guth *et al.*, 2009). Alveolar macrophages also express high levels of CD205 which is generally expressed by some populations of DCs but not expressed in macrophages (Bilyk *et al.*, 1991). Other studies show that alveolar macrophages from bronchoalveolar lavage (BAL) of normal mice lungs are phenotypically distinct from tissue macrophages (Gonzalez-Juarrero *et al.*, 2001; Bosio *et al.*, 2005; von Garnier *et al.*, 2005). Macrophages are generally CD11b positive with high expression of MHC II. However, alveolar macrophages are CD11b negative unless stimulated (Guth *et al.*, 2009). These unique characteristics of alveolar macrophages have been associated with their unique environment: the cells are constantly

under high oxygen tension, bathed in high levels of GM-CSF and surfactant proteins such as SP-A and SP-D, which can modulate their properties (Wright, 2005; Guth *et al.*, 2009).

Alveolar macrophages are the first defence mechanism once an organism reaches the lower airway (Guth *et al.*, 2009) . They are involved in phagocytosis of invading pathogens as well as secretion of cytokines and enzymes (Guth *et al.*, 2009).

Although macrophages can phagocytose and kill bacteria, during PA infection, they are shown to enhance the recognition of the bacteria and triggering inflammation by releasing cytokines and chemokines (Lavoie *et al.*, 2011b). Eloise and colleagues (Eloïse *et al.*, 2009) demonstrated that murine alveolar macrophages in culture secrete the chemokine: CXCL1 as a result of TLR-4 activation by LPS and the cytokines TNF-α and IL-6 as a result of TLR-5 activation by flagellin. Furthermore, macrophages can secrete interleukin 1β (IL-1β), which can consequently cause the release of neutrophil chemokines by epithelial cells (Mijares *et al.*, 2011a). Hashimoto and his colleagues showed that depletion of alveolar macrophages in mouse and rat models of acute PA lung infections, led to a diminished chemokine (such as macrophage inflammatory protein-2 (MIP-2) and CXCL-1(KC)) secretion, decreased recruitment of neutrophils and clearance of PA (Silva, 2010b). Kooguchi and colleagues (Kooguchi *et al.*, 1998) also reported decrease early recruitment of neutrophils when lung macrophages were depleted (Kooguchi *et al.*, 1998). A recent study by Singh and colleagues (Singh *et al.*, 2015) showed that human macrophages under non-opsonic conditions could not themselves control PA growth but release a number of signals that could enhance the recruitment of other immune cells to infection sites. Together, these studies demonstrate that, macrophages enhance the recruitment of neutrophils to infection site and coordinate immune response against PA

c. Neutrophils

Neutrophils originate from the bone marrow from myeloid precursor cells and constitute about 50–70% of the circulating white blood cells in humans, but only 10–25% in mice (Doeing *et al.*, 2003; Mestas *et al.*, 2004). Neutrophil proliferation and differentiation within the bone marrow is controlled by the granulocyte colony stimulating factor (G-CSF) (Lieschke *et al.*, 1994) in response to cytokines such as interleukin 17 A (IL-17A). IL-17A can be synthesised by neutrophil-regulating T cells including Th-17 cells, γδ T cells, natural killer (NK) T cells (Ley *et al.*, 2006) and a subset of $CD8^+$ cytotoxic T cells (Kondo *et al.*, 2009). The production of IL-17A is also controlled by IL-23, which originates from dendritic cells and resident tissue macrophages (Kolaczkowska *et al.*, 2013). Upon infection, matured neutrophils are recruited to the infection sites (Balamayooran *et al.*, 2010) . Neutrophil initial recruitment, activation and survival is facilitated by airway epithelial cells and macrophages through the release of G-CSF, GM-CSF, IL-8, IL-17A and TNF-α. Also, neutrophils themselves can cause the recruitment of more neutrophils at the site of infection by releasing IL-17A and IL-8 (Linden *et al.*, 2005; Mantovani *et al.*, 2011). As the neutrophils reach sites of inflammation, they become activated, producing free radicals, releasing the content of their granules and engulfing invading organisms. Thus during inflammation, neutrophil numbers increase in tissues but as time progresses, they undergo apoptosis and are eliminated by macrophages and dendritic cells. This process subsequently down regulates IL-23 and reduce the release of G-CSF (Stark *et al.*, 2005; Ley *et al.*, 2006).

Circulating mature neutrophils are between 7–10 μm in diameter, with segmented nucleus and a cytoplasm enriched in granules and secretory vesicles (Borregaard, 2010). There are three types of neutrophil granules: primary, secondary and tertiary granules, which contain inflammatory molecules and enzymes (Borregaard, 2010; Galli *et al.*, 2011). The primary granules, also known as azurophilic granules, contain myeloperoxidase (MPO) and antimicrobial peptides that in humans can be sub-divided into $defensins^{hi}$ and $defensins^{low}$. The secondary granules, known as specific granules, contain lactoferrin and in humans can

be sub-divided into four subtypes (lactoferrin^{hi}, gelatinase^{hi}, ficolin1^{hi} and cysteine-rich secretory protein 3^{hi} (CRISP3) containing granules (Borregaard, 2010)). The tertiary granules, known as gelatinase granules, contain matrix metalloproteinase 9 (MMP9) also called gelatinase B (Kolaczkowska *et al.*, 2013). Neutrophils can clear invading pathogens in different ways. Just like other phagocytes (i.e. macrophages and DCs), neutrophils phagocytose target particles under both opsonic and non-opsonic conditions (Lee *et al.*, 2003). The uptake of particles is however enhanced under opsonic conditions (Lee *et al.*, 2003). In the latter case, neutrophils can engulf opsonised pathogens by using either Fc receptors (such as FcγRIIA (CD32) and FcγRIIIb (CD16)) which bind to immunoglobulin-coated particles, or β2 integrins (such as Macrophage-1 antigen (MAC1), consisting of CD11b and CD18 integrins) which bind to complement-coated particles (McKenzie *et al.*, 1998). It is suggested that particles opsonised by complement components are internalized by sinking into the cell to form the phagosome, while particles opsonised by immunoglobulin lead to ligation of Fcγ recepters with a vigorous extension of pseudopods that enclose and finally trap the particle into a phagosome (Greenberg *et al.*, 2002). The phagosome subsequently matures by undergoing a series of remodelling events including acidification and increased presence of degrading enzymes (Lee *et al.*, 2003). A detailed process of neutrophil phagocytosis is discussed by Lee and colleagues (Lee *et al.*, 2003).

Within the phagosomes, microbes are killed through the action of reactive oxygen species (NADPH oxygenase-dependent) or antibacterial proteins (lysozyme, defensins, lactoferrin and neutrophil proteases) (Borregaard *et al.*, 1990; Hager *et al.*, 2010). For instance, lysozyme is an anti-microbial protein which disrupts the peptidoglycan component of the bacteria making the bacteria more permeable, while defensins are cationic antimicrobial proteins that attack the cell membranes of bacteria (Lee *et al.*, 2003; Mayer-Scholl *et al.*, 2004). Lactoferrin binds iron, reducing availability of iron as iron is critical for bacteria growth. Neutrophil proteases such as neutrophil elastase and cathepsins degrade bacteria virulence factors (Lee *et al.*, 2003; Mayer-Scholl *et al.*, 2004). These antibacterial proteins either act on intracellular or

extracellular pathogens when released into the phagosomes or the extracellular environment, respectively (Kolaczkowska *et al.*, 2013).

Additionally, neutrophils can clear extracellular pathogens by releasing neutrophil extracellular traps (NETs) (Kolaczkowska *et al.*, 2013). NETs are made up of core DNA complexed with histones, proteins such as cathepsins and lactoferrin, and enzymes such as neutrophil elastase and MPO released from neutrophil granules (Kolaczkowska *et al.*, 2013). NETs can immobilise pathogens, hence enhancing further phagocytosis of the trapped microbes (Kolaczkowska *et al.*, 2013). NETs can also kill pathogens directly through antimicrobial proteases and by releasing histones. The antimicrobial properties of histones were recognised long ago, but the mechanism of their antimicrobial action is still unclear (Papayannopoulos *et al.*, 2009; Mesa *et al.*, 2013). A recent study by Floyd and colleagues (Floyd *et al.*, 2016) showed the importance of the PA flagella for NETs induction during PA infection.

Several studies have demonstrated the role of neutrophils in the control of PA infection. For instance, neutrophil-depleted mice were more susceptible to PA infection even when very low numbers of bacteria were used (Koh *et al.*, 2009). Tsai and colleagues (Tsai *et al.*, 2000) showed that neutrophil depletion results in higher mortality of mice with PA-induced pneumonia and Fujimoto and colleagues (Fujimoto *et al.*, 2002) revealed the importance of neutrophils in the defence against PA infection using murine models of PA induced sepsis.

d. Lymphocytes

Lymphocytes are white blood cells consisting of NK cells, B and T cells. NK cells are innate immune cells and function in cell-mediated cytotoxicity while the B and T cells are fundamental for adaptive immune responses. B cells are responsible for humoral immunity, whereas T cells facilitate cell-mediated immune responses (Zheng, 2013). T lymphocytes can be subdivided into helper (Th, CD4+) and cytotoxic T cells (CD8+), and helper cells can be subdivided into Th-1, Th-2, Th-17, Th-9 , Th-22 and Tregs (Bayes *et al.*, 2010; Zheng, 2013) depending on their pattern of cytokine production. More details on T cell subtypes is covered in Chapter 4.

The role for T-lymphocytes in PA control is not well understood. There is some evidence of T cells involved in immunomodulation during PA infection especially in chronic infections (Dunkley *et al.*, 1994). Different strains of mice show different T cell response to PA infection and T cell responses correlate with susceptibility to infection (Dunkley *et al.*, 1994). The mice susceptible to PA show a predominant Th-2 pulmonary response whereas the resistant mouse strains show a predominant Th-1 response (Dunkley *et al.*, 1994). This pattern of T cell response has been reported by other researchers (Moser *et al.*, 1999; Tam *et al.*, 1999). Predominant Th-1 responses also correlate with better CF lung function compared to Th-2 responses in chronic PA infected CF patients (Moser *et al.*, 2000; Moser *et al.*, 2002).

Th-17 cells predominate in endobronchial biopsies of patients with CF and non-CF bronchiectasis compared to healthy controls. Likewise, the Th-17 cytokine IL-17 is high in BAL of established CF patients, with a positive correlation between IL-17 levels and neutrophil numbers (Tan *et al.*, 2011a).

A recent study by Bayes and colleagues (Bayes et al., 2016) however shows that the principal cytokine of Th-17 cells (i.e. IL-17) is essential for protection against PA induced chronic pulmonary infection, in mouse models infected with PA strains from CF patients (Bayes et al., 2016). Thus, the outcome of infections with PA depends on the type of T cell response.

B cell (antibody- mediated) immune responses are not beneficial in PA infections, especially in CF patients (Koch *et al.*, 1993). Although healthy individuals have some level of anti-PA antibodies (Mauch *et al.*, 2014), chronically infected CF patients develop a vigorous humoral response against PA (Mauch *et al.*, 2014) and this has been used in CF treatment centres to distinguish a chronic infection from a colonisation (Hoiby *et al.*, 1977; Pressler *et al.*, 2009). Researchers showed that PA-specific antibodies in CF patients react against LPS, flagella and elastase, and they tend to form immune complexes, which result in complement activation and opsonisation and neutrophil activation which indirectly induces tissue damage (Hoiby *et al.*, 1977; Koch *et al.*, 1993).

1.8 Altered immune response in Cystic Fibrosis

An effective immune response is as a result of the synergistic contribution of epithelial cells, resident and recruited immune cells including neutrophils, macrophages, DCs and T cells. Whether CFTR plays a direct role in the function of immune cells, is still blurred and controversial, as there is evidence of a normal immune function of CF patients at different sites except for the lung (Cohen *et al.*, 2012).

Other studies show that CFTR acts as a receptor for PA in AECs and that a defective CFTR function may affect AECs function (Cohen *et al.*, 2012). Even though some studies have suggested a similar expression of TLRs between CF and non-CF cells (Muir *et al.*, 2004; Greene *et al.*, 2005), changes in localization of the receptors may lead to significant differences in the onset of TLR signalling (Cohen *et al.*, 2012). There is increased TLR2 and TLR5 expression in human AECs from CF, which correlates with higher inflammatory responses (Muir *et al.*, 2004; Greene *et al.*, 2005). Furthermore, mutations in CFTR may lead to defective glycosylation of secreted epithelial glycoconjugates (such as proteoglycans and glycoproteins). Defects in glycosylation affect host pathogen interactions, mucus composition and bacteria clearance (Barasch *et al.*, 1991; Saiman *et al.*, 1993). Airway epithelium also produces antioxidants including thiocyanate (SCN^-) and glutathione (GSH) which are trafficked into the epithelial lining fluid (Cantin *et al.*, 1987), these aid in controlling damage caused by oxidative stress (Cantin *et al.*, 1987) and reverse the viscosity of the airway mucus (Kelly, 1999; Hudson, 2001). There is evidence of high reduction of glutathione in both CF patients (Roum *et al.*, 1993) and CF mouse models (Velsor *et al.*, 2001) due to defective protein trafficking in CF. The defective CFTR affects trafficking of GSH into the epithelial lining fluid but not the production of GSH (Gao *et al.*, 1999; Kogan *et al.*, 2003). Thus mutations in CFTR affect these antioxidants and lead to a high oxidative stress (Bartling *et al.*, 2009). Similarly, the trafficking of thiocyanate is also reduced in mutant CFTR epithelial cells with consequent decrease in pathogen clearance such as *S. aureus and* PA (Moskwa *et al.*, 2007).

The excessive pro-inflammatory signalling observed in macrophages from both CF patients and murine CF models may be partly due to sustained signalling of TLR4 observed in early endosome, which leads to the activation of NF-κB and the interferon pathways (Cohen *et al.*, 2012).

Some researchers have shown that CF neutrophils and macrophages from humans have a lower ability to kill phagocytosed bacteria (Hartl *et al.*, 2007; Painter *et al.*, 2008) when compared with non CF controls. These findings however, have been challenged by Kingma (Kingma, 2010) who found no significant difference in the phagocytic function of neutrophils using confirmed CF new-borns and normal controls.

A theory supported by many CF researchers however is that there is a slower neutrophil apoptotic rate in CF patients (McKeon *et al.*, 2008; Moriceau *et al.*, 2010), which in part is due to resistance of CF neutrophils to the pro-apoptotic cytokines TNF-α and GM-CSF. GM-CSF can function as an anti-apoptotic cytokine and can be produced through activation of the NF-κB pathway by epithelial cells and GM-CSF is found in much higher quantities in CF than in normal controls (Saba *et al.*, 2002). These findings imply that neutrophils in CF persist in the airway and contribute to inflammation.

Furthermore, the large quantities of neutrophil proteases hinder effective phagocytosis (Vandivier *et al.*, 2002; Sedor *et al.*, 2007), by cleavage of macrophage receptors, defective opsonisation and diminished killing of bacteria (Cohen *et al.*, 2012). These observations suggest that even if CFTR mutations have no direct effect on the function of phagocytic cells, they could perhaps have an effect on the kinetics of neutrophil degradation, the release of DNA and proteases which may have an additional effect on the exaggerated inflammation in the airway of CF patients (Cohen et al., 2012).

Both CF patients and mice have impaired process of autophagy with a consequent aggregation of p62 (Cormet-Boyaka et al., 2016). Autophagy is a physiological process that is highly regulated and involves the degradation of pathogens, non-functional cells and protein

aggregates (Cormet-Boyaka et al., 2016). The process of autophagy turns over energy during starvation and stress (Cormet-Boyaka et al., 2016). The autophagy process is in charge of degrading p62 and thus impairment in autophagy leads to accumulation of p62 which subsequently form aggregates (Fua et al., 2009; Cormet-Boyaka et al., 2016). Indeed, CF macrophages have accumulated amounts of p62 which enhance mutant CFTR sequestration (Cormet-Boyaka et al., 2016). The presence of p62 aggregates also lead to the consumption of autophagosome proteins (Cormet-Boyaka et al., 2016).

Moreover, the sustained inflammation in CF airways has also been associated with a reduced A20 in CF (Kelly et al., 2013). A20 is an enzyme that negatively regulates the NF-κB pathway and has an important role in the homeostasis of the immune system (McCallum K et al., 2016). Thus the absence of A20 in CF airway epithelial cells contributes to a sustained activation of NF-κB, with a subsequent sustained inflammatory state (Kelly et al., 2013). This has led to A20 been targeted as an anti-inflammatory drug (Reihill JA et al., 2015; Malcomson et al., 2016).

1.9 Rationale of the project

This study was designed to investigate the cooperation between phagocytic cells (macrophages and neutrophils), in controlling *Pseudomonas aeruginosa* (PA) infection and how infection outcome is affected by prevalent inflammatory conditions. The study stems from a previous work in our laboratory (Singh, 2012) where it was observed that interferon (IFN)-γ production by peripheral blood mononuclear cells (PBMCs) from CF patients positively correlated with lung function, particularly in patients chronically infected with PA, whereas IL-17A levels inclined to correlate negatively with lung function with this trend becoming significant in CF patients chronically infected with PA (Singh *et al.*, 2015). These results were in agreement with IFN-γ and IL-17A playing protective and detrimental roles respectively in CF. To explore the protective effect of IFN-γ in CF, the effect of IFN-γ alone or in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) on the ability of human macrophages to control PA growth, resist the cytotoxicity induced by PA and to promote or inhibit inflammation was investigated (Singh *et al.*, 2015). Treatment of macrophages with IFN-γ in the presence and absence of GM-CSF did not alter bacterial growth or macrophage survival upon PA infection, but made the macrophages more pro-inflammatory (Singh *et al.*, 2015) (Figure 1.5). As neutrophils have been described as the key effector cells for the control of PA infection (Sadikot *et al.*, 2005; Hirche *et al.*, 2008; Koh *et al.*, 2009; Williams *et al.*, 2010), there was therefore the need to include neutrophils as the effector phagocytes in the infection assays (Figure 1.5) to determine how the cooperation between these cells and inflammatory conditions affect the control of PA.

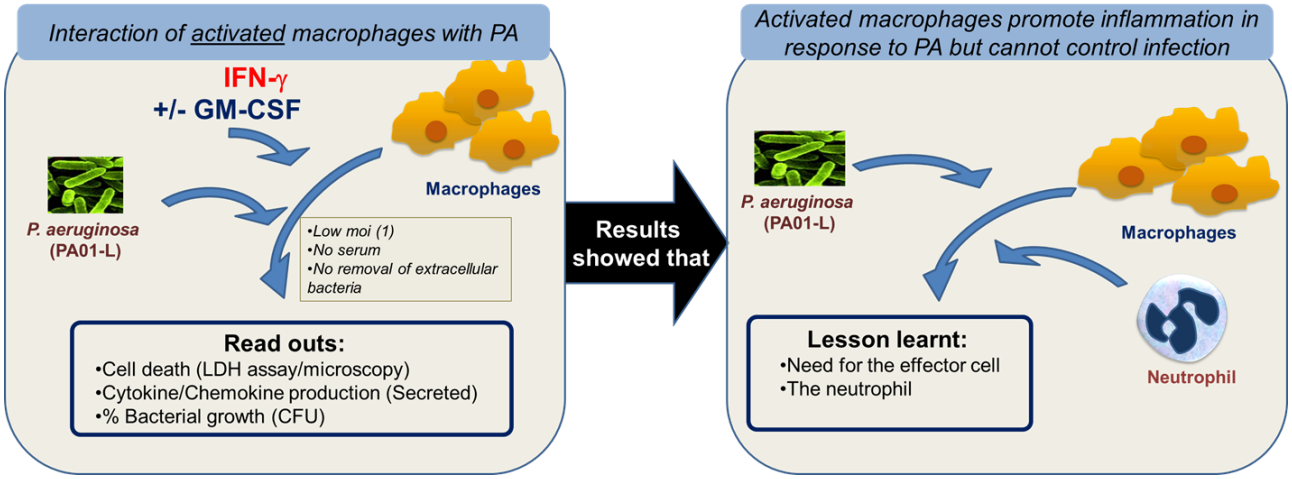


Figure 1.5: Previous results leading to this project.
In a previous study in our laboratory, treatment of human monocyte-derived macrophages with IFN-γ in the presence and absence of GM-CSF made the macrophages more pro-inflammatory based on their secreted cytokines with IFN-γ + GM-CSF treatment being more pro-inflammatory compared to IFN-γ only treatment. However, all of these treatments of the macrophages failed to alter bacteria growth or macrophage survival upon infection with PA. As neutrophils are the effector cells in controlling PA infection, there was the need to include neutrophils as the effector phagocytes in the infection assay to determine how both phagocytes collaborate in controlling PA under different inflammatory conditions.

1.10 Hypothesis of project

IFN-γ is beneficial in PA infections despite not promoting bacterial clearance by promoting a more balanced neutrophilic inflammatory response through its direct effect on IL-1β production.

1.11 General aim of the project

To establish a macrophage and neutrophil co-culture model which will be suitable to investigate human anti-bacterial immunity and how it is affected by prevalent inflammatory conditions.

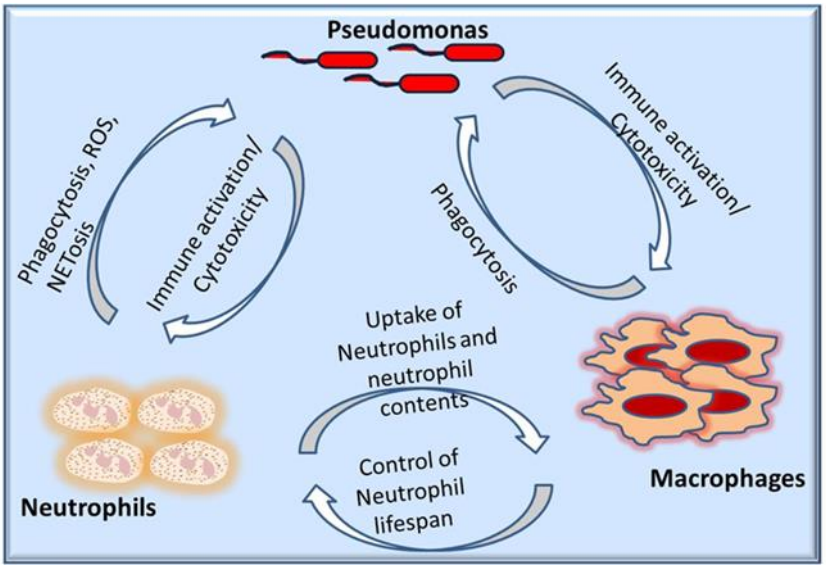


Figure 1.6: Potential interactions between macrophages and neutrophils during PA infection.
Upon PA invasion, the bacteria could either exert cytotoxic effects on the immune system by the release of virulence factors or cause immune activation. The latter consequently stimulate an immune response for the elimination of the bacteria. Focusing on the cooperation between macrophages and neutrophils, macrophages could cause the release of inflammatory mediators to attract other immune cells such as neutrophils to the infection site. The neutrophils could phagocytose and kill bacteria using their cytotoxic neutrophilic granule molecules. Neutrophils could also destroy bacteria by the release of reactive oxygen species (ROS) or through the formation of neutrophil extracellular traps (NETs). However, due to the cytotoxic nature of neutrophil granule molecules, their activity needs to be well controlled to avoid an over exuberant inflammatory process and tissue damage. Thus, macrophages ability to control neutrophil's life span and their uptake of apoptotic neutrophils and neutrophil content, help to control the inflammatory process to reduce tissue damage: as neutrophils are taken up before they lyse, hence avoiding the release of the neutrophil's content into the surrounding tissue. Thus, macrophage and neutrophil co-cultures will provide an appropriate model of investigating anti-bacterial immunity and how it could be affected by major inflammatory conditions.

1.12 Specific aims of the project

1. To establish a macrophage/neutrophil co-culture infection model of PA

2. To investigate the ability of human macrophage/neutrophil co-cultures to control PA growth, promote or inhibit inflammation and cause tissue damage.

3. To determine how the presence of the pro-inflammatory cytokines IFN-γ and IL-17A affect the outcome(s) of the infection of macrophage/neutrophil co-cultures with PA.

Chapter 2

General materials and methods

Chapter 2: General materials and methods

2.1 Ethical clearance

Ethical approval for the study was obtained from the University of Nottingham Research Ethics Committee (Ethics Reference No: BT17012011).

2.2 Inclusion criteria

Healthy blood donors who have not consumed any alcohol or any medication in the past 24-72 hours were used during the course of this study.

2.3 Informed consent

Informed consent was sought from all healthy volunteers and a consent form was issued to him/her to read, sign and return to the investigator.

2.4 Phlebotomy

Intravenous blood was drawn by the QMC phlebotomist service into sterile EDTA BD vacutainers (BD Diagnostics-Preanalytical Systems, UK, REF: 367525) for whole blood samples. For serum samples, blood was drawn into sterile plain serum tubes (Scientific Laboratory Supplies, REF: BE04-744Q).

2.5 Bacteria strain

Pseudomonas aeruginosa strain PAO1 subline Lausanne (PAO1-L) has been employed in this study. PAO1 was originally isolated by Holloway in 1955 (Holloway, 1955) but studies have shown the divergence of different sublines from the original PAO1 (Klockgether *et al.*, 2010). PAO1 lacks the ExoU of the T3SS but expresses the ExoS, ExoT, and ExoY (Stover *et al.*, 2000; Vance *et al.*, 2005). Based on the LPS O antigen classification, PAO1-L belongs to the serogroup O2/O5 and has moderate virulence (Priebe *et al.*, 2002; Singh *et al.*, 2015). Gene sequence for PAO1-L has shown it is closely related to the original PAO1 strain isolated by Holloway (Singh *et al.*, 2015).

PAO1-L expressing green fluorescent protein (GFP) was obtained from Jean Dubern (University of Nottingham). A mini-Tn7 system which facilitates integration of single-copy genes into the PAO1-L chromosomes was used to generate the PAO1-L-GFP strain (Lambertsen; Unpublished).

2.6 Preparation of PA inoculum

To prepare PA inoculum for the various infection assays, PA from -80^{o}C stock prepared in 50% glycerol in Lysogeny broth (LB) was streaked onto a LB agar plate and incubated at 37^{o}C overnight. A single colony was picked from the plate the next day into 5 ml X-Vivo 15 medium (without phenol red and gentamicin, Lonza) and incubated overnight at 37^{o}C, 200/220 rpm. The next day 1% inoculum with optical density at 600nm (OD_{600nm}) =1 (thus final OD_{600nm}=0.01) was prepared and incubated for 3 h at 37^{o}C, 200/220 rpm to obtain a mid-log phase PA culture. PA was then collected, washed with ice cold Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} without phenol red (HBSS with Ca^{2+} and Mg^{2+}, Invitrogen) and diluted in HBSS complete medium [HBSS with Ca^{2+} and Mg^{2+} containing 5% human serum albumin (HSA, Sera labs)] to appropriate number of bacteria needed for the assay. To calculate the amount of bacteria, an aliquot of the inoculum was taken for counting using a Thoma Helber counting chamber. The aliquot for counting was first diluted 1:10 with HBSS with Ca^{2+} and Mg^{2+} without phenol red and mixed 1:1 with 4% paraformaldehyde (Electron Microscopy Sciences, lot: 160205) to fix bacteria. 2 µl of the fixed bacteria was loaded onto the Thoma Helber counting chamber and observed under a phase contrast microscope (Axiovert 40 C, Zeiss). The number of the bacteria was calculated using the formula: Bacteria / ml= {Total number of bacteria counted x 2 x 10^{7} x dilution factor (usually 20)} / {Total number of smallest squares counted (usually 32)}.

Alternatively, the bacteria number was calculated by first, measuring OD_{600nm} of the final suspension after all washes, then the concentration extrapolated from a previous graph of known OD_{600nm} versus colony forming units (CFU) of the same bacteria strain prepared in the same way as above. Concentrations of bacteria in assays were always confirmed by assessing CFU in inoculum (as described in Chapter 2.18).

2.7 Processing of fresh whole blood for preparation of normal human serum and heat-inactivated serum

Fresh blood was taken into plain serum vacutainer tubes (Scientific Laboratory Supplies, BE04-744Q) which have been kept in a 50 ml falcon tube (BD Biosciences, UK) and placed on ice between the period of blood collection and transport to the laboratory. The serum vacutainer tube was removed and kept stationery in a rack for one hour at 4°C to allow blood coagulation. The tube was then centrifuged at 1500 x g, 4°C, medium acceleration and deceleration for 10 min. The sera (normal human serum, NHS) were collected and aliquoted into 2 ml centrifuge tubes and stored at -80°C for future use. Aliquots to be used immediately were kept on ice throughout the experiment to avoid complement inactivation. The sera for which complement were to be inactivated were incubated at 56°C for 30 min and then transferred to ice.

2.8 Selection of serum for opsonic conditions

To select the serum appropriate for assays under opsonic conditions, PA ($1x10^5$/100µl) was pre-opsonised at 37°C for 20 + 120 min (to match cell-mediated killing assays) on a rotating wheel (Boekel Scientific) at 8 rpm in 10% NHS or heat-inactivated serum at 37°C, 8 rpm. Cultures were then tested for viable bacteria.

2.9 Quantification of anti-PA antibodies in human sera

PA inoculum was prepared as described in Chapter 2.6 but with some modifications. The modifications included: (i) a single colony of PA from the overnight culture on LB plate was taken into a 5 ml of sterile LB liquid medium for the overnight liquid culture, (ii) the next day, 1% inoculum of OD_{600nm} =1 (thus final OD_{600nm}=0.01) was prepared with LB liquid medium and incubated for 4 h at 37ºC, 200rpm, (iii) all washes after the 4h incubation of PA were done with PBS with Ca^{2+} and Mg^{2+} (Gibco), (iv) washed PA was fixed by resuspending the bacteria in 3.7% paraformaldehyde on ice and (v) the OD_{600nm} of fixed bacteria was adjusted to 0.5 in PBS with Ca^{2+} and Mg^{2+}. Fixed bacteria (100 µl of suspension with OD_{600nm}=0.5) was added

to each well of a 96 well plate (Thermo Scientific F-96 CERT.MAXISORP NUNC IMMUNO plate 439454) and incubated overnight at 4°C to coat the wells. The next day, the coated wells were washed with 300 µl of PBS without Ca^{2+} and Mg^{2+} (Sigma D8537) three times. Wells were then blocked with 3% bovine serum albumin [protease-free BSA (Alpha Diagnostic) in PBS without Ca^{2+} and Mg^{2+}: i.e. the blocking buffer] at room temperature for 1 h before washing three times with 300 µl of PBS without Ca^{2+} and Mg^{2+}. Test samples (NHS) or rabbit anti- PA sera (R and D Systems, used as positive control) both serially diluted in blocking buffer, or the negative control (i.e. PBS without Ca^{2+} and $Mg^{2+)}$ were loaded in triplicate wells (100 µl/well). NHS was obtained from either Complement Technology Incorporative, Catalogue number: NHS, or from healthy volunteers as indicated in Chapter 3.3.1.7 and Figure 3.10. The plate was then incubated for 2 h at room temperature followed by another wash process with 300 µl of PBS without Ca^{2+} and Mg^{2+}. To each well was then added 100 µl of the corresponding secondary antibody (i.e. Goat anti-rabbit IgG conjugated with HRP (R and D Systems) diluted 1: 1,000 in blocking buffer was added to wells containing primary rabbit anti-PA sera and anti-human IgG Fc-specific conjugated with alkaline phosphatase (Sigma-Aldrich) diluted 1:1000 in blocking buffer was added to the NHS wells) and these were incubated for 1 h at room temperature. This incubation was followed by another wash process as described earlier. Then 100 µl of the appropriate substrate solutions were added to corresponding wells (referred in here as substrate 1 and substrate 2) and the plate was incubated in the dark for 20 min at room temperature. Substrate 1: {hydrogen peroxide and tetramethylbenzidine (TMB) (denoted as solution A and B were brought to room temperature and equal volumes mixed to be used immediately or within 15 minutes for the rabbit anti-PA wells (R and D Systems, catalogue number: DY999). Substrate 2: {1 tablet of tris buffer in 20 mls of HPLC water (Fisher Scientific, mw18.02) and 1 tablet of p- nitrophenyl added to be used immediately for the NHS wells (Sigma-Aldrich, Code: 1002316844).

The optical densities were read at 405 nm for the wells treated with alkaline phosphatase-conjugated secondary antibody. For wells treated with HRP-conjugated secondary antibody,

50 µl of stop solution (2N H_2SO_4, R and D Systems, catalogue number: DY994) were added before the optical densities were read at 450 nm at room temperature with wavelength correction at 540 nm. A microplate reader (Multiskan FC, Thermo Scientific) was used to read optical densities (ODs). Graphs of the values for the ODs were plotted on Y-axis against NHS serial dilutions on the X-axis using either GraphPad Prism 6 or Microsoft excel 2013 version. These graphs provided the equation of the linear regression and R^2 values. Accuracy was increased by removing data points outside the linear part of the graph. The equations of the regression lines were then used to calculate the antibody titres (Thus, the means of the triplicate optical density values for the secondary antibody only control was multiplied by 2 to obtain the OD values of the tires, then this was substituted for Y in the equation of the line to calculate for X, the X values represent the anti-PA IgG concentration titres).

2.10 Quantification of Human TNF-α by ELISA

Human TNF-α was quantified in supernatants from some of the preliminary macrophage/neutrophil co-cultures infections with PA, using a Human TNF-α DuoSet ELISA Development System (R and D Systems, catalogue number: DY210-05) and following the manufacturer's protocol.

In brief, the wells of a 96 well microplate were coated with capture antibody and incubated overnight. The next day, the wells were washed with 400 µl of wash buffer three times and then 100 µl of test sample or standards were added to appropriate wells and plate covered with adhesive strip and incubated for 2 h at room temperature. This was followed by another wash with 400 µl of wash buffer three times. 100 µl of the detection antibody diluted to a working concentration of 500ng/ml in reagent diluent was added to each well and the plate was again covered with another fresh adhesive strip and incubated for 2 h at room temperature. This was followed by another wash as described earlier and a working dilution of streptavidin conjugated with HRP was added to each well. The plate was covered and incubated in the dark for 20 minutes at room temperature followed by another wash process (same as described earlier). Then 100 µl of substrate solution was added to each well and

incubated in the dark for 20 minutes at room temperature followed by the addition of 50 µl of stop solution to each well. The optical density of each well was read immediately with a microplate reader (Multiskan FC, Thermo Scientific) at 450 nm with wavelength correction at 540 nm or 570 nm.

2.11 Preparation of monocyte-derived macrophages

Monocyte-derived macrophages were generated using either leucocytes cones or buffy coats from the Blood Transfusion Service, Sheffield, UK. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, UK). For leukocyte cones, HBSS without Ca^{2+} and Mg^{2+} (Invitrogene) was added to dilute the cells (3:1, thus 30ml of HBSS per 10 ml of cells). The diluted mixture was layered onto Histopaque-1077 as follows: 15 ml of histopaque for 20 ml of the diluted cells) in 50 ml falcon tubes (BD Biosciences, UK or CellStar, UK). In the case of buffy coats, bags were cut and drained into a T75 flask (which usually amount to 50 mls, and diluted up to 140 ml with Dulbecco's Phosphate Buffered saline (DPBS) without Ca^{2+} and Mg^{2+} (Invitrogene). Then 35 ml of the diluted buffy coat was layered onto 15ml Histopaque-1077 in 50 ml falcon tubes.

Samples were centrifuged for 30 min at 800 x g, low acceleration and deceleration, at 23 ^{o}C using an Allegra X- 15R centrifuge (Beckman Coulter). After the centrifugation, the PBMC layer was collected, washed and the monocyte (CD14+ cells) fraction was obtained by positive selection using human CD14 MicroBeads (Miltenyi Biotec, UK) and LS MACS columns (Miltenyi Biotec, UK), following the manufacturer's instructions .The purified monocytes were suspended in RPMI complete medium [RPMI-1640 (Sigma-Aldrich, UK) containing 15% human AB serum (PAA Laboratories Ltd, UK), 2 mM L-glutamine (Sigma-Aldrich, UK), and 10 mM HEPES (Invitrogen, UK)] containing 10 ng/ml rhGM-CSF (Premium grade, catalogue number 130-050-201, Miltenyi Biotec, UK) at a density of 2 x 10^6 monocytes/ml. 5 ml of the above cell suspension were seeded per Teflon bottle [either Nalgene from Fisher Scientific, UK or Chemware Teflon PFA wide mouth jars (6185G30/D1069024) from Thomas scientific] and incubated at 37^{o}C, 5% CO_2 for 7 days. On Day 3, 1.5 ml of fresh RPMI complete medium

containing 10ng/ml rhGM-CSF was added to each bottle. Macrophages were harvested on day 7 by swirling the Teflon bottles and transferring the content into 15 ml falcon tubes (BD Biosciences, UK or CellStar, UK) and placed on ice to avoid adherence of the cells to the falcon tubes.

Alternatively, ultra-low attachment 24 well plates flat bottom with lids (Corning NY14831, Corning incorporated, USA) were used for macrophages differentiation. In this case, 500 µl of monocytes re-suspended in RPMI complete medium containing 10 ng/ml rhGM-CSF at a density of 1.5 x 10^6 monocytes/ml were seeded in each well (thus 750,000 cells per well). On Day 3, 500 µl of fresh RPMI complete medium containing 10 ng/ml rhGM-CSF was added to each well. To collect macrophages at Day 7, the low attachment 24 well plates were placed on ice for 20 min, then using 1 ml pipette, the cells were gently flushed and transferred into 15 ml falcon tubes and placed on ice. Wells were observed microscopically to ensure complete harvest of the macrophages. If there were any cells still attached to the wells, then 500 µl of the medium to be used for the infection assay was added to the wells and plate was incubated for 5 min on ice. Then cells were flushed off gently with 1 ml pipette and transferred to the 15 ml falcon tube. In all cases, cell suspensions in the 15 ml falcon tubes were centrifuged at 350 x g, 4^{o}C, acceleration 3, deceleration 3, for 5 min to pellet macrophages. These were then washed 3 times in serum-free medium before re-suspended in appropriate medium per assay. Cells were counted and adjusted to densities as appropriate per assay.

2.12 Preparation of human neutrophils

To isolate neutrophils, 12 ml of Histopaque-1077 (Sigma-Aldrich, UK) was added to a 50 ml falcon tube (BD Biosciences, UK) and 12 ml of Histopaque-1119 (Sigma-Aldrich, UK) was then carefully layered beneath the Histopaque-1077. Fresh whole blood (12 ml) collected in EDTA vacutainers (BD Diagnostics-Preanalytical Systems) was diluted 1:1 with RPMI 1640 (Sigma-Aldrich, UK), layered over the Histopaque-1077 layer and centrifuged at 700 x g, acceleration slow, no brake for 30 min at room temperature. Neutrophils were collected and washed once with RPMI 1640. Red blood cells were lysed in 0.2 % saline (Sigma-Aldrich, UK)

in distilled water at room temperature for 1 min. An equal volume of 1.6% saline in distilled water was added to the cells to equilibrate the solution, and cells were washed once with RPMI 1640. Two more washes were carried out in the buffer or medium required for the assay. Trypan blue staining (Chapter 2.13) was used always to determine total viable cell counts and cytospins (Chapter 2.14) were mostly used to assess purity of neutrophil preparations.

2.13 Trypan blue staining for determining cell viability

To determine the total viable cells (i.e. of macrophages or neutrophils) yielded and the percentage viability of cells, the isolated neutrophils or macrophages were resuspended in appropriate medium per assay before taking 50µl of the cell suspension into an eppendorf tube containing 50µl of 0.4% trypan blue stock solution (Sigma-Aldrich) (i.e. 1:1 making a dilution factor of 2). This is mixed and then 10µl of the mixture used in filling the chamber of a Hemocytometer (NanoEnTek) and cells made to settle for about a minute. The dead cells will take up the blue dye while the viable cells will not (Strober, 2001). The cells were counted from 4 different squares (i.e. 1 x 1 mm squares) of the Hemocytometer taking note of live and dead cells. These values were used to calculate for the total viable cells yielded and the percentage cell viability.

To calculate for the total viable cells = (Total viable cell count ⁄ the number of quadrants counted which is usually 4) X (Dilution factor which is usually 2) X (10^4 as the Hemocytometer factor) X (total volume of the cell suspension in millilitres). To calculate for the percentage viable cells = (Total viable cell count⁄Total cell count including viable and non-viable) X 100.

2.14 Cytospins preparation

Cell suspensions were placed onto a glass slide already inserted into cytofunnels (EZ Single Cytofunnel, Ref: A78710020, Fisher Scientific). This was then spin at 200 rpm, 20 min, at low acceleration using a cytospin (Cytospin 2, Shandon). Slides were air dried, labelled with Hemacolor (MERCK) following the manufacturer's procedure (solution 1: applied for 30 seconds, solution 2 for 3 seconds and solution 3 for 6 seconds), air dried again, mounted with DPX (Sigma-Aldrich) and examined using a bright field inverted microscope (Nikon Diaphot 300). In some instances, cells were previously fixed with 2% or 1% paraformaldehyde in HBSS complete medium and kept on ice for at least 15 min before being processed for microscopy using either the manufacturer's Hemacolor staining procedure or a modified Hemacolor protocol (solution 1: fixative was skipped since the cells were fixed, solution 2 was applied for 6 seconds and solution 3 for 3 seconds), also see Chapter 0. The stained slides were examined under a bright field inverted microscope (Nikon Diaphot 300) fitted with a camera (Diagnostics instrument, Model: 25.4) and an in-built software (Sport RTS) which allows images to be captured and analysed on a computer screen.

2.15 Reactive oxygen species (ROS) assay

Freshly isolated neutrophils were exposed to either a mid-log phase PA inoculum or Zymosan (heat/ chemically killed yeast bioparticles of *Saccharomyces cerevisiae*, Molecular Probes) at multiplicity of infection (MOI) of 20. This was done in the presence of Buffer A {HBSS with Ca^{2+} and Mg^{2+} without phenol red (Gibco) containing 20 mM HEPES (Invitrogen, UK) and 0.1% HSA} and 4 X Buffer B {268 µg/ml luminol (Sigma-Aldrich) dissolved in Buffer A}. Assay reagents, PA or Zymosan were loaded according to plate layout and the plate edges were sealed with parafilm and immediately taken for reading using a luminometer Fluo star Omega (BMG LABTECH).

2.16 Flow cytometry analysis

Isolated neutrophils were resuspended in 5% foetal bovine serum (FBS) in HBSS without Ca^{2+} and Mg^{2+} with 0.1% sodium azide solution at a density of 4 x 10^6 cells/ml. The cells were labelled with the antibodies CD3-FITC, CD14-ECD, CD16-PE, CD11b-FITC or their corresponding isotype controls (IgG1-FITC for CD3- and CD11b-FITC, IgG2a-ECD for CD14-ECD, IgG1-PE for CD16-PE) all from Beckman Coulter, UK. The cells were analysed with Beckman Coulter FC500 flow cytometer collecting 50,000 events and the data were analysed using Weasel v 3.0.2.

2.17 Macrophages and neutrophils-PA infection assay

To assess the collaborative efforts of macrophages and neutrophils to control PA, PA inoculum was prepared as described in Chapter 2.6 and desired concentrations made. The appropriate inoculum of PA in 100 µl was incubated at 37^{o}C on a rotating wheel at 8 rpm in the presence or absence of 10% NHS in HBSS complete medium for 20 min, depending on assay conditions (i.e. opsonic or non-opsonic conditions) in 2 ml centrifuge tubes. Monocyte-derived macrophages prepared as described above in Chapter 2.11 (2 x 10^5 macrophages in HBSS complete medium) were infected with PA at MOI of 1 under opsonic or non-opsonic conditions. Infected macrophages were incubated at 37^{o}C at 8 rpm for 2 h. Then half of the samples were collected for CFU analysis and supernatants frozen for further analysis. This time point will give information on the PA only and PA with macrophages conditions at 2 h post-infection (hpi).

Purified neutrophils (5 x 10^5 in HBSS complete medium, ratio of macrophage: neutrophil = 1:5) were then added accordingly; this will give PA only, PA with neutrophils and PA with macrophages and neutrophils conditions. Samples were incubated for 30 min at 37^{o}C at 8 rpm, after which they were analysed for CFU and supernatants were collected and frozen for further analysis. The process for the co-culture infection assay is summarised in Figure 2.1.

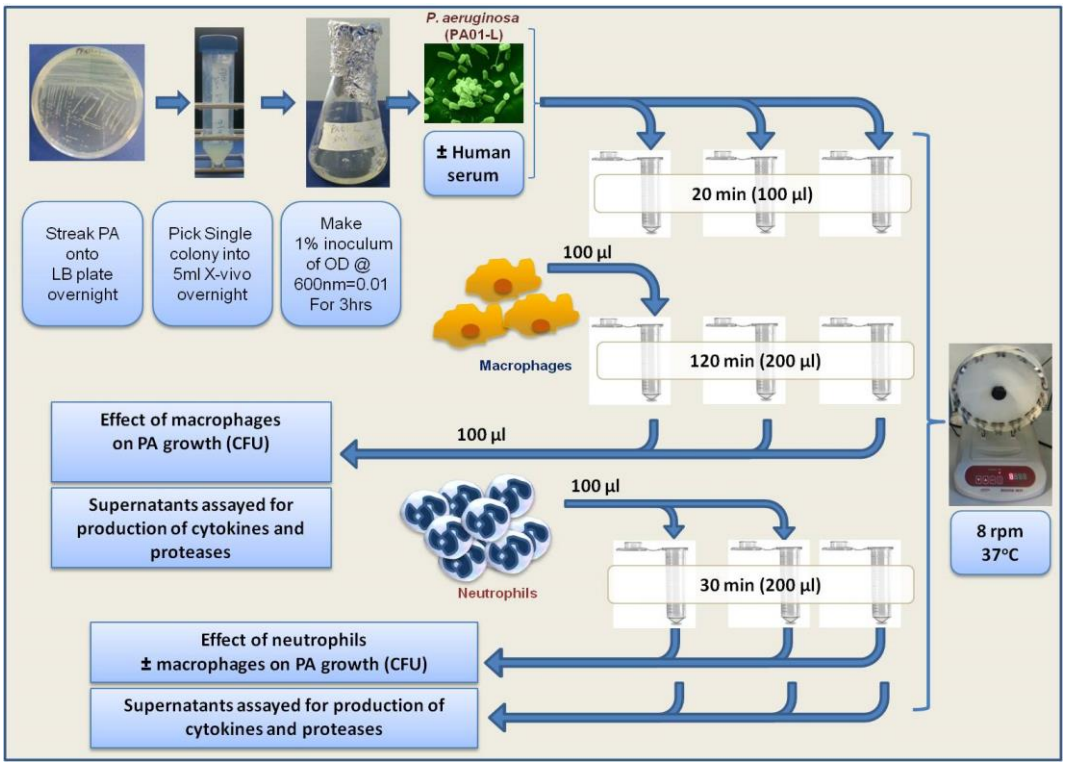


Figure 2.1: Macrophages and Neutrophils co-culture infection assay.
For the macrophage/neutrophil co-culture infection model, PA from -80oC stock was streaked onto LB agar plate and incubated at 37ºC overnight. A single colony was picked from the plate the next day into 5ml X-Vivo 15 medium and incubated again overnight at 37ºC, 200/220 rpm. The next day, 1% inoculum of OD_{600nm}=1 (final OD_{600nm} =0.01) was prepared and incubated for 3 hours at 37ºC, 200/220 rpm to obtain a log-phase PA culture. PA was then washed and appropriate inoculum made. PA was either pre-opsonised with 10% NHS or not, depending on assay conditions. PA was incubated initially with macrophages for 2 hours and samples were obtained 2 hpi for CFU and preparation of supernatants. Freshly prepared neutrophils were added accordingly and incubated for 30 min. Samples were then processed for CFU and supernatants.

To test the effect of IFN-γ and IL-17A on infection outcome, the same procedure described above (Chapter 2.17) was followed except that, macrophages were pre-treated for 24 hours with the cytokine of interest [either rhIFN-γ (10 ng/ml, 285-IF-100, R&D Systems, Inc.) or rhIL-17A/CF (15 ng/ml, 7955-IL-025/CF, R&D Systems, Inc.) in the presence of GM-CSF (Premium grade, catalogue number 130-050-201, Miltenyi Biotec, UK) on day 6 and the macrophage/neutrophil co-cultures were incubated for 30 or 60 min under opsonic conditions (10% NHS) (see Figure 2.2). Cytokine and serum concentrations were maintained throughout the experiment.

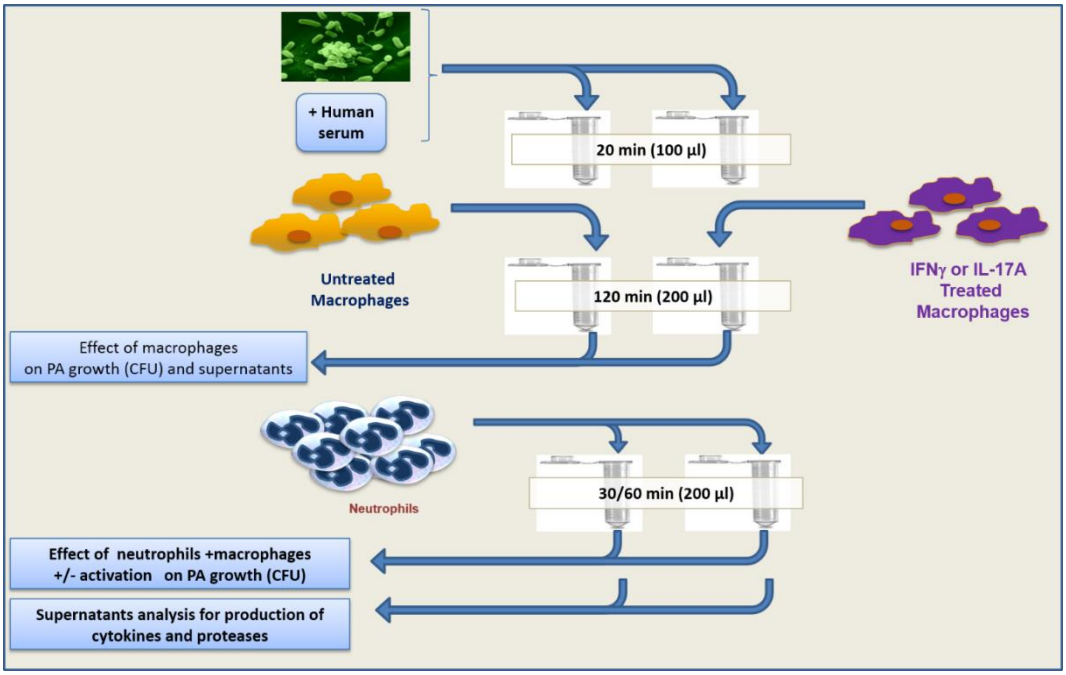


Figure 2.2: Effect of pro-inflammatory cytokines on the Macrophages and Neutrophil co-culture infection assay.
To test the effect of inflammatory mediators such as IFN-γ and IL-17A on the ability of macrophage/neutrophil co-cultures to control PA, macrophages were pre-treated with the cytokine of interest for 24 hours prior to the infection assay. Treated or untreated macrophages were then used as described above in Figure 2.1.

2.18 Colony forming units and preparation of supernatants

Samples from the macrophage/neutrophil infection assays were processed for CFU analysis and preparation of supernatants as follows.

For CFU, half of the samples were diluted 1:10 in water, passed 5 times through a 25G syringe and 10-fold serial dilutions in PBS with Ca^{2+} and Mg^{2+} (Gibco) in sterile 96-well U-bottom plates were made. 100 µl of 4 consecutive dilutions were spread onto 4 separate LB agar plates using a sterile spreader and then incubated at 37^{o}C overnight. PA colonies on each plate were counted the following day. The absolute count of CFU was calculated as the number of CFU X dilution factor (i.e. if for example 300 colonies were counted at 10^{-3} dilution, then the total CFU will be $300X10^{3}$).

To calculate for the percentage of bacterial CFU remaining in infected neutrophils compared to PA only conditions, the total CFU counts of PA only conditions were taken to be 100%. Then the percentage of bacteria remaining in the infected neutrophil cultures will be = (Total CFU counts in (PA + neutrophils) conditions / Total CFU counts in PA only conditions) X 100.

Also to calculate for the percentage of bacterial CFU remaining in infected macrophage/neutrophil co-culture conditions compared to infected macrophages only conditions, the total CFU counts in infected macrophages only conditions were taken to be 100%. Then the percentage of bacteria remaining in the infected macrophage/neutrophil cultures will be = (Total CFU counts in (PA + macrophage/neutrophil) conditions / Total CFU counts in PA + macrophages conditions) X 100.

For supernatants, samples were initially centrifuged at 300 g for 5 min at 4^{o}C, supernatants were collected in fresh sterile 1.5 ml tubes and further cleared by centrifugation at 13,000 rpm for 10 min at 4^{o}C. Supernatants were collected, aliquoted and stored at -80^{o}C until analysis.

2.19 Neutrophil elastase assay

Neutrophil elastase was quantified using a neutrophil elastase activity assay kit (Cayman Chemicals) following the manufacture's protocol. In brief, 100ul of serially diluted standards were added to corresponding wells to obtain a standard curve. Culture supernatants (neat and diluted 1:10 in assay buffer) were added to corresponding wells. Neutrophil elastase substrate was added to each well and the plate incubated at 37^{o}C for 90 min. Fluorescence was read after the incubation in a Fluoroskan Ascent FL (Thermo Labsystems) at an excitation and emission wavelengths of 485nm and 525nm respectively.

2.20 Cytokine quantification

Culture supernatants were tested for presence of cytokines using Magnetic Luminex Screening kit (R and D Systems, Catalogue number: LXSAHM) following the manufacture's procedure.

In summary, antibodies specific to the analytes of interest were pre-coated onto magnetic microparticles which were coded with distinct colours. Known standards, culture supernatants and microparticles were added accordingly to allow the antibodies to bind to appropriate analytes of interest. Wells were washed to remove any unbound material. This was followed by the addition of a biotinylated antibody cocktail to each well. This biotinylated antibody cocktail was specific to the analytes of interest and then a wash to remove any unbound biotinylated antibody was performed. Subsequently, Streptavidin conjugated with phycoerythrin (PE) was added to each well to bind the biotinylated antibody followed by a wash to remove any unbound Streptavidin-PE. Finally, all standards and samples were suspended in buffer and read using a Bio-Rad Bio-Plex 200 system.

2.21 Neutrophil infection assay in the presence and absence of IL-17A/ IFN-γ

Mid-log phase PAO1 stock cultures prepared as described in Chapter 2.6 were diluted to 2x10^{7} bacteria/ml in HBSS complete medium. Bacteria were opsonised by incubation for 20 min at

8 rpm at 37°C in the presence of 10% NHS in a 2 ml centrifuge tube. Neutrophils (4×10^5/80 µl in HBSS complete medium) were primed with or without the cytokine of interest (either IL-17A at 15 ng/ml, or IFN-γ at 10 ng/ml, or complete medium only) for 30 min at 37°C at 8 rpm. Primed neutrophils were added to bacteria (MOI = 5) and gently pipetted up and down. To control tubes (i.e. PA only cultures), HBSS complete medium was added to make up the final volume as is in the test tubes. Additional NHS was added to achieve a final concentration of 10% NHS in all tubes. All the tubes were incubated at 8 rpm for 30 min at 37°C and analysed for CFU as above in Chapter 2.18.

2.22 Statistical analysis

Statistical analysis was performed in GraphPad Prism v 6.02. Significance was calculated by one-way ANOVA with Tukey's post-test when comparing more than two groups. Paired two-tailed Student's *t* test was used for direct comparison between two conditions.

Chapter 3

Establishment of a macrophage/neutrophil co-culture infection model and analysis of their ability to control *Pseudomonas aeruginosa*

Chapter 3: Establishment of a macrophage/neutrophil co-culture infection model and analysis of its ability to control *Pseudomonas aeruginosa* (PA)

3.1 Introduction

Neutrophils are key for a protective immune response against PA infection (Hirche *et al.*, 2008; Williams *et al.*, 2010) and IL-17-driven responses, which promote neutrophil-mediated inflammation are associated with resistance to PA infection (Andrews *et al.*, 2003), further enforcing the central role of neutrophils in protection against PA. PA is an opportunistic pathogen that causes infections in patients with tissue damage or compromised immunity such as patients suffering from neutropenia, CF, thermal injury and those undergoing organ transplants or in intensive care units (Lyczak *et al.*, 2000). PA infections are of utmost concern due to the difficulty in treatment in susceptible individuals.

Upon infection of the airways by PA, neutrophils are quickly recruited to the infection site to aid clearance. Available evidence support the essential role for neutrophils in defence against PA infection and this seems to outweigh the contribution of other immune cells especially during acute infections (Ojielo *et al.*, 2003). For example, neutrophil depleted mice are more susceptible to PA infection even at very low bacterial dose (Koh *et al.*, 2009). Tsai and colleagues (Tsai *et al.*, 2000) also showed that neutrophil depletion resulted in higher mortality of PA-infected mice. However, there are multiple examples of PA overcoming neutrophil-mediated killing *in vitro* and also reports of PA using neutrophil-released DNA to form biofilms (Laharrague *et al.*, 1984; Walker *et al.*, 2005). Considering the clinical relevance of this opportunist, it is essential to explore avenues of infection control and therapeutic targets; hence the need for further investigation into the interplay between PA and neutrophils.

The outcome of PA infection however does not only depend on the rapid recruitment of appropriate neutrophil numbers to the infection site, but also on the killing ability of these neutrophils, which can be affected by both bacterial and host factors (Shaver *et al.*, 2007; Conway Morris *et al.*, 2009). For instance, a classical scenario observed in CF patients

chronically infected with PA is exaggerated numbers but yet dysfunctional neutrophils that further contribute to damage of lung tissues (Smith, 1994). Although neutrophils are essential for protection against extracellular bacteria, neutrophil-dominated inflammatory responses can also compromise organ function through tissue damage. It is therefore necessary to find measures of improving neutrophil efficiency while reducing tissue damage, which is the underlying principle of this project.

Neutrophils do not work in isolation but cooperate with other immune cells such as epithelial cells, macrophages, dendritic cells and T cells (Gellatly *et al.*, 2013). Although macrophages can engulf and kill bacteria, during PA infection, their principal role is to enhance the recognition and clearance of the bacteria by releasing cytokines and chemokines that promote recruitment and activation of neutrophils (Eloïse *et al.*, 2009; Lavoie *et al.*, 2011b). In agreement with these notion, a previous study by Singh and colleagues using PBMCs from CF patients, shows that human macrophages could not themselves significantly control PA growth but release a number of signals that could potentially enhance the recruitment of other immune cells to infection sites (Singh, 2012; Singh *et al.*, 2015). Additionally, macrophages play an important role by phagocytosing dying neutrophils which in turn promotes resolution of inflammation and tissue repair (Knapp *et al.*, 2003). Because of the cross-talk between macrophages and neutrophils during the immune response to infection, if using *in vitro* models, it should comprise of at least both cell types to possibly provide a suitable setting to study human antimicrobial immunity.

This part of the study aimed to establish a macrophage and neutrophil co-culture infection model of PA to assess how these two phagocytes collaborate in controlling PA infection.

3.2 Materials and methods

To establish a suitable model to assess the ability of macrophage/neutrophil co-cultures to control PA, a number of optimisation assays were performed including methodology for neutrophil preparation, serum selection, reactive oxygen species (ROS) quantification, processing of infected cultures for CFU quantification and cell preparations using cytospins. Each of these optimised methods have been detailed in the general materials and methods section (Chapters 2.8, 2.12, 2.14, 2.15 and 2.18). The final co-culture infection assay which is also detailed in the general materials and methods (Chapter 2.17) was used to assess the ability of the macrophage/neutrophil co-cultures to control PA. Infections were performed in the absence or presence of 10% normal human serum (NHS) as a source of complement.

3.3 Results

3.3.1 Optimisation assays

3.3.1.1 Isolation of human neutrophils using histopaque density gradient centrifugation

Even though a protocol for neutrophil isolation was available in the laboratory (Singh, 2012) there was a need for optimisation since there was variability among donors with regards to ROS production by neutrophils in response to PA infection, the reason for which was unclear. A reliably fast technique for neutrophils isolation is essential due to their short half-life (Summers *et al.*, 2010). The isolation technique should also yield neutrophils of high purity with minimal or no activation (Oh *et al.*, 2008b). Two density gradient techniques were initially considered. One using a discontinuous gradient of Histopaque-1077 and Histopaque-1119 (Singh, 2012) which are a mixture of polysucrose and sodium diatrizoate at densities 1.077g/ml and1.119g/ml, respectively. The other technique involved using a single neutrophil isolation medium called lymphocyte®-Poly (Cedarlane, Catalogue number: CL5070) which is a mixture of sodium metrizoate and Dextran 500 (Oh *et al.*, 2008b). Gradient formation using

the neutrophil isolation medium was however not reproducible among donors, whereas the histopaque method gave reproducible gradient formation.

The histopaque technique (Figure 3.1) was therefore chosen in all subsequent assays but with strict donor selection as indicated in the inclusion criteria (Chapter 2.2) and the use of human serum albumin (HSA) and Hank's buffer without Ca^{2+} and Mg^{2+}. Flow cytometry analysis (Figure 3.2) and microscopy (Figure 3.3) of isolated neutrophils showed high purity. For the Flow cytometry data, the absolute percentages of the various antibody staining were calculated as the percentage of positive cells for a particular antibody staining minus the isotype control for that corresponding antibody. The flow cytometry analysis (Figure 3.2) showed there was minimal T cell contamination **(i.e. Figure 3.2, F-E which gives 2.23 - 0.89 = 1.34%).** It also showed that 94.96% of the cells in the preparations were $CD16^+$ **(i.e. Figure 3.2, L-K which gives 95.3 - 0.34 = 94.96%)** and **97.31%** being $CD11b^+$ **(i.e. Figure 3.2, Q-P which gives 98.2 - 0.89 = 97.31%).** There were 95% of cells being double positive for CD16 and CD11b **(i.e. Figure 3.2, U = 95%)**. Forward versus side scatter analysis of the population showed that in the presence of anti-CD16, anti-CD11b or both antibodies **(i.e. H, N and S respectively)**, neutrophils appeared to be activated as cellular characteristics changed with cells displaying higher forward scatter (FSC) and side scatter (SSC).

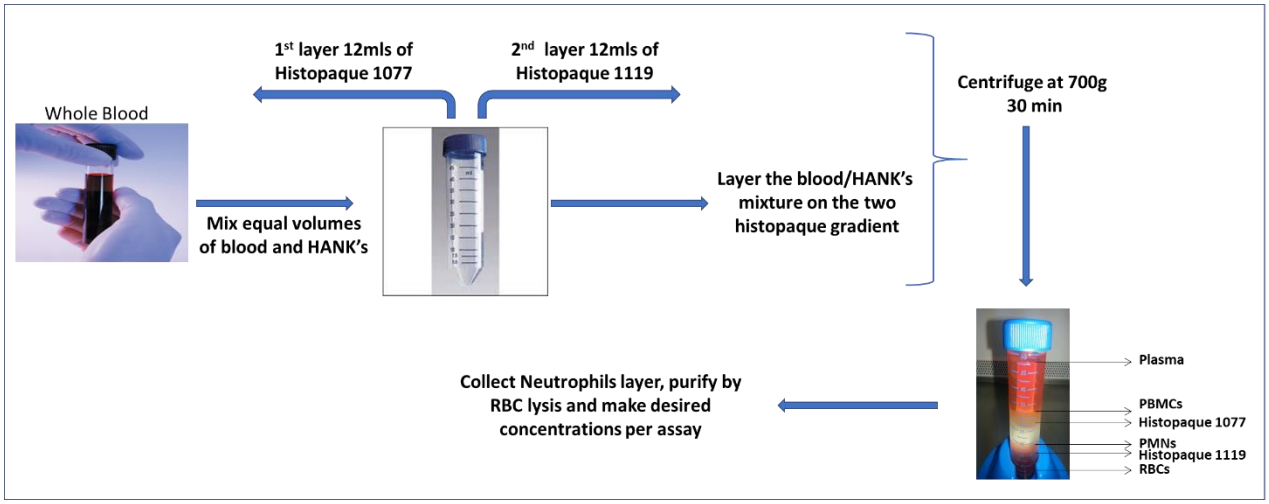


Figure 3.1: Neutrophil Isolation procedure.
A discontinuous gradient of histopaque 1077 and 1119 was made in a 50ml falcon tube with the aid of a glass Pasteur pipette. Then a mixture of blood and Hank's buffer was layered onto it. The entire gradient was centrifuged at 700 xg, 30 min at low acceleration and deceleration. Neutrophil layer was collected and enriched by RBC lysis. Neutrophil suspensions were prepared as required per assay. PMNs: Polymorphonuclear neutrophils (neutrophils), PBMCs: Peripheral blood mononuclear cells, RBCs: Red blood cells.

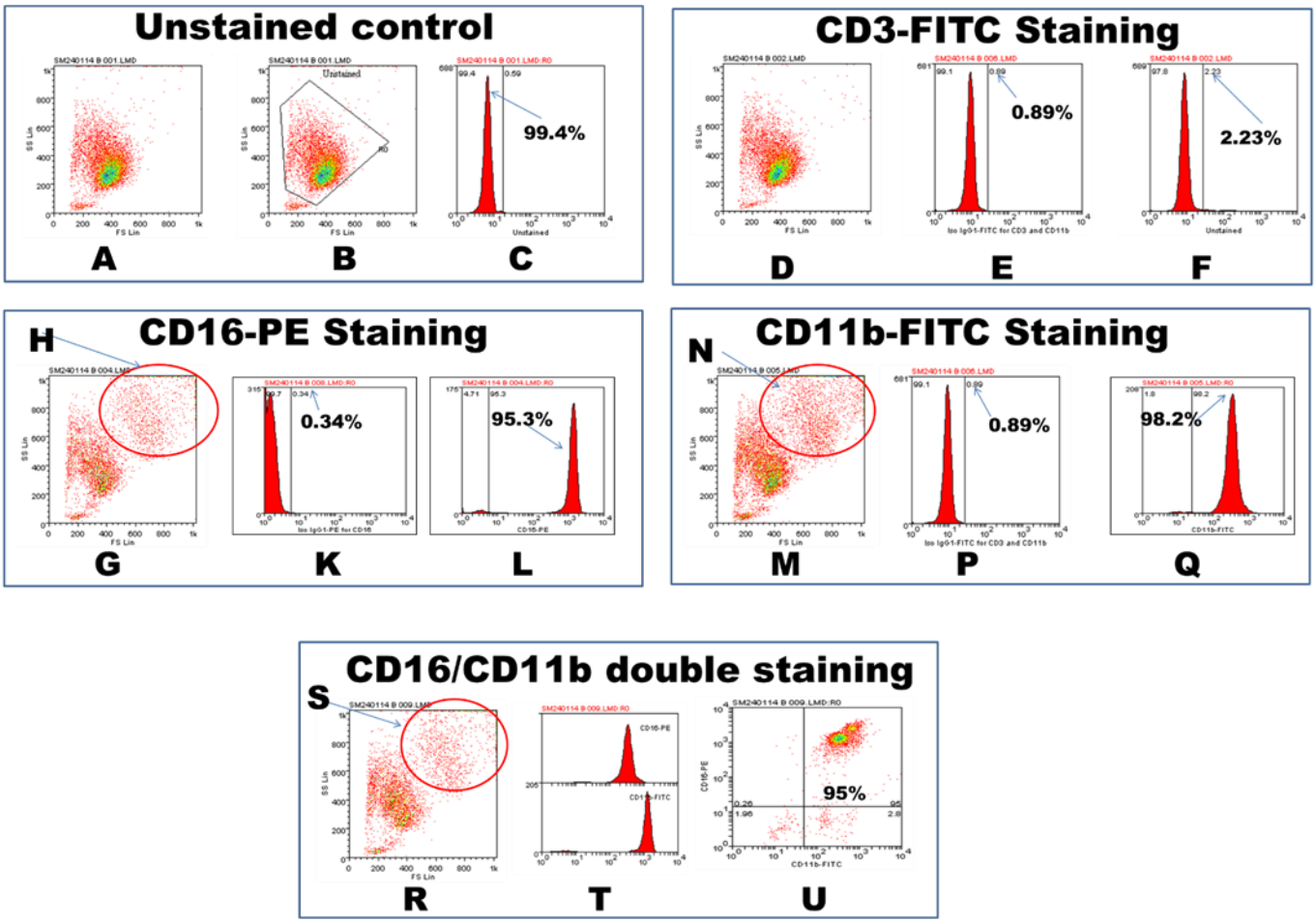


Figure 3.2: Flow cytometry analysis of PMNs purified from discontinuous density gradient centrifugation using histopaque.

Isolated neutrophils were either unstained or stained with various antibodies or their corresponding isotype controls. In the unstained control **A, B and C: (A):** Represents the forward versus side scatter, **(B):** The gating and **(C):** The fluorescence of unlabelled cells in the FL-1 channel. In the **CD3 staining**, **D, E and F** represent the forward versus side scatter, the isotype control for CD3-FITC and the percentage CD3 positive cells, respectively. In the **CD16 staining, G, K and L** represent the forward versus side scatter, the isotype control for CD16-PE and the percentage of CD16 positive cells, respectively**.** In the **CD11b-FITC staining, M, P and Q** represent the forward versus side scatter, the isotype control for CD11b-FITC and the percentage CD11b-FITC positive cells, respectively**.** In the **double staining for CD16-PE and CD11b-FITC, R, T and U** represent the forward versus side scatter of the double positive cells, the histogram for double positive cells and the dot plot for percentage double positive cells, respectively**.** Cell granularity in the presence of antibody to CD16, CD11b, or both are represented as **H, N and S** respectively.

3.3.1.2 Phenotypic characterisation of isolated neutrophils

Isolated neutrophils were placed onto glass slides using cytospin and stained with Hemacolor following the manufacture's protocol (solution 1: applied for 30 seconds, solution 2 for 3 seconds and solution 3 for 6 seconds) without prior fixation**.** This seemed to show some cell damage even in the absence of bacteria (Figure 3.3, A). Since cell damage was one of the experimental parameters under investigation, the cells were fixed with paraformaldehyde prior centrifugation. Thus, 2% paraformaldehyde was initially used to fix the cells and cells were stained with Hemacolor following the manufacture's protocol. This resulted in the cells looking healthier but the characteristic neutrophil phenotype was less obvious (Figure 3.3, B). This was thought to be due to over fixation of the cells since they were both fixed and stained with the Hemacolor protocol which involved the first step of fixation again. Thus the percentage of paraformaldehyde for fixation was reduced and the fixation step was eliminated in the staining protocol. Thus 1% paraformaldehyde was used for fixation and cells stained with a Hemacolor modified protocol (solution 1: fixative, was skipped since the cells were already fixed, solution 2 was applied for 6 seconds and solution 3 for 3 seconds). This gave cells that displayed the characteristic neutrophil phenotype (Figure 3.3, C) and hence 1% paraformaldehyde and Hemacolor modified protocol was chosen for subsequent microscopic characterisation.

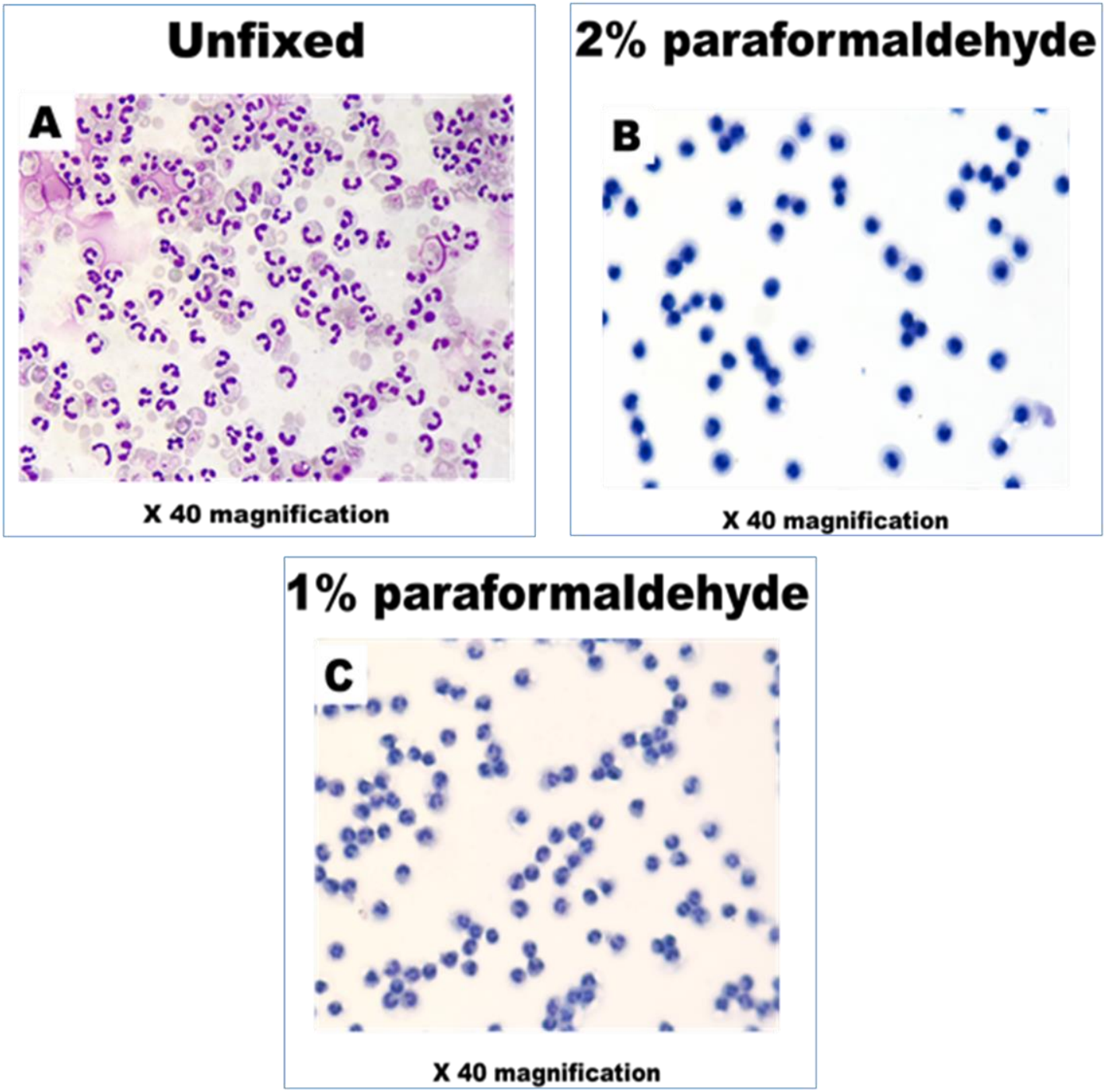


Figure 3.3: Microscopy analysis of isolated neutrophils.
Microscopic examination of neutrophils stained with Hemacolor and observed under a bright field inverted microscope. **(A):** Represents neutrophils stained with Hemacolor following manufacturer's protocol without prior fixation. **(B):** Represents neutrophils fixed with 2% paraformaldehyde prior centrifugation and stained with Hemacolor following manufacturer's protocol. **(C)**: Represents neutrophils fixed with 1% paraformaldehyde before centrifugation and stained with a Hemacolor modified protocol. Pictures were taken with the same magnification (X40). Microscopic examination was performed in selected subsequent assays but the above data are from a single representative assay.

3.3.1.3 PA does not produce ROS on its own

To rule out the possibility of PA interfering with ROS assay by its self, ROS production by cultures of PA only and cultures of neutrophils incubated with PA or Zymosan was investigated. The results showed that PA did not produce ROS in any of the assays. Neutrophils from all four donors tested produced ROS in the presence of PA and zymosan although there were different patterns and degree of response among the different donors (Figure 3.4). These differences in response of PA infection among the different donors could be attributed to the difference in individual immune responses. Another important observation was that isolated neutrophils did not release ROS in the absence of stimuli, which implied that the isolation technique did not activate the neutrophils to the extent of affecting this functional assays.

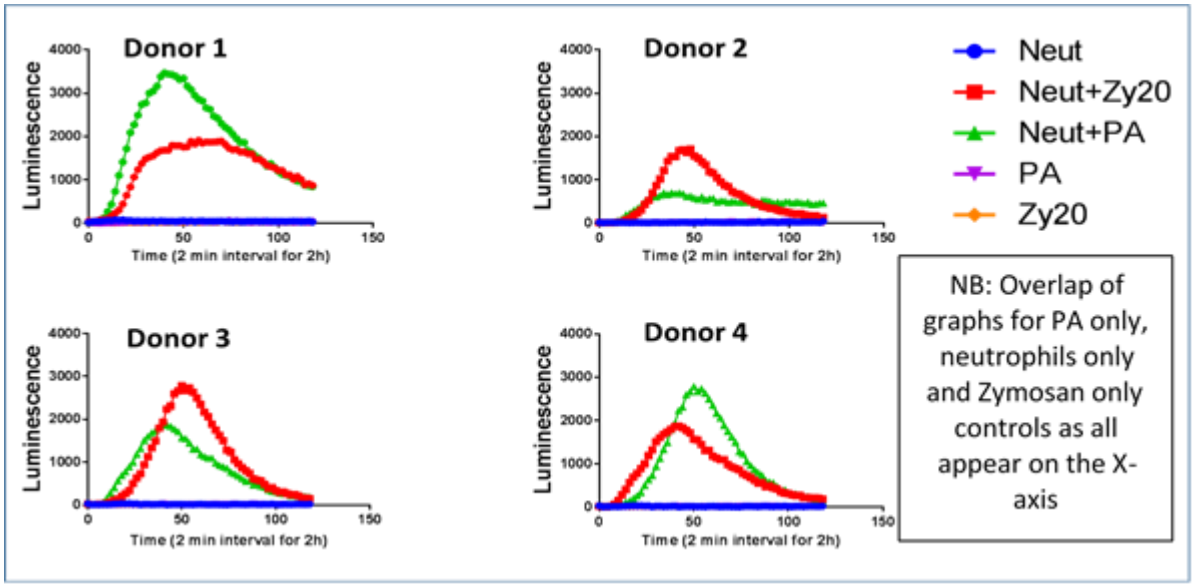


Figure 3.4: PA only does not produce ROS.
Isolated neutrophils were either infected with mid-log phase PA or treated with Zymosan both at MOI of 20 and examined for ROS production as described in materials and methods. The figure represents the ROS production by neutrophils in response to PA and Zymosan in 4 independent donors. Neut: Neutrophils, PA: *P. aeruginosa*, Zy20: Zymosan at MOI 20. These results are from 4 independent experimental repeats.

3.3.1.4 Cell lysis conditions for quantification of total bacteria

Triton X-100 at a final concentration of 0.1% (v/v) (Mishra *et al.*, 2012) was initially used to lyse neutrophils in infection assays to quantify total bacteria. Preliminary results showed reduction in PA numbers after incubation with neutrophils under serum free conditions. To validate these assays, 0.1% Triton X-100 was added to PA only cultures and a similar reduction in PA numbers was observed which suggested the detergent had a direct effect on PA viability.

We then used 0.01% Triton X-100 (Young *et al.*, 2011), 0.05% saponin (Hampton *et al.*, 1994) and distilled water (Singh, 2012). When these various lysing solutions were added to mid-log phase PA cultures, there was reduction in CFU with 0.1% Triton X-100 and 0.05% saponin (Figure 3.5, A) and a less reduction with 0.01% Triton X-100 (Figure 3.5, A) observed, while water did not affect CFU values (Figure 3.5, B).

There was the need to also ensure that the lysing solution could lyse neutrophils efficiently to quantify intracellular bacteria. Hence, the various lysing solutions were tested for their ability to lyse neutrophils. The result shows that 0.1% Triton X-100 (Figure 3.6, C) and 0.05% saponin (Figure 3.6, D) lysed the cells very well. However, 0.01% Triton X-100 could not lyse the cells efficiently (Figure 3.6, B). Distilled water on the other hand lysed the neutrophils well but the bacteria formed clumps (Figure 3.6, E). The bacteria were however, well dispersed as shown in (Figure 3.6, F) after lysis with distilled water and passing through 25G needle. Distilled water was therefore chosen as the most suitable solution for lysing neutrophils but bacteria dispersion had to be aided using a 25G needle.

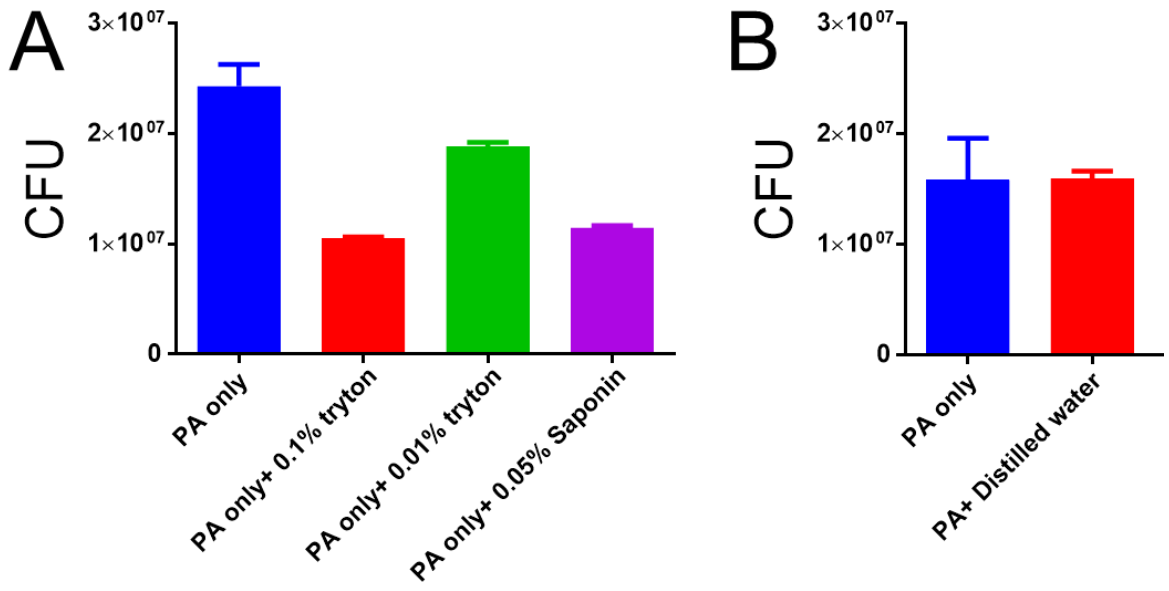


Figure 3.5: Effect of different lysing solutions on PA viability.
Unopsonised mid-log phase PA cultures were treated with various lysing solutions and incubated for 30 min at 8 rpm at 37°C, serial dilutions were made and plated on LB agar plates and then incubated at 37°C overnight. **(A):** The effect of 0.1% Triton X-100, 0.05% saponin and 0.01% Triton X-100 on PA viability. **(B):** The effect of Water on PA viability. Data are from duplicates of two independent experiments.

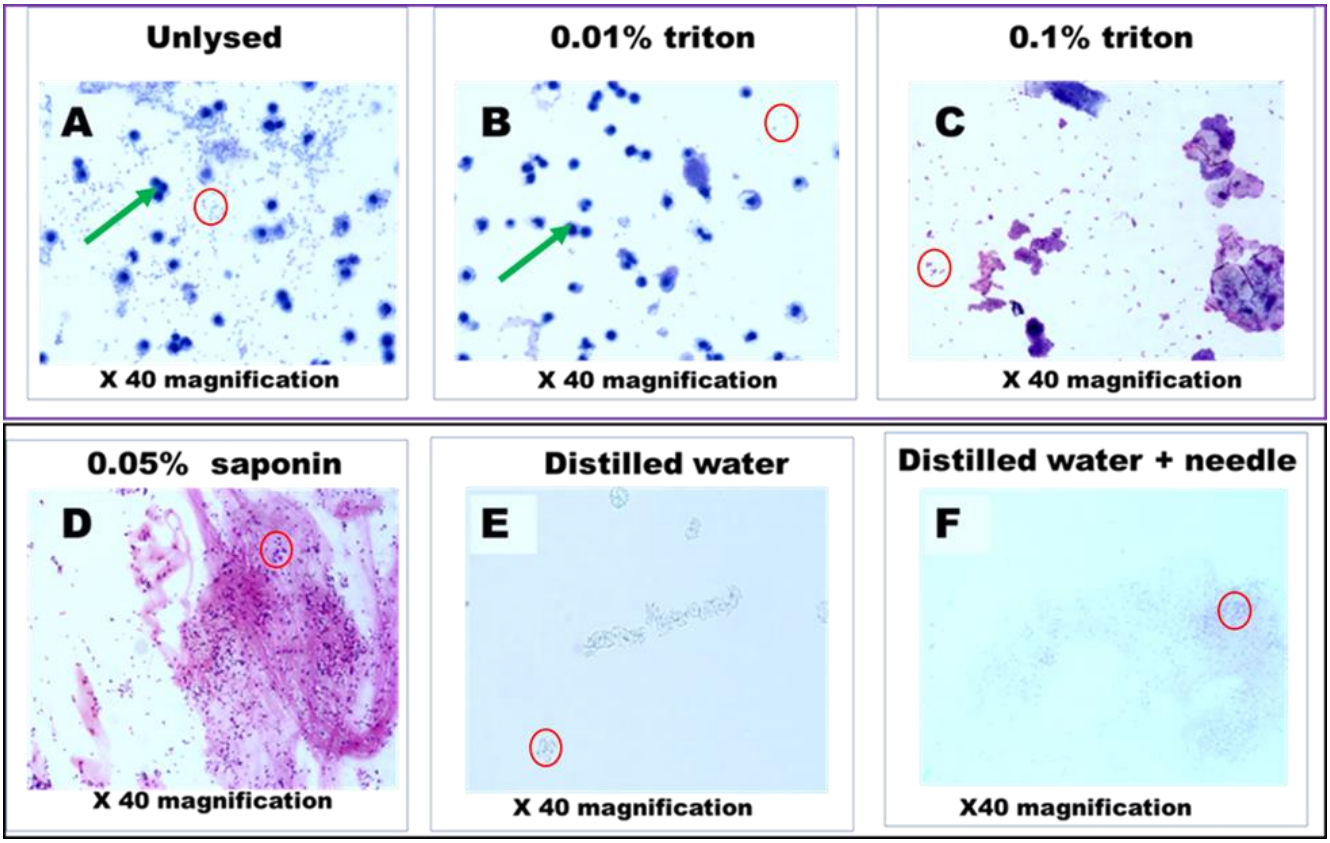


Figure 3.6: Effect of various lysing solutions on neutrophils lysis and bacteria dispersion. Microscopic examination of neutrophils infected with PA either unlysed **(A)** or treated with various lysing solutions such as 0.01% Triton-X100 **(B)**, 0.1% Triton-X100 **(C)**, 0.05% saponin **(D)** and distilled water **(E). (F):** Represents cells lysed with distilled water and passed through 25G needle 5 times for bacteria dispersion. Green arrows point to intact neutrophils while red circles show bacteria. The above is a representative image from a single independent experiment.

3.3.1.5 Control of PA infection by neutrophils

The ability of isolated human neutrophils to phagocytose PA when incubated at different cell densities was evaluated. For this, unopsonised PAO1-L expressing a green fluorescent protein (GFP) was incubated with neutrophils at varied concentrations ($2x10^6$, $4x10^6$ or $8x10^6$ cells/ml) while maintaining the multiplicity of infection (MOI=10) constant.

Infected cultures were analysed by the staff of Amnis Corporation using the Flow Sight system as part of their demonstration and training on how the system works. The neutrophils phagocytosed either one or more bacteria as shown by the number of dots within cells (Figure 3.7, A). The percentage of PA phagocytosis by neutrophils increased by increasing both neutrophil and PA numbers while maintaining MOI as shown in (Figure 3.7, B). Thus, 400,000 neutrophils or more was chosen for all subsequent assays.

Finally, to determine neutrophil mediated killing of PA, either opsonised or unopsonised mid-log phase PA were incubated with neutrophils for 30 min at 8 rpm, 37°C. To obtain total bacteria, distilled water was added to all tubes to a final dilution of the cultures at 1:10 and lysates were passed through a 25G needle five times to lyse cells and disperse bacteria respectively. Serial dilutions were made with PBS with Ca^{2+} and Mg^{2+} and plated on LB agar before incubation overnight for CFU quantification. The results showed that PA CFU were reduced under both the opsonised and unopsonised conditions but with statistical significance achieved only in opsonised conditions (Figure 3.8). This particular batch of human serum promoted bacterial growth which in part accounted for the significant difference in the opsonic versus non-opsonic conditions. This was a commercial sera obtained from Complement Technology, Inc. (Catalogue number: NHS) which was certified for its complement activity from the certificate of analysis. However, because of the variability in the ability of different sera from this source to kill PA (as it was observed that there was increase in bacterial CFU in the presence of this sera and a drastic decrease in bacterial CFU in the presence of another batch of sera obtained from the same company with the same catalogue number), a serum selection procedure was optimised in our lab (see Chapter 3.3.1.6).

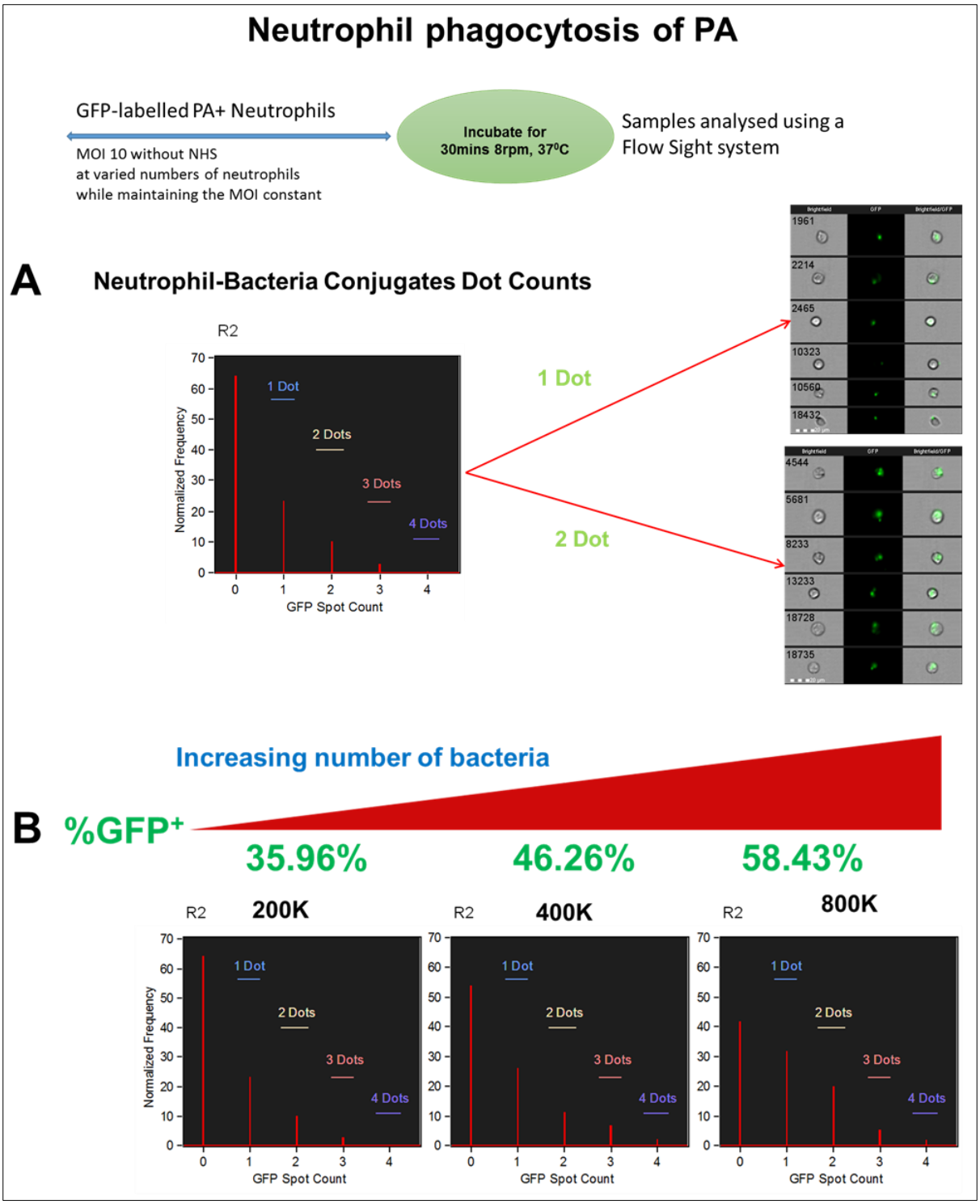


Figure 3.7: Effect of cell density on neutrophil phagocytosis of PA.
Different concentrations of isolated neutrophils (100 µl of $2x10^6$, $4x10^6$, or $8x10^6$ cells/ml) were infected with GFP expressing mid-log phase PA at the same MOI (MOI 10) in the absence of serum. The cultures were incubated for 30 min at 8 rpm, 37°C and analysed using a Flow Sight system. **(A):** Represents the experimental set up and the number of bacteria phagocytosed by neutrophils represented by the number of Dots **(1,2,3 or 4 Dots)**. **(B):** Represents the percentage of phagocytosed bacteria. The data were analysed using a Flow Sight software by the staff of Amnis Corporation (Barry Lewis, Owen Hughes, Lars Ohl, and Priya Santhalingam). Results are from a single independent experiment.

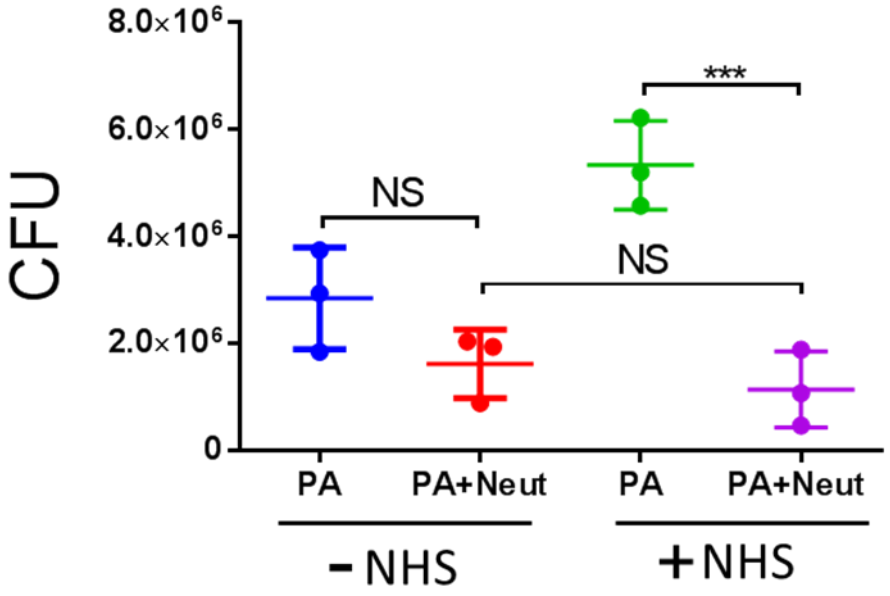


Figure 3.8: Reduction of PA infection by neutrophils.
Isolated neutrophils were infected with mid-log phase PA in the presence and absence of 10% normal human serum (NHS) and incubated for 30 min at 8 rpm, 37°C. Distilled water was added to all samples and lysates were passed through 25G needle five times to ensure cell lysis and bacteria dispersal. Serial dilutions were made and plated on LB agar. Plates were incubated overnight for assessment of CFU. Data analysed using one-way ANOVA. Data from three independent experiments. *** = $p<0.001$, NS = Not significant

3.3.1.6 Establishing a serum screening procedure for opsonic conditions

As mentioned above, at the initial stages of this study, serum was obtained from Complement Technology, Inc. (Catalogue number: NHS). It was observed that the first batch of serum purchased promoted PA growth as shown in (Figure 3.8). Intriguingly, another batch of serum from the same company with the same catalogue number showed an enhanced ability to kill PA even in the absence of phagocytic cells in some of the infection assays, so much so that no viable bacteria could be recorded. Hence for experiments under opsonic conditions, it was necessary to investigate the effect of serum only on PA growth and select which serum to be employed in the subsequent opsonic assays.

This study was performed by Kara Mayor (BMedSci student) under my supervision. The effect of complement was tested by heat inactivating the serum at 56°C for 30 min. Results showed different patterns of PA killing by sera from 7 different voluntary donors (Figure 3.9, A). In all instances, heat inactivation of serum increased CFU of PA, but some normal human serum samples had a drastic effect on PA viability while others promoted PA growth (Figure 3.9, A).

From these patterns, sera with a pattern in which PA was mildly sensitive to serum-mediated killing (such as donors 1 and 4) were selected for further tests. Assays were repeated few weeks later using fresh serum samples from donors 1 and 4. Interestingly, the data showed similar pattern but different degrees of PA clearance (Figure 3.9, B). Thus, not only do complement activity varied among donors, but also fluctuated among different preparations from the same donor. The massive clearance of PA by the serum from donor 1 made it unsuitable for the subsequent phagocyte-mediated killing assays. Donor 4 sera were initially employed in subsequent opsonic assays while more donors were screened and selected accordingly for future opsonic assays.

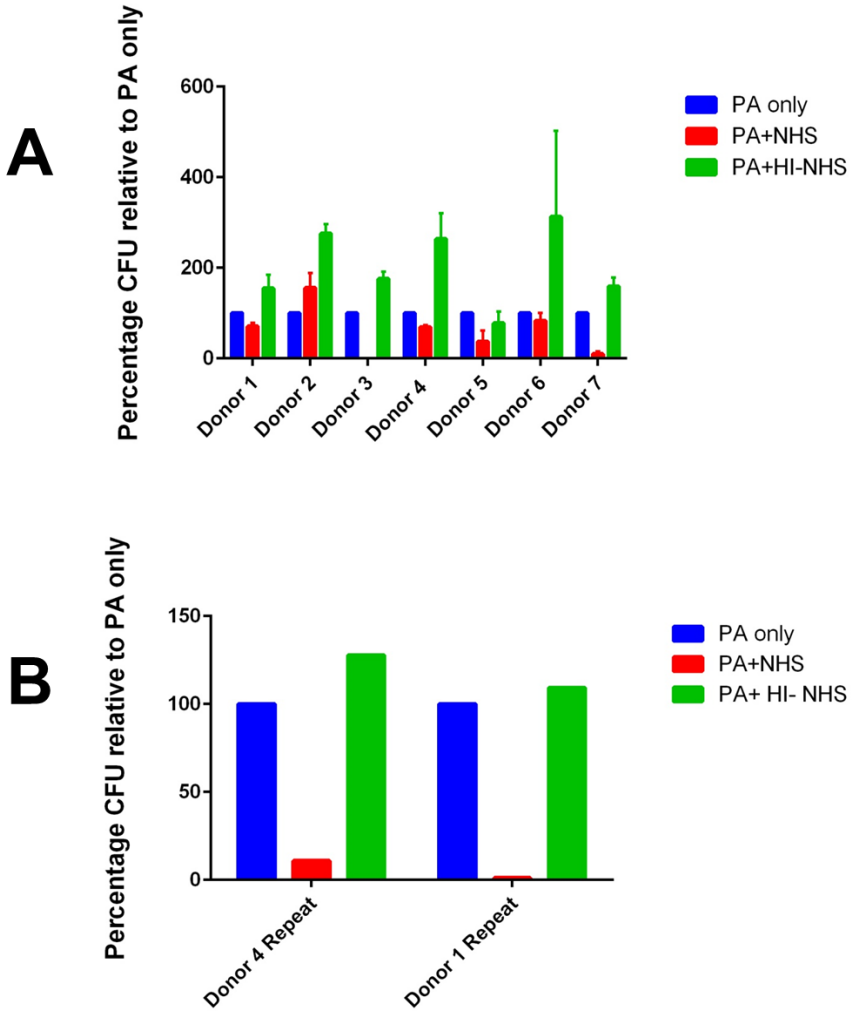


Figure 3.9: Effect of normal human serum on PA viability is highly variable among donors and time of blood collection.
Serum only-mediated killing of PA was determined by incubating PA ($1x10^6$/ml) in the absence or presence of 10% of either normal human serum (NHS) or heat inactivated serum (HI-NHS) for 20+120 min before taking samples for CFU analysis and the results were as follows: **(A):** Represents the variation in serum only-mediated killing of PA among donors, **(B):** Represents the variation in serum only mediated killing of PA within donor 1 and donor 4 where fresh blood was collected approximately two months later.

3.3.1.7 Quantification of PA antibodies from healthy volunteers

To determine if anti-PA antibodies could play a role in the ability of sera to kill PA, the sera from donors 1 to 7 discussed under (Chapter 3.3.1.6 and Figure 3.9 A) were tested for anti-PA IgG antibody by Kara Mayor under my supervision.

Antibodies against PA (specifically anti-PA IgG) in the various sera were quantified by ELISA following the procedure described in Chapter 2.9. In all instances, sera from the donors 1 to 7 contained high amounts of anti-PA IgG as shown by the graph of the optical density measurement against the serial dilutions of the various sera (Figure 3.10). Linear regression lines were plotted with GraphPad Prism 6 for the various donors with OD on the Y-axis and sera serial dilutions on the X-axis (Figure 3.11). The equations from the linear regression analysis from Figure 3.11 were used to calculate the antibody titres. The calculations for the various donor sera is shown in

Table 3.1 and indicate that the titres for all 7 donors fell within the same range (10^{-5}). These observations indicate that the variation in PA clearance among these donors was likely not dependent on anti-PA antibodies nor on the classical complement pathway, perhaps more dependent on the other complement pathways (i.e. Alternative and Lectin pathways).

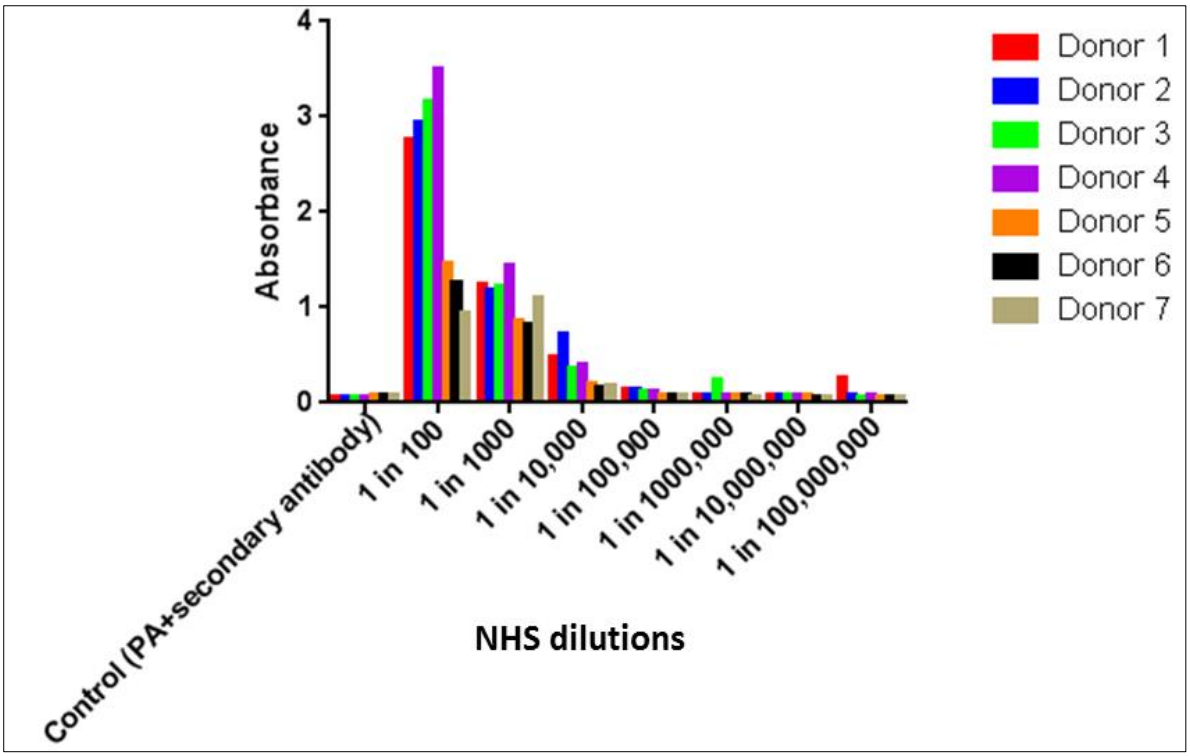


Figure 3.10: Anti-PA IgG antibody quantification in sera of healthy volunteers.
Sera from healthy volunteers were tested for anti-PA IgG by ELISA. The graph of optical density against serial dilutions of sera is shown above (Donor 1 to 7). Each column represents the mean of a single dilution done in triplicate.

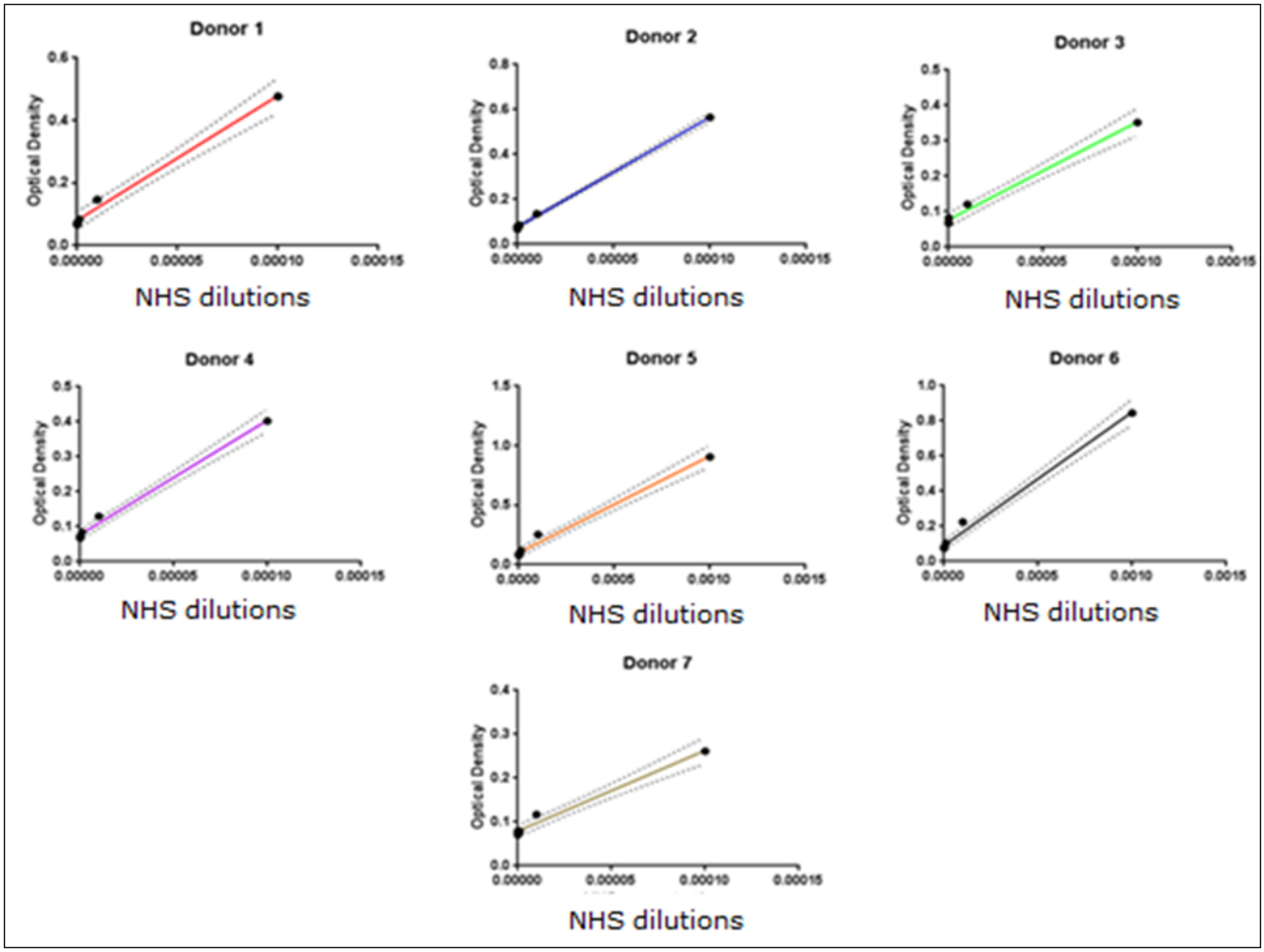


Figure 3.11: Linear regression lines for the determination of anti-PA IgG titres from healthy volunteers.

The individual regression line for each of the donors was plotted and equation of the line and R^2 values were obtained. Each of these regression lines had an $R^2 > 0.98$ as values above the linear part of the curve were excluded. Each point represents the mean of a single dilution done in triplicate. The equation of the regression lines were used in calculating the antibody titres as shown in Table 3.1.

Table 3.1: Antibody titres for all the donors are within 10^{-5} range

The equations of the regression lines obtained from Figure 3.11 were used to calculate the antibody titres. Two times the value of the optical density obtained with the negative control is substituted into the equation for Y to calculate X. The X values represent the anti-PA IgG antibody titres. Table 3.1 shows the R^2 values, the equations of the regression lines and the antibody titres which remained within the same range for all the 7 donor sera (10^{-5}).

Donor number	R^2	Equation	Antibody Titre
1	0.9924	$y = 3988x + 0.08046$	1.359×10^{-5}
2	0.9989	$y = 4888x + 0.07721$	1.175×10^{-5}
3	0.9924	$y = 2765x + 0.07711$	2.082×10^{-5}
4	0.9936	$y = 3272x + 0.07713$	1.758×10^{-5}
5	0.9883	$y = 812x + 0.1011$	6.925×10^{-5}
6	0.9914	$y = 759x + 0.09112$	8.724×10^{-5}
7	0.9825	$y = 1837x + 0.07921$	4.253×10^{-5}

3.3.1.8 Quantification of PA antibodies from commercial sera

In one of the preliminary experiments, anti-PA IgG antibodies was quantified from the commercial sera obtained from Complement Technology, Inc. (Catalogue number: NHS) following exactly the procedure described in Chapter 2.9. A graph of the optical density measurement against the serial dilutions plotted in GraphPad Prism 6 of the commercial sera (NHS) and the positive control (Rabbit anti-PA) is shown in (Figure 3.12, A) and that of the NHS alone shown in (Figure 3.12, B). These showed a decrease in optical density of either the NHS or the rabbit anti-PA serum as the dilutions increases. A linear graph plotted in Microsoft Excel 2013 with the optical densities on the Y-axis and the dilutions on the X-axis displays the equation of the linear graph and the R^2 values (Figure 3.13). The equations of the lines were used to calculate the antibody titres as described above. The anti-PA IgG tires were calculated to be $3.6337x10^{-2}$ for the Rabbit anti-PA positive control and $6.935x10^{-3}$ for the commercially obtained NHS as shown in Figure 3.13.

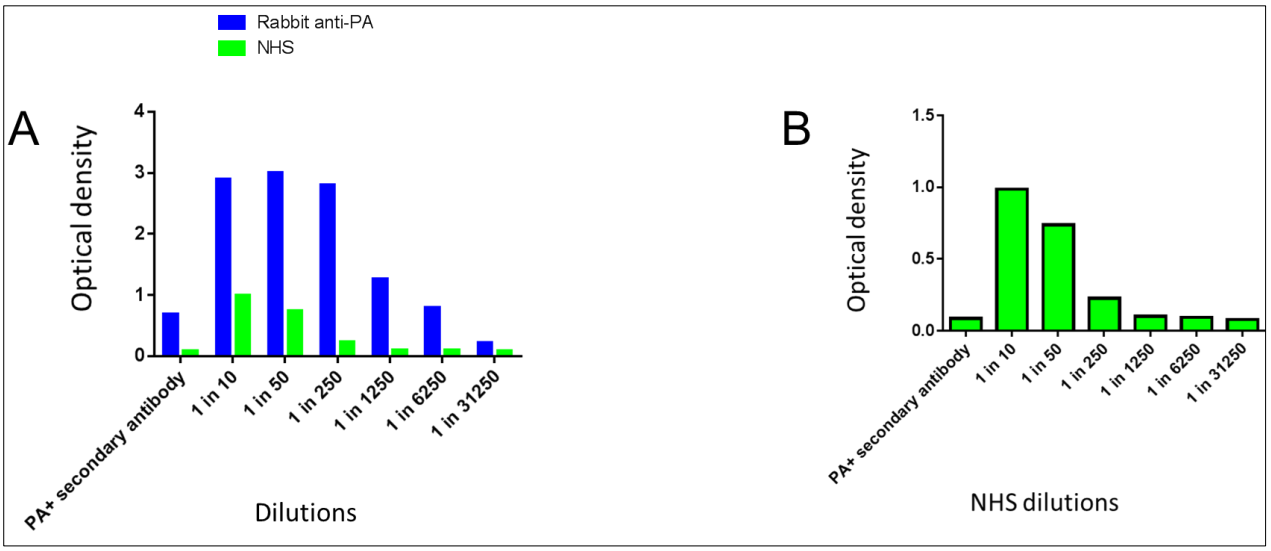


Figure 3.12: Anti-PA IgG antibody quantification in commercial sera and the positive rabbit anti-PA control.
Anti-PA IgG was quantified from a commercially obtained NHS and a positive control consisting of rabbit anti-PA antibody by ELISA. The graph of optical density against the serial dilutions plotted with Microsoft Excel 2013 for either the NHS or the Rabbit anti-PA **(A)** or the NHS only **(B)**. Each column represents the mean of a single dilution done in triplicate.

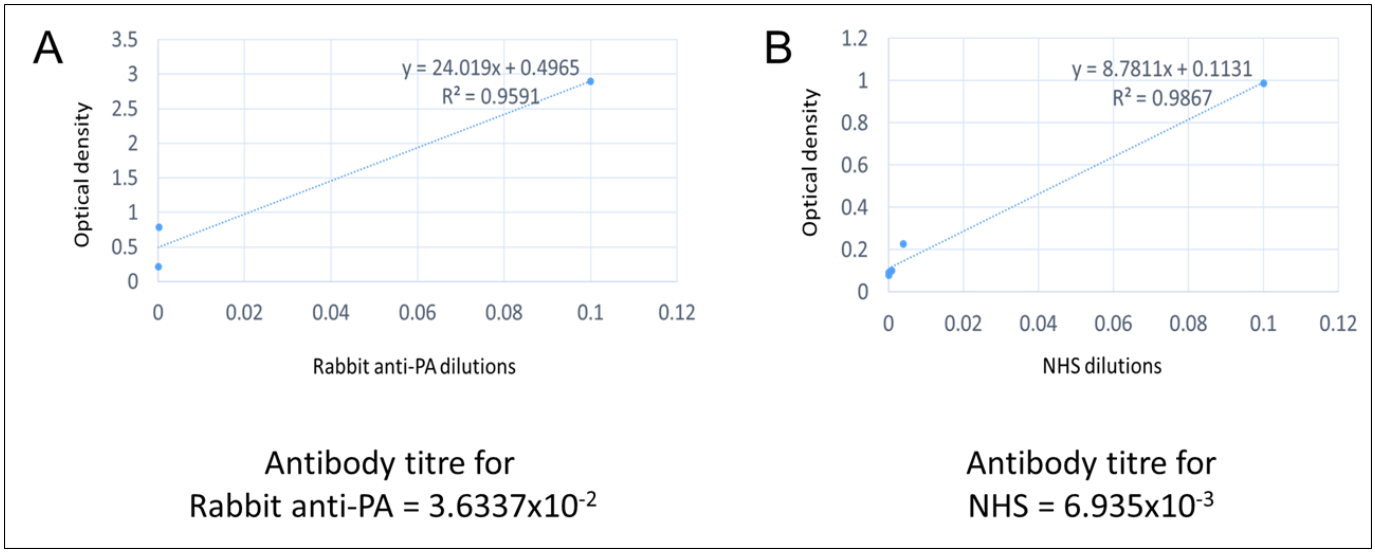


Figure 3.13: Determination of anti-PA IgG antibody titres from commercial sera and Rabbit anti-PA.
A linear graph of optical density against dilutions were plotted for either the Rabbit anti-PA **(A)** or the NHS **(B)** in Microsoft Excel 2013 version which provided the equation of the line and R^2 values. Each point represents the mean of a single dilution done in triplicate. The equation of the lines were used in calculating the antibody titres as shown above.

3.3.2 Optimised macrophage/neutrophil infection model with PA

After the optimisation assays were completed, the final protocol for the macrophage/neutrophil PA infection assay was established as detailed in Chapter 2.17.

3.3.3 Macrophage/neutrophil co-culture mediated killing of PA under opsonic and non-opsonic conditions

To assess the ability of the macrophage/neutrophil co-cultures to control PA, the co-culture infection assay detailed in Chapter 2.17 was used. Briefly, macrophages were infected with PA and samples obtained 2 hpi for CFU counts and preparation of supernatants. Freshly prepared neutrophils were added at 2 hpi and incubated further for 30 min. Samples were then again processed for CFU counts and supernatant analysis.

The results showed that, under non opsonic conditions, macrophages could not significantly reduce PA growth (120 min samples) (Figure 3.14, A). Adding neutrophils significantly reduced PA growth, both in the absence and presence of macrophages (Figure 3.14, B). In addition, there was a significant reduction of CFU in the macrophage and neutrophils co-culture samples compared to neutrophils only (Figure 3.14, B).

As expected, the addition of serum to the macrophage/neutrophil co-culture infection model reduced overall PA growth (note different scales between graphs in Figures 3.14 and 3.15). Again, the macrophages alone were not able to significantly reduce PA growth even under opsonic conditions (Figure 3.15, A). Also, PA clearance was significantly increased in the presence of neutrophils (Figure 3.15, B). However, there was no significant difference in PA clearance between neutrophils only versus macrophages and neutrophils co-culture under opsonic conditions (Figure 3.15, B).

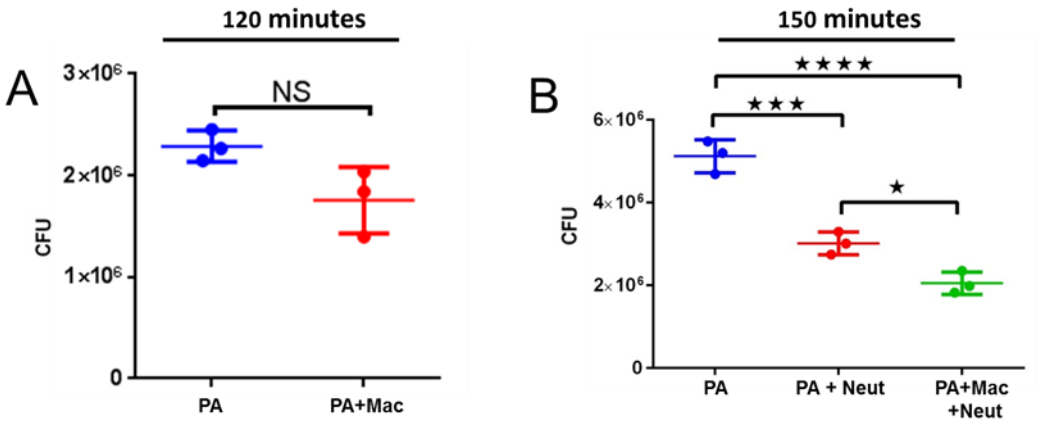


Figure 3.14: PA survival in the presence of macrophages and/or neutrophils under non-opsonic conditions.
Macrophages (Mac) were infected with PA under non opsonic conditions. After 2 hpi samples were obtained for CFU assessment and neutrophils (Neut) were added. Cultures were incubated further for 30 min and samples were then collected for CFU. **(A):** Represents the CFU at 2 hpi. **(B):** Represents the CFU at 2.5 hpi. Results from three independent repeats. Data analysed using one-way ANOVA **(B)** or Paired two-tailed t-test **(A)**. * p<0.05, *** = p<0.001, **** = p<0.0001.

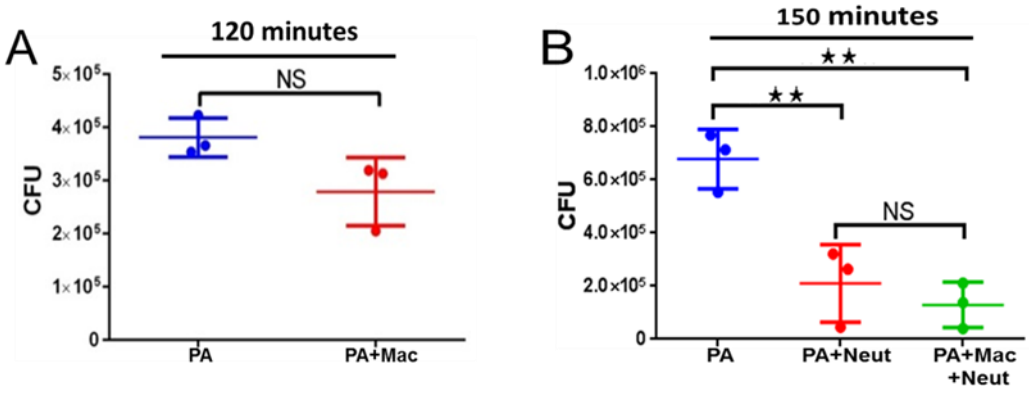


Figure 3.15: PA survival in the presence of macrophages and/or neutrophils under opsonic conditions.
Macrophages (Mac) were infected with PA under opsonic conditions. After 2 hpi samples were obtained for CFU assessment and neutrophils (Neut) were added. Cultures were incubated further for 30 min and samples were then collected for CFU. Serum concentration was maintained at 10% throughout the experimental procedure. **(A):** Represents the CFU counts at 2 hpi. **(B):** Represents the CFU counts at 2.5 hpi. Results from three independent repeats. Data analysed using one-way ANOVA **(B)** or Paired two-tailed t-test **(A)**. ** = p<0.01, NS = Not significant.

3.3.4 Neutrophil elastase production by macrophage/neutrophil co-cultures infected with PA

With the purpose of testing for the release of neutrophil elastase by macrophage/neutrophil co-cultures infected with PA, supernatants from the experiments of macrophage/neutrophil infections with or without 10% serum described in Chapter 3.3.3 were tested for the release of neutrophil elastase following the procedure described in Chapter 2.19. The purposes for testing for the release of neutrophil elastase was to serve as a marker for tissue damage, and also to decipher a potential mechanism of IL-1β processing (i.e. as neutrophil elastase has been shown to have the ability of processing inactive pro-IL-1β to the active form. see Chapter 5 for details on neutrophil elastase and IL-1β processing)

PA only without any neutrophils were used as control conditions, and as expected, there was no neutrophil elastase release by PA only both under non-opsonic and opsonic conditions (Figure 3.16). Neutrophil elastase was released by infected neutrophils and macrophage/neutrophil co-cultures (2.5 hpi) performed either with or without serum (Figure 3.16). There was no difference in the amount of neutrophil elastase released by infected neutrophils only cultures compared to macrophage/neutrophil co-cultures. Thus macrophages did not appear to influence the release of neutrophil elastase under our experimental conditions. The lack of detection of neutrophil elastase in the PA only (Figure 3.16) as well as in macrophage only conditions in Chapter 4 (Figure 4.2) demonstrate the specificity of the assay.

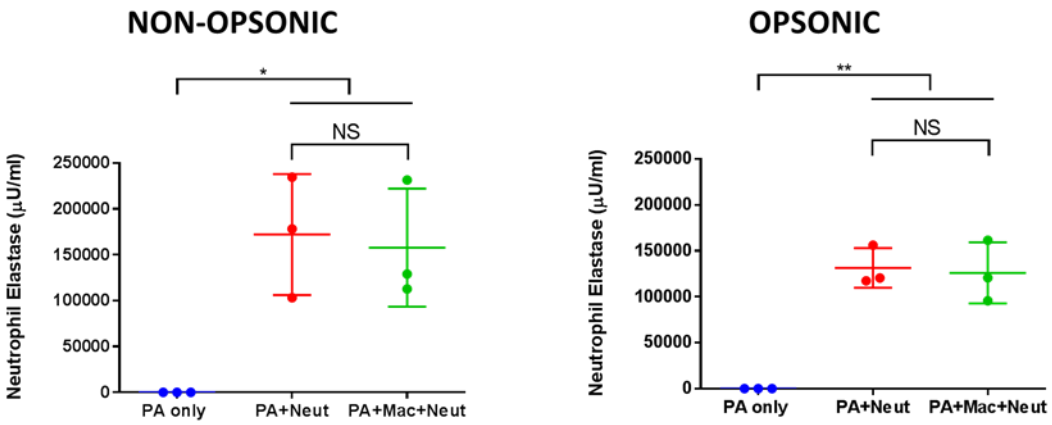


Figure 3.16: Release of neutrophil elastase by neutrophils and macrophage/ neutrophil co-cultures infected with PA under non-opsonic and opsonic conditions.
Human macrophages were infected with PA at MOI 1 in presence or absence of human serum. At 2 hpi half of the culture was collected and processed for CFU quantification and preparation of supernatants and freshly purified human neutrophils were added. Co-cultures were incubated for 30 min and processed for CFU quantification and preparation of supernatants. Supernatants from PA-infected macrophage/neutrophil co-cultures and neutrophil only cultures (2.5 hpi) were tested for the presence of neutrophil elastase following the manufacturer's protocol as described in Chapter 2.19. PA: *P. aeruginosa*, Mac: macrophages; Neut: Neutrophils. Results from three independent repeats. Data analysed using one-way ANOVA. * = $p<0.05$, ** = $p<0.01$.

3.3.5 TNF-α production from macrophages and/or neutrophils infected with PA under non-opsonic conditions

To test the influence of macrophages/neutrophil co-cultures on the production of cytokines, supernatants from the macrophage/neutrophil co-culture infections performed under non-opsonic conditions were initially tested for the release of TNF-α by ELISA following the manufacturer's procedure as described in Chapter 2.10. For this, supernatants from the 2.5 hpi were used to compare TNF-α production by infected neutrophils only versus infected macrophage/neutrophil co-cultures.

The results showed no TNF- α detection in the PA only control and by PA infections of neutrophils (Figure 3.17). However, TNF-α production was detected in infected macrophage/neutrophil co-cultures (Figure 3.17).

The absence of TNF- α in the supernatants from infected neutrophils led to the decision of testing for other cytokines but with the use of a method which is more efficient in minimising the amount of supernatant needed for the various cytokines to be analysed. Thus the Luminex assay for cytokine detection (described in Chapter 2.20) was chosen for the subsequent cytokine analysis (detailed in Chapter 4 and 5), as this allowed for testing a number of cytokines with only a small amount of supernatant per sample.

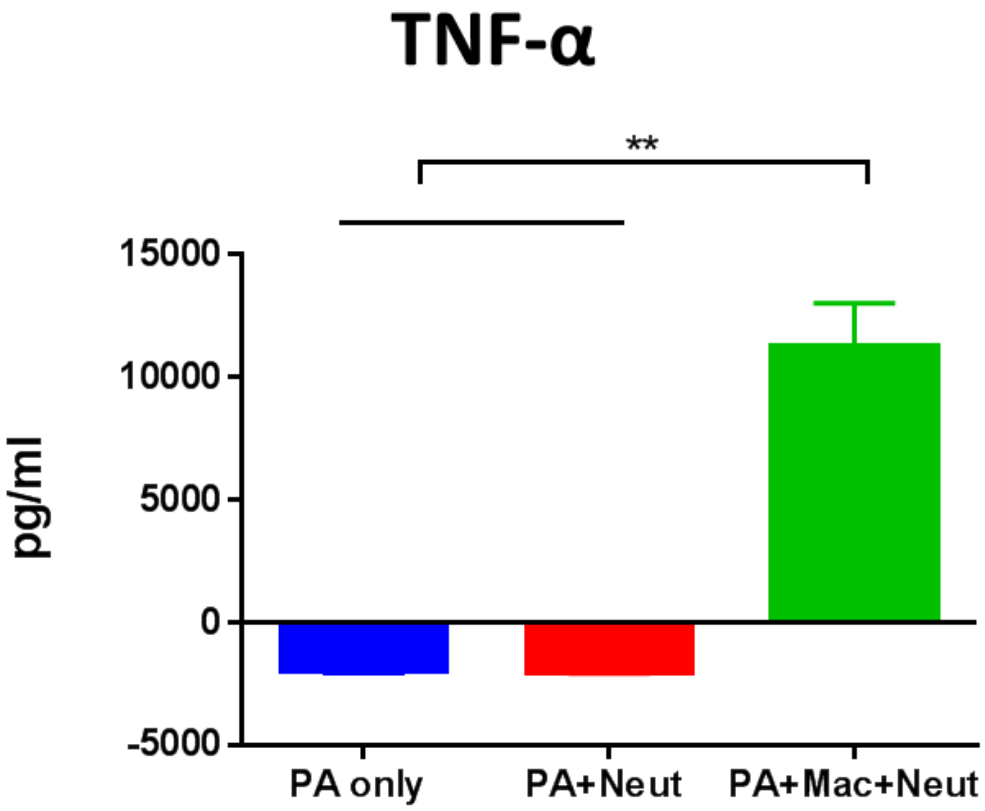


Figure 3.17: Detection of TNF-α in PA infections of macrophage/neutrophil co-cultures. Macrophages (Mac) were infected with PA under non-opsonic conditions. After 2 h samples were obtained for assessment of CFU and neutrophils (Neut) were added. Cultures were again incubated for 30 min and samples were then collected for CFU and supernatants. Supernatants obtained 2.5 hpi from PA-infected macrophage/neutrophil co-cultures and PA-infected neutrophils were tested for the presence of TNF-α following the manufacturer's protocol. PA: *P. aeruginosa*, Mac: macrophages; Neut: Neutrophils. ** = $p<0.01$.

3.4 Discussion

3.4.1 Optimisation of neutrophil isolation protocol

Based on the study by Hana et al (Oh *et al.*, 2008a) showing that alcohol had an effect on neutrophil function and that donor consumption of alcohol or medication even up to 72 hours can affect neutrophil isolation (Oh *et al.*, 2008b), donors included in this study were selected strictly based on the inclusion criteria (healthy donors who have not taken any medication or alcohol in the past 24 to 72 h) to increase the possibility of reproducibly obtaining healthy neutrophils.

Although neutrophil activation during their isolation process is almost inevitable due to their high sensitivity (Kuijpers *et al.*, 1991), efforts were made to prevent it. The isolated neutrophils were functional, as shown by their stimulation with both Zymosan and PAO1-L for ROS production (Figure 3.4). Although there was still variability in ROS production by the different neutrophils from different donors (Figure 3.4), the lack of ROS release by neutrophils only controls implies that the cells were not spontaneously activated to the extent of affecting this function. Whereas it was not possible to perform a flow cytometry analysis on all preparations of neutrophils before they were used for subsequent assays, microscopic examination of isolated neutrophils was performed in selected subsequent assays and the cells mostly displayed neutrophil characteristics. Trypan blue staining of isolated neutrophils showed over 95% viability. Flow cytometry analysis and microscopy also indicated purity of over 95% which is similar to the study of Hana et al (Oh *et al.*, 2008a) who reported over 95% purity of isolated neutrophils. This result is also consistent with the study of Alois Strasser et al (Strasser *et al.*, 1998) who found over 95% purity and viability on isolation of neutrophils from dog blood using the density gradient technique with the same isolation medium used in this study (discontinuous gradient using histopaque 1077 and 1119). Another study by Stejskal and colleagues (Stejskal *et al.*, 2010) also reported a 90.6% purity of human neutrophils using

the discontinuous density gradient with histopaque 1077 and 1119 (Stejskal *et al.*, 2010). Thus this technique was the preferred choice for neutrophil isolation.

Isolated neutrophils were resuspended in medium containing human serum (HSA) albumin. Albumin is a key component of blood plasma and functions as a carrier protein for fatty acids, steroids and hormones, it also serves as an antioxidant by binding to free radicals and enhances cell survival (Francis, 2010). We chose HSA instead of BSA because, there are species-specific differences between the two proteins such as chemical and thermal stability, binding properties and electrophoretic behaviour among others (Michnik *et al.*, 2006b). Also, in a study by Michnik and colleagues (Michnik *et al.*, 2006a), they found that the overall stability of HSA was much higher than that of BSA. Since we were using human cells in our assays, HSA was the preferred choice. Microscopic examinations of neutrophils resuspended in medium containing HSA appeared healthy (Figure 3.3), suggesting that the histopaque density gradient technique with the optimisations used yielded healthy neutrophils of high purity with minimal activation.

3.4.2 Neutrophil-mediated killing of PA

This project is the first in our laboratory to look at neutrophil mediated killing of PA hence, there was the need to first establish a reproducible experimental assay on neutrophils ability or inability to kill PA. To do this, a number of published manuscripts on how to perform a neutrophil killing assay of PA (Hampton *et al.*, 1994; Painter *et al.*, 2008; Young *et al.*, 2011; Mishra *et al.*, 2012) were referred to.

In one of the preliminary experiments, neutrophils were initially incubated with PA for either 30 min or 1 h, but there were no major differences in CFU reductions between the 30 min and 1 h conditions, hence the 30 min incubation was chosen for subsequent assays. Also, neutrophils were initially infected with PA at different MOIs (including MOI at 1, 5, 10 and 20), but significant reduction of CFU of PA were only observed at MOI of 1 and 5. Hence MOI of 5 was chosen for the neutrophil only mediated killing of PA assays so we could ascertain any

effect of the prevalent cytokines tested. Preliminary results also showed that distilled water as a lysis agent was suitable because of its ability to lyse neutrophils without affecting PA viability (Figure 3.5 and Figure 3.6) and was therefore the preferred choice in all subsequent assays.

In this study, infection of neutrophils with PA led to a reduction in PA CFU under both opsonic and non-opsonic conditions (Figure 3.8). However, the reduction was not statistically significant in the absence of opsonisation. This finding might be due to a number of factors. First, the higher numbers of bacteria in opsonised versus unopsonised conditions, as the serum employed in this particular assay surprisingly promoted PA growth and there was no difference in CFU when comparing (PA+Neutrophils with serum) versus (PA+Neutrophils without serum) (Thus Figure 3.8, red versus purple graphs). The increase in PA growth in the presence of this serum sample was unexpected but might be due to the particular serum used as other sera in future assays did not have the same effect. It is however reported that the ability of PA to produce pyoverdin is critical for PA growth in human serum, and that medium with minimal glucose levels and iron retarded PA growth in serum (Ankenbauer *et al.*, 1985). The medium used in this assay (i.e. HBSS complete medium is rich in glucose and might promote pyoverdin production by PA with a consequent increase in PA growth.

Nonetheless, the increased growth of PA in the presence of this serum was reverted by the presence of neutrophils (Figure 3.8). Hence, a more physiologically relevant reason for the significant reduction in PA growth in the opsonised conditions compared to the non-opsonic conditions may be bacterial opsonisation through the activation of the complement system. The complement system is a group of serum proteins, as well as cell surface proteins which play a critical role in host immune defence (Muller-Eberhard, 1988). Complement can be activated through three pathways; the classical, alternative, and lectin pathways. The activation of the classical pathway is mediated by C1q binding to complexes of antigens with antibody, whereas the alternative pathway is stimulated by the binding of C3 to bacteria or viral surfaces, and the lectin pathway is initiated by the binding of mannose binding lectin to microbial surfaces (Janeway *et al.*, 2001c). The activation of these pathways lead to the

activation and cleavage of C3 and consequently forms the membrane attach complex (MAC; C5b-9), which can directly kill the invading pathogen (Janeway *et al.*, 2001c). Besides the direct killing by MAC, complement components and their fragments also play a crucial role in opsonisation and phagocytosis through recognition of complement component 3b (C3b) by complement receptor 1 (CR1), CR3, and CR4. They also have a role in inflammatory cell migration and activation through the production of C3a and C5a and amplification of immune response through the production of C3dg (Muller-Eberhard, 1988; Janeway *et al.*, 2001c).

Thus the more significant killing of PA by neutrophils in the presence of serum may also be due to the complementary direct killing effect of complement through MAC formation and/or the formation of complement mediated opsonins which aided in the opsonisation of the bacteria to enhance its uptake by the neutrophils. Future assays involving the use of C5 depleted serum to avoid MAC formation and hence direct complement mediated killing of PA might be useful in deciphering this.

Indeed, the essential role of the complement system in host immune defence has been demonstrated by a number of researchers (Muller-Eberhard, 1988; Kohl, 2001). It has been reported that mice deficient in C5 and C5a receptor are more susceptible to infections with PA in spite of high neutrophil recruitment to infected lungs (Larsen *et al.*, 1982; Cerquetti *et al.*, 1986; Hopken *et al.*, 1996). Although the high numbers of neutrophil recruited in the deficient mice were not expected and the reasons for this observation were unclear, it goes to suggest that neutrophil function and not numbers are more important at infection sites. All the complement pathways cleave C5 to produce C5a (an anaphylatoxin) and C5b that forms the MAC (C5b-9, which can kill invading pathogens directly) (Janeway *et al.*, 2001c). C5a interacts with the receptors C5aR and C5L2 that are expressed on the surfaces of both immune and non-immune cells to cause a major pro-inflammatory response against invading pathogens (Woodruff *et al.*, 2011).

Muller and colleagues (Mueller-Ortiz *et al.*, 2004) have also shown the importance of the alternative complement pathway in the defence against PA. Thus, the possibility of the activation of the complement system through various pathways being a reason for the findings in this study is highly likely.

Additionally, antibodies against PA in the serum could be involved in opsonisation of the bacteria which might also enhance the phagocytic ability of the neutrophils through the interaction of the Fc portion of the antibodies with the Fc receptors, such as CD16, expressed by neutrophils (Ravetch, 1994). Indeed, there was a high titre of anti-PA antibodies in this batch of serum as indicated in Figure 3.13 which further implicates the involvement of Fc receptors. Fc receptor-mediated phagocytosis is one of the mechanisms used by phagocytes to degrade invading pathogens that would have ordinarily resisted phagocytosis (Janeway *et al.*, 2001a). Furthermore, coating of bacteria surfaces by antibodies can also activate the complement system leading to complement components being bound to the surfaces of bacteria (Janeway *et al.*, 2001a). As mentioned above complement components on the surface of opsonised bacteria can interact with complement receptor on phagocytes surfaces (such as CR1). Thus receptors of complement and Fc receptors co-localised at phagocyte surfaces act synergistically for the uptake of microorganisms (Janeway *et al.*, 2001a). Hence, a bacteria coated with both immunoglobulin and complement component will be more easily degraded than those with single coatings of either immunoglobulin or complement component.

While the reduction in CFU of PA in the unopsonised conditions was not statistically significant, results from the flow sight demonstration clearly show the ability of neutrophils to phagocytose PA under non-opsonic conditions (Figure 3.7), although their killing ability could not really be deciphered from this assay. Thus the neutrophils might still be able to control PA to some extent under the non-opsonic conditions but a much enhanced control would be expected under opsonic conditions.

Looking at the experimental setting established in this study (i.e. neutrophils infected with PA at MOI 5 with 10% NHS, Figure 3.8), about 4-fold reduction in the number of bacteria was observed in the presence of neutrophils. There are very limited studies on the role of human neutrophils in protection against PA. In this study however, neutrophils ability to reduce bacterial CFU was lower compared to that observed by Li and colleague (Li *et al.*, 2002) in which between one to three log reductions of bacterial CFU was observed using human neutrophils infected with *S. epidermidis* H753 in the presence of 75% human serum. This difference might be due to the differences in experimental conditions such as the percentage of serum and bacteria species in that PA might be generally more resistant to neutrophil-mediated killing. Also in a study by Mishra (Mishra *et al.*, 2012) they showed increased ROS production in response to PA in the presence of 10% human serum when compared to no serum conditions. We used 10% human serum in our assay conditions based on previous studies (Hampton *et al.*, 1994; Mishra *et al.*, 2012). Thus increasing the percentage of serum in the current assay may increase opsonophagocytosis as well as increase ROS release by the neutrophils which would lead to higher bacteria killing. It may also be possible to increase the rate of PA killing by adding PA specific antibodies to our assay although increasing the amount of serum might serve this purpose since the serum contained abundant anti-PA antibodies (

Table 3.1 and Figure 3.12). Nevertheless, the ability of neutrophils to control PA infection has been shown in this study by the combined results from both the CFU and the flow sight analysis.

3.4.3 Effect of normal human serum on PA viability is highly variable among donors and time of blood collection

Preliminary results showed that neutrophil-mediated clearance of PA was more significant in the presence of serum. For this reason non-opsonic versus opsonic conditions were compared in the macrophage/neutrophil co-culture PA infection model. It was found that for the purposes of opsonic assay conditions, it was necessary to screen sera from different donors for the ability of serum only to directly kill PA. Our results showed a high variability in PA killing among sera from different donors and even within the same donors when samples were collected at different times.

However, the PA antibody titres were similar among all the donors screened (within the range of 10^5 as shown in

Table 3.1). These high titres of PA antibodies among presumably healthy donors were unexpected. The reasons for this finding might be that, these individuals had come in contact with PA in the environment and their immune system was induced to produce an antibody-mediated response against PA or perhaps these individuals had antibodies against other pathogens which could cross react with PA as suggested by Mauch and colleagues who also detected the existence of high PA antibody tires in healthy donors (Mauch *et al.*, 2014).

Since the PA antibody titres were similar among all donors, the differences in PA clearance were considered more likely due to differences in complement activity.

The findings of variation in complement activity among donors in this study is in agreement with the work of Ritchie and his colleagues (Ritchie *et al.*, 2004), who investigated the levels of C3 and C4 in fifty five thousand Caucasians. Their results showed that not only did the levels of C3 change with age and sex of individuals but also, there was a positive correlation between oestrogen and C3 levels. Additionally, in a study by Yang and colleagues (Yang *et al.*, 2003) where correlation between phenotypic and genotypic differences in C4 was investigated, they showed that persons with shorter gene length but higher copy numbers of C4 genes had higher levels of serum C4 protein, with a consequent higher innate immunity. Thus our findings are consistent with previous literature.

Furthermore, the findings of the variation within same individuals (Donor 1 and 4) in this study could be attributed to a number of factors such as the experimental procedure. The second repeat of the experiment (for Donor 1 and 4) included a stage where the culture samples were passed through a 25G needle 5 times before serial dilutions were made for CFU, this was done to maintain consistent experimental conditions between the serum only infection assay and the neutrophils and macrophages infection assay where cells were lysed following this procedure. This passage through syringe was lacking in the initial study.

Even more relevantly, blood samples (for donor 1 and 4 repeats in Figure 3.9, B) were collected approximately two months later. Studies have shown the existence of differences in

complement activity within the same individuals at different times (Cannon *et al.*, 1994). Similarly in a study by Bouvenot and colleagues (Bouvenot *et al.*, 1984) in 40 apparently healthy males, they observed that C3 proteins displayed a significant circadian rhythm. Thus, the difference in serum clearance of PA within same individuals might not only be due to the different days of sample collection but also the time of the day as well. Therefore, it was necessary to maintain consistency in the time of day for sample collection from our voluntary blood donors.

3.4.4 Macrophages and neutrophils co-cultures reduce PA infection

The macrophage/neutrophil co-culture infection model described in Chapter 2.17 involved the infection of monocyte-derived macrophages generated in the presence of GM-CSF with PA at MOI of 1 for 2 h for CFU and supernatants analysis, and neutrophils added at ratios of macrophage: neutrophil being 1:5 with further incubation for 30 min and samples again obtained 2.5 hpi for CFU and supernatants analysis. Assays were performed with or without 10% NHS.

This assay conditions represents an acute *in vitro* infection model of PA. The monocyte derived macrophages were generated in the presence of GM-CSF to produce macrophages with a classical M1-like phenotype (Verreck *et al.*, 2004). GM-CSF is highly produced by infected tissues, and is capable of directing macrophages to differentiate into more microbicidal and pro-inflammatory types (Hamilton, 2008). Assays were performed at low MOI (MOI 1) because an initial study in our lab had shown that the macrophages could not withstand the bacterial infection at higher MOI, the macrophages however could survive infection for up to 2 hpi and then started dying at later time point of infection (4hpi and 6hpi) even with the MOI of 1 (Singh, 2012). In addition, the production of inflammatory mediators were already shown at 2 hpi (Singh, 2012). Thus MOI 1 was chosen for 2 h infection with the macrophages to enable us to analyse the contribution of viable macrophages in controlling PA infection in our co-culture model. The sampling at 2 hpi gives a clue of the macrophages only

response to PA infection and the inflammatory mediators released which could affect neutrophil function in the co-culture model.

The addition of neutrophils to the already infected macrophages symbolises the initial involvement of macrophages as sensors and triggers of inflammation and of neutrophils as efficient microbicidal cells whose activity if not properly controlled can lead to an exaggerated inflammatory response and hence tissue damage (Serhan, 2010; Silva, 2010a; Soehnlein *et al.*, 2010; Jenner *et al.*, 2014). It was therefore our expectation that the inflammatory mediators released by the infected macrophages could influence the neutrophilic response.

The ratio of macrophage to neutrophils takes into consideration the higher numbers of neutrophils than macrophages at infection sites. Even though 1:5 was nowhere close to the massive numbers of neutrophils at infection sites *in vivo*, it was chosen based on feasibility in the numbers of neutrophils isolated per assay. Knowing the MOI of bacteria and ratios of macrophage: neutrophil in our assays will also allow us to analyse various factors that could affect the response of neutrophils to PA infections under diverse inflammatory conditions.

Neutrophils added 2 hpi were further incubated for 30 min because neutrophils are short lived (Summers *et al.*, 2010). The neutrophil isolation process had already taken 2 h or more to complete and moreover a preliminary experiment did not show any significant reduction in bacterial CFU when 30 min and 60 min incubation of PA with neutrophils were compared. The sampling at 2.5 hpi gives an indication of the response of neutrophils only and macrophage/neutrophil co-cultures to PA infection and the possible inflammatory mediators released. Finally, the use of NHS under opsonic assay conditions takes into consideration the involvement of complement activation in bacteria opsonisation and killing and the percentage of NHS (10%) was chosen based on published literature (Hampton *et al.*, 1994; Mishra *et al.*, 2012).

This model however has its limitations in the fact that, it neither considers the influence of macrophages on neutrophil migration which *in vivo* would lead to phenotypic changes of

neutrophils nor does it consider the huge increase in neutrophil numbers observed at inflammatory sites *in vivo* (Soehnlein *et al.*, 2010). It also employed only PAO1-L strain, but this is just a starting point and that other strains of PA or even other pathogens, could be tested using this model in the future.

Meanwhile, results from this study using the macrophage/neutrophil co-culture infection model show the collaboration of macrophages and neutrophils in controlling PA infection particularly under non-opsonic conditions as discussed below:

a. Macrophages partially restrict PA growth

The macrophages alone were not able to significantly reduce PA growth, but a minor restriction in PA growth under opsonic and non-opsonic conditions was observed. These results are comparable with the findings of Singh and colleagues (Singh *et al.*, 2015) where human macrophages were found neither able to significantly control PA growth under non-opsonic conditions, nor survive PA infection although the culture conditions were different and macrophages were plated instead of in suspension. Singh and colleagues also showed that human macrophages release signals that could potentially enhance the recruitment of other immune cells to infection sites (Singh, 2012; Singh *et al.*, 2015). Our results from culture supernatants showing the production of large amounts of TNF-α from the macrophage/neutrophil co-cultures which was lacking in the neutrophil only conditions (Figure 3.17) further supports the potential immunomodulatory role of macrophages. The inability of macrophages only to significantly control PA infections is however in contrast with the study of Sharon and colleagues (McClellan *et al.*, 2003) who reported the ability of macrophages to control PA corneal infections in mice. Findings from this study suggest that although macrophages did not directly reduce PA growth significantly (Figure 3.14 and Figure 3.15), they are probably essential in controlling PA as they recruit neutrophils by releasing a number of cytokines such as TNF- α (Figure 3.17).

b. Neutrophils reduce PA growth by themselves and in co-cultures with macrophages

Human neutrophils on the other hand were able to significantly reduce PA growth by themselves and in co-culture with macrophages.

Studies have shown that macrophages cooperate with neutrophils at infectious sites during an innate immune response (Silva, 2010a; Soehnlein *et al.*, 2010; Silva, 2011). Novais and colleagues (Novais *et al.*, 2009) have also shown the cooperation of neutrophils and macrophages to resist infections of *Leishmania braziliensis* using mouse models. Indeed, various researchers have shown the potential cooperative efforts of both cell types in microbial clearance (Novais *et al.*, 2009; Silva, 2010a; Soehnlein *et al.*, 2010; Silva, 2011).

Using a novel *in vitro* human macrophage/neutrophil PA infection model to assess the collaboration of these phagocytes in controlling PA infections, we have observed a marginal additive effect in the control of PA by human macrophages and neutrophils co-cultures under our experimental conditions, which is more apparent under non-opsonic conditions. Thus exposure to inflammatory mediators produced by macrophages does not drastically affect the ability of neutrophils to clear PA, both in the absence and presence of complement under our experimental conditions.

3.5 Summary and conclusions

This Chapter describes assays to optimise experimental conditions and a two-cell model of PA infection involving a macrophage/neutrophil co-culture. For this, a number of optimisation assays were carried out and the final macrophage neutrophil co-culture model was established. To test the ability of neutrophils only to control PA, healthy donors who have neither consumed any alcohol or medication in the past 72 hours were used, neutrophils were isolated using discontinuous density gradient centrifugation with two Histopaques at densities 1.077 g/ml, and 1.119 g/ml, and the cells resuspended in appropriate medium (e.g. Buffer A for ROS assay or HBSS with Ca^{2+} and Mg^{2+} with HSA for all infection assays). The neutrophils were infected with mid-log phase PA with or without 10% normal human serum and plated for CFU or analysed for phagocytosis.

To test the ability of macrophage and neutrophil co-cultures to control PA, monocytes were isolated and differentiated into macrophages in the presence of GM-CSF. Macrophages were infected with PA for 2 hours (MOI 1) under non-opsonic or opsonic conditions. Samples were obtained for CFU at 2 hpi and neutrophils were added (5 neutrophils per macrophage) at that stage. Macrophage/neutrophil co-cultures were incubated for 30 min and samples obtained for CFU and supernatants. Serum concentration was also maintained at 10% throughout the opsonic experimental procedure. From the data obtained, the following conclusions were drawn:

1. Discontinuous density gradient centrifugation using Histopaque (1077 and 1119) yields neutrophils of high purity with minimal activation.
2. PA strain (PAO1-L) is not capable of producing ROS.
3. Distilled water sufficiently lyses neutrophils without affecting PA viability
4. Neutrophils could clear PA infection.
5. Neutrophils and macrophages collaborate in clearing PA infection, particularly under non-opsonic conditions.

Chapter 4

Comparison of the effect of IFN-γ and IL-17A on the ability of human phagocytes to control *Pseudomonas aeruginosa*

Chapter 4: Comparison of the effect of IFN-γ and IL-17A on the ability of human phagocytes to control *Pseudomonas aeruginosa* (PA)

4.1 Introduction

This Chapter describes studies exploiting the macrophage/neutrophil co-culture infection model described in Chapter 3 to determine the effect of Th-1 and Th-17 dominated immune response on the cross-talk between human macrophages and neutrophils during infections with PA. The original concept was that infection of macrophage/neutrophil co-cultures in the presence of cytokines associated to Th-1 or Th-17 responses will provide an indication of the reasons behind the positive association between IFN-γ production and lung function and the negative association between IL-17A production and lung function in CF (Moser *et al.*, 2005; Singh *et al.*, 2015). To test this concept, infection assays were performed with untreated macrophages or treated with either IFN-γ or IL-17A, the signature cytokines produced by Th-1 and Th-17 cells respectively. Both cytokines were maintained throughout the infection assay. Bacteria clearance, cytokine production and release of neutrophil elastase were quantified. The direct effect of IL-17A and IFN-γ on the ability of neutrophils to kill PA was also measured.

T-cells developed from the thymus enter into circulation as naive T-cells until they encounter a specific antigen (Janeway *et al.*, 2001b). Upon encounter of a specific antigen, they proliferate and differentiate into effector T-cells, which become ready to mount an immune response upon getting into contact with their target organisms (Janeway *et al.*, 2001b). T-cells play an important role in shaping the immune response to infection but their activation relies on the prior activation of the innate immune response. For instance, tissue resident dendritic cells (DCs) become activated when they encounter antigens and are able to trigger PRRs. Activated DCs become effective antigen-presenting cells (APCs) which present MHC-peptide complexes on their surfaces to naive T-cells to help in initiating them to differentiate into effector T-cells. Both activated macrophages and B cells can also act as APCs by expressing co-stimulatory and MHC class II molecules even though they are less powerful compared to

the activated DCs (Janeway *et al.*, 2001b). The activation of T cells is highly regulated and involves several receptor interactions including T-cell receptor (TCR), co-stimulatory receptors as well as a suitable signalling molecule with a consequent clonal expansion and differentiation of effector T cells and cytokine production (Curtsinger *et al.*, 2010). Thus T-cell activation has been proposed to require 3 signals (Curtsinger *et al.*, 2010). The first signal is the MHC-peptide complexes (Signal 1) and involves the interaction of T-cells with APCs when APCs present specific antigens to T-cells through their MHC-complexes (Shahinian *et al.*, 1993). The second signal (signal 2) is a co-stimulatory signal (Shahinian *et al.*, 1993) and involves the binding of co-stimulatory receptor for instance CD28 receptor on the T-cells to co-stimulatory molecules on the APCs (e.g. CD80 and CD86) (Shahinian *et al.*, 1993). When T-cells are presented with signal 1 without the signal 2, they become anergic (i.e. the T cells become incapable of mounting a specific response to their antigens) (Curtsinger *et al.*, 2010). The third signal (signal 3) was initially proposed for only CD8 T- cells but there is now evidence showing that the CD4 T-cells also require signal 3 (Curtsinger *et al.*, 2010). Cytokines produced by macrophages and DCs are also required for an effective generation of effector and memory T cells as well as the T-cell survival (Mescher *et al.*, 2006; Curtsinger *et al.*, 2010). Signals provided by these cytokines (mostly IL12, IFN-α and IFN-β for CD8 T-cells and IL-1 for CD4 T-cells) are termed signal 3 (Curtsinger *et al.*, 2010).

As indicated in Chapter 1, the effector T-cells are divided into different types based on the types of peptide antigens. Cytoplasmic intracellular pathogen peptides are presented to CD8 T cells via MHC class I molecules on their cell surfaces. These T-cells will differentiate into cytotoxic T cells which directly kill their infected target cells (Janeway *et al.*, 2001b). Intracellular vesicular peptides and ingested extracellular bacterial peptides and toxins are presented to CD4 T cells via MHC class II molecules. These T-cells were initially thought to differentiate into helper T cells 1 and 2 (Th-1 and Th-2) (Janeway *et al.*, 2001b).

Mosmann and his colleague initiated the idea of T-cell sub-division depending on the cytokine profile released by T-cells with IFN-γ being considered a Th-1 cytokine while IL-4 and IL-5

considered Th-2 cytokines (Mosmann *et al.*, 1986; Locksley *et al.*, 1987). However, current advances in T cell research have shown that the T-helper cells can actually be subdivided into more groups: when naive CD4 T-cells become activated, they can differentiate into either T helper cells (such as Th-1, Th-2, Th-9, Th-17 or Th-22), regulatory T-cells (Tregs) or follicular helper T cells (Tfh) depending on the cytokine milieu (Bayes *et al.*, 2010; Luckheeram *et al.*, 2012; Zheng, 2013). Studies have shown that the differentiation towards Th-1 cells is activated by IFN-γ and IL-12 (Trinchieri *et al.*, 2003; Luckheeram *et al.*, 2012). Th-1 cells are involved in cell-mediated immune response and produce pro-inflammatory cytokines such as IFN-γ, TNF-α and TNF-β (Trinchieri *et al.*, 2003; Luckheeram *et al.*, 2012). The differentiation towards Th-2 cells is activated by IL-4 and IL-2 (Luckheeram *et al.*, 2012). Th-2 cells promote humoral immune responses and produce cytokines such as IL-4, IL-5, IL-13 and IL-15 (Luckheeram *et al.*, 2012). Th-2 cells are also associated with promoting allergic responses (Sokol *et al.*, 2009). Similarly, the differentiation towards Th-17 cells is promoted by TGF-β, IL-6, IL-21 and IL-23 (Luckheeram *et al.*, 2012). Th-17 cells are involved in immune response to many extracellular pathogens including bacteria, fungi, and viruses and are implicated in auto-immune diseases (Brustle *et al.*, 2007). Th-17 cells produce cytokines such as IL-17, IL-21, IL-22 and IL-26 (Luckheeram *et al.*, 2012; Meller *et al.*, 2015). Differentiation towards Th-22 cells is activated by IL-6 (Jia *et al.*, 2014). Th-22 cells are associated to mucosal immune responses as well as inflammatory diseases and produce the cytokine IL-22 (Jia *et al.*, 2014). Differentiation towards Th-9 on the other hand is activated by TGF-β and IL-4 (Veldhoen *et al.*, 2008). Th-9 cells interact with other cell types such as B cells for humoral immune response and produce IL-9 (Luckheeram *et al.*, 2012; Kaplan *et al.*, 2015). Th-9 cells are also involved in allergic disease development (Xing *et al.*, 2011). Furthermore, Tfh cells are activated by IL-6 and IL-21 (Vogelzang *et al.*, 2008), they help B cells to mount antigen specific immune responses and can produce IL-4 and IL-21 (Vinuesa *et al.*, 2005). Tregs are activated by TGF-β and IL-2, are involved in maintaining self-tolerance and produce TGF-β and IL-10 (Luckheeram *et al.*, 2012). More details on T-cell differentiation can be found in these references (Zhu *et al.*, 2010; Luckheeram *et al.*, 2012; Zheng, 2013; Geginat *et al.*, 2014).

As mentioned in Chapter 1, the role for T-lymphocytes in PA control is not well understood but there are some evidence of T cells involved in immunomodulation during PA infection mainly in chronic infections (Dunkley *et al.*, 1994). The general concept is that Th-2 response is associated with susceptibility to PA whereas Th-1 response is associated with resistance to PA (Dunkley *et al.*, 1994; Moser *et al.*, 1999; Tam *et al.*, 1999) as detailed earlier in Chapter 1. It has also been demonstrated that Th-1 response predominantly correlates with better CF lung function compared to Th-2 responses in chronic PA infected CF patients (Moser *et al.*, 2000; Moser *et al.*, 2002) which suggests that the infection outcome with PA depends on the type of T cell response.

Effective clearance of PA is a concerted effort between both the innate and the adaptive immune response (Sadikot *et al.*, 2005). Macrophages and neutrophils are both influenced by cytokines provided by the adaptive immunity (Walsh *et al.*, 2013). In particular, Th-17 cells through the release of IL-17A and GM-CSF, promote a neutrophilic inflammatory response best suited for clearance of extracellular bacteria and fungi (Walsh *et al.*, 2013). Th-1 cell-mediated inflammation dominated by IFN-γ, is generally associated with protection against intracellular pathogens (Walsh *et al.*, 2013).

In agreement with literature (Moser *et al.*, 2005), previous studies in our laboratory showed a positive correlation between IFN-γ production by PBMCs from CF patients with lung function, particularly in patients chronically infected with PA. In contrast, IL-17A levels tended to correlate negatively with lung function with this trend becoming significant in patients chronically infected with PA (Singh *et al.*, 2015). These results were in agreement with IFN-γ and IL-17A playing protective and detrimental roles respectively in CF (Singh *et al.*, 2015). Mulcahy and colleagues (Mulcahy *et al.*, 2015) have also shown a negative correlation between Th-17 cells and lung function in CF. To explore the protective effect of IFN-γ during PA infection, the effect of IFN-γ alone or in combination with GM-CSF on the ability of human macrophages to control PA growth, resist the cytotoxicity induced by PA or promote inflammation was investigated (Singh *et al.*, 2015). Treatment of macrophages with IFN-γ in

the presence and absence of GM-CSF, failed to alter bacterial growth or macrophage survival upon PA infection, but altered the cytokine response to infection (Singh *et al.*, 2015). It was therefore proposed that IFN-γ could be beneficial in CF by promoting a more balanced neutrophilic inflammatory response during infection with extracellular bacteria that could protect organ function and that a macrophage/neutrophil co-culture will provide a suitable model to investigate this possibility.

4.2 Materials and methods

To assess the effect of inflammatory mediators such as IFN-γ and IL-17A on the ability of macrophages and neutrophils to control PA infection, infection assays were performed using macrophages untreated or treated with either cytokine for 24 h prior infection as described in Chapter 2.17 and Figure 2.2. In brief, macrophages were either left untreated or pre-treated with IFN-γ (10 ng/ml) or IL-17A (15 ng/ml) for 24 h prior to infections. Untreated and treated macrophages were collected and infected with PA under opsonic conditions (10% NHS, Figure 2.2). Samples were obtained 2 hpi for assessment of CFU and analysis of cytokines and proteases. Neutrophils were added 2 hpi and cultures incubated further for 30 min and samples were collected again for CFU and supernatants for the analysis of cytokines and proteases. Serum and cytokine concentrations were maintained throughout the experimental procedure.

To test if IL-17A or IFN-γ had a direct effect on the ability of neutrophils to control PA, neutrophils were either untreated or pre-treated with IL-17A or IFN-γ before being infected with PA and analysed as described in Chapter 2.21. In some instances, samples were observed under a bright field inverted microscope to investigate possible morphological changes upon infection with PA in the presence and absence of IL-17A (see Chapter 4.3.6).

4.3 Results

4.3.1 Effect of IFN-γ on the ability of macrophage/neutrophil co-cultures to reduce PA growth

To assess the effect of IFN-γ on the ability of macrophage/neutrophil co-cultures to reduce PA growth, macrophages untreated or pre-treated with IFN-γ for 24 h were infected with PA under opsonic conditions for 2 h at which time neutrophils were added to a macrophage: neutrophil ratio of 1:5. Co-cultures were further incubated for 30 min. Samples were obtained again at 2.5 hpi for CFU analysis and supernatants for analysis of cytokines and proteases (see Chapter 2.18 and Figure 2.2). Results showed that IFN-γ did not affect the ability of macrophages to clear PA infections (Figure 4.1, A, blue graphs). There was a significant increase in the bacterial load in IFN-γ treated co-cultures measured as total CFU (Figure 4.1, A) and as percentage of remaining bacteria (Figure 4.1, B). Thus, IFN-γ decreased the ability of macrophage/neutrophil co-cultures to clear PA infection.

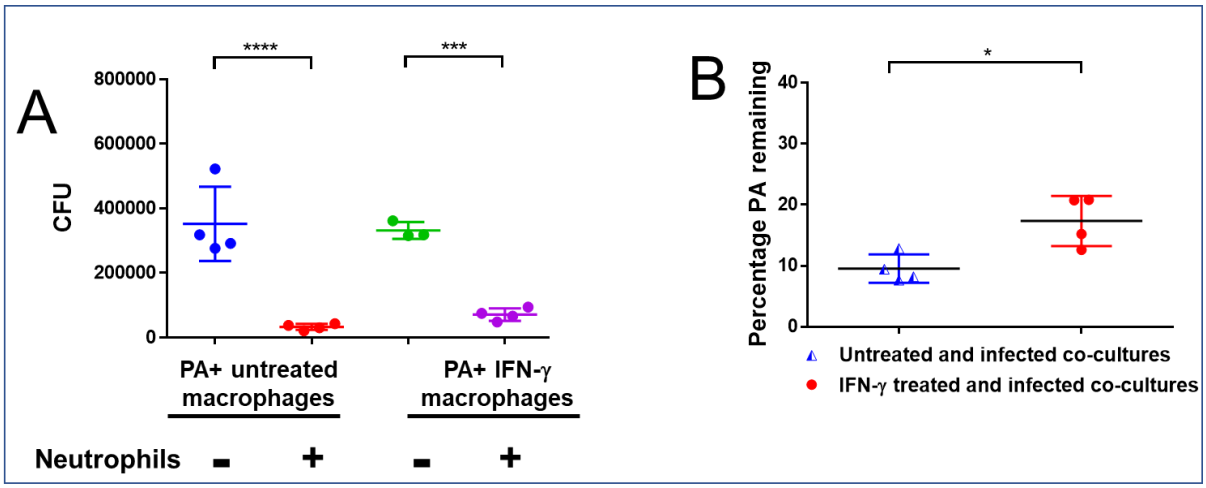


Figure 4.1: Effect of IFN-γ on the ability of macrophage/neutrophil co-cultures to reduce PA growth.

Human macrophages (2 x10^5) generated in the presence of GM-CSF were left untreated (Unt) or treated at day 6 with IFN-γ. On the following day, macrophages were collected and infected with PA at MOI 1 in the presence of human serum. At 2 hpi, half of the culture was collected and processed for CFU quantification and preparation of supernatants and freshly purified human neutrophils were added (5 x10^5, macrophage: neutrophil ratio 1:5). Co-cultures were incubated for 30 min and processed for CFU quantification and preparation of supernatants. (**A):** Effect of IFN-γ on the ability of macrophages and macrophage/neutrophil co-cultures to reduce PA growth. (**B**)**:** Effect of IFN-γ on the percentage of PA remaining in the macrophage/neutrophil co-cultures (2.5 hpi) compared to the macrophage only cultures (2 hpi). Mac: macrophages, Neut: neutrophils. Results from four independent repeats. Data analysed using either one-way ANOVA **(A)** or Paired two-tailed t-test **(B)**. * = $p<0.05$, *** = $p<0.001$, **** = $p<0.0001$, NS = Not significant.

4.3.2 Effect of IFN-γ on the release of neutrophil elastase by macrophage/neutrophil co-cultures infected with PA

To test the ability of IFN-γ to modulate the release of neutrophil elastase by macrophage/neutrophil co-cultures infected with PA, supernatants from the experiments described above (Chapter 4.3.1, Figure 4.1) were tested for the release of neutrophil elastase following the procedure described in Chapter 2.19. As shown in Figure 4.2, there was no release of neutrophil elastase by infected macrophages only cultures (2 hpi) either untreated or treated with IFN-γ which again proves the specificity of the assay. Meanwhile, infected macrophage/neutrophil co-cultures released neutrophil elastase but there was no difference in the amount released by either untreated or IFN-γ-treated cultures (Figure 4.2). Thus IFN-γ did not influence the release of neutrophil elastase under our experimental conditions.

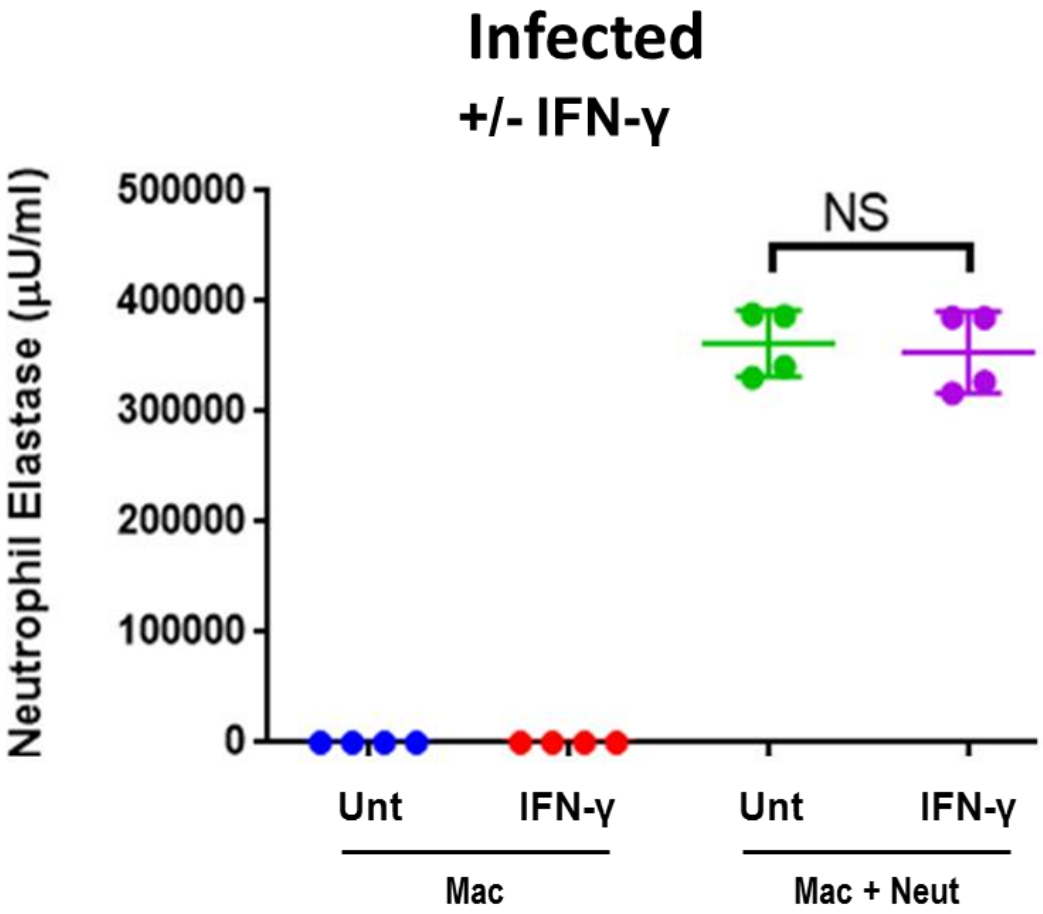


Figure 4.2: IFN-γ does not affect the release of neutrophil elastase by macrophage/neutrophil co-cultures infected with PA.
Human macrophages (2 x10^5) generated in the presence of GM-CSF were left untreated (Unt.) or treated at day 6 with IFN-γ. On the following day, macrophages were collected and infected with PA at MOI 1 in presence of human serum. At 2 hpi half of the culture was collected and processed for CFU quantification and preparation of supernatants and freshly purified human neutrophils were added (5 x10^5, macrophage: neutrophil ratio 1:5). Co-cultures were incubated for 30 min and processed for CFU quantification and preparation of supernatants. IFN-γ and serum concentrations were maintained throughout the experimental process. Supernatants from PA-infected macrophage only (2 hpi) and macrophage/neutrophil co-cultures (2.5 hpi) were tested for the presence of neutrophil elastase following the manufacturer's protocol as described in materials and methods Chapter 2.19. Mac: macrophages, Neut: neutrophils. Results from four independent repeats. Data analysed using Paired two-tailed t-test. NS = Not significant.

4.3.3 Effect of IFN-γ on cytokine production by macrophage/neutrophil co-cultures infected with PA

To determine the effect of IFN-γ on the ability of infected human macrophage/neutrophil co-cultures to promote or inhibit inflammation, supernatants from the experiment described above (Figure 4.2) were tested for the presence of cytokines as described in Chapter 2.20. Supernatants from infected macrophages only (2 hpi) and macrophage/neutrophil co-cultures (2.5 hpi) were analysed for the presence of IL-1β, IL-1α, MIP-1α, MCP-1, IL-8, TNF-α, IL-10 and IL-1RA (Figure 4.3 and Figure 4.4). Graphs of the various cytokines tested from the effect of IFN-γ treatment (Figure 4.3) on cytokine production were presented using the same scale as those from the effect of IL-17A treatment (Figure 4.13) for easy comparison between the two treatment conditions.

With IFN-γ treatment, there was significant increase in IL-8 production by infected macrophages only (2 hpi) but reduced in the macrophage/neutrophil co-cultures (2.5 hpi) (Figure 4.3, IL-8 graph) perhaps due to the dilution of supernatants at 2 hpi by the addition of neutrophils. IL-1β was increased with neutrophil addition to the macrophage infections but significantly reduced in the presence of IFN-γ treatment (Figure 4.3, IL-1β graph). IL-1α had a trend towards an increase with neutrophil addition to the macrophage infections but this was not significant (Figure 4.3, IL-1α graph). MCP-1 and TNF-α had a tendency to increase with IFN-γ treatment in the macrophage infections and the trend was maintained with neutrophil addition to the macrophage cultures (Figure 4.3, MCP-1 and TNF-α graph) and there was no effect seen on MIP-1α production (Figure 4.3, MIP-1α graph).

IL-1RA levels were above the range of the standard of the assay in all cases while IL-10 levels were below the range of the standard of the assay (Figure 4.4)

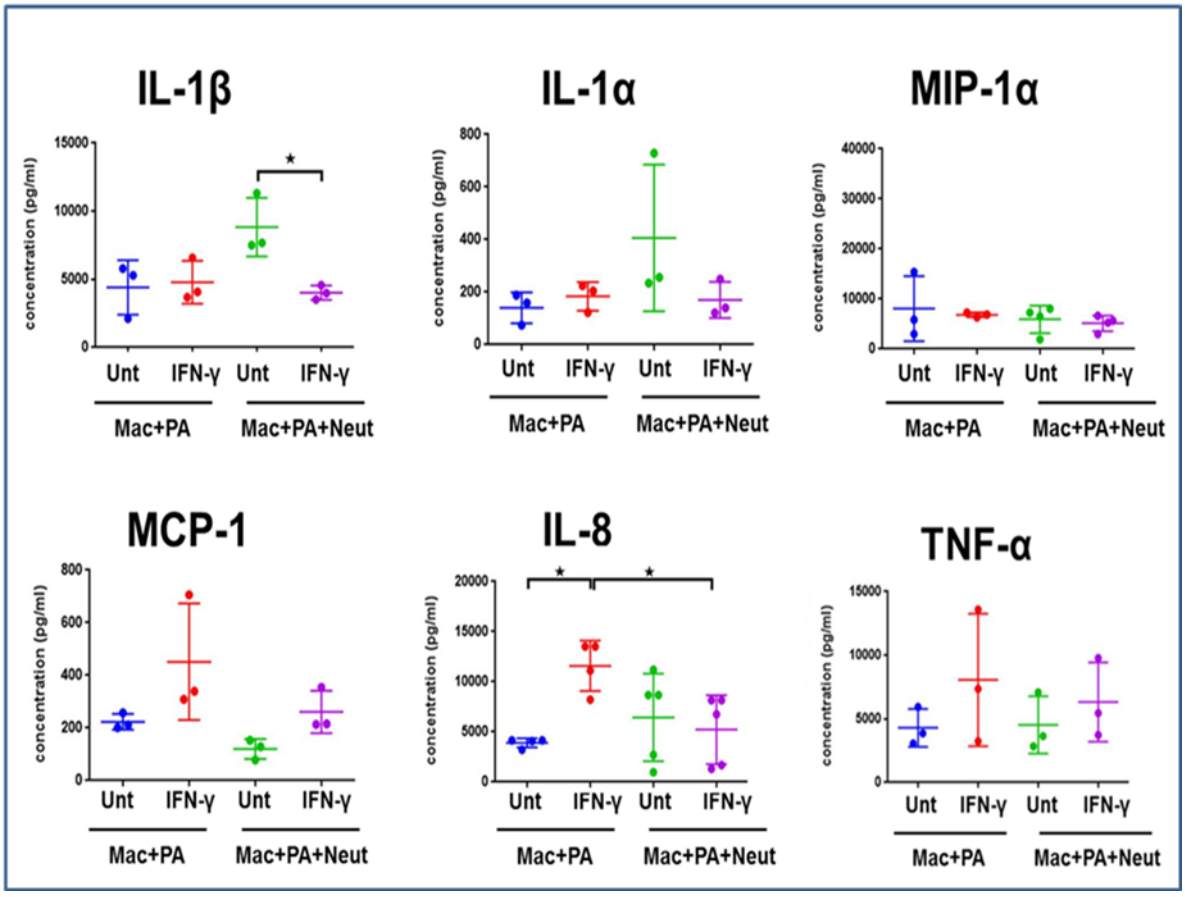


Figure 4.3: Effect of IFN-γ on cytokine production by macrophage/neutrophil co-cultures infected with PA.
Human macrophages (2 x10^5) generated in the presence of GM-CSF were left untreated (Unt.) or treated at day 6 with IFN-γ. On the following day, macrophages were collected and infected with PA at MOI 1 in the presence of human serum. At 2 hpi half of the culture was collected processed for CFU quantification and preparation of supernatants and freshly purified human neutrophils were added (5 x10^5, macrophage: neutrophil ratio 1:5). Co-cultures were incubated for 30 min and processed for CFU quantification and preparation of supernatants. IFN-γ and serum concentrations were maintained throughout the experimental process. Supernatants were analysed for cytokines using the magnetic Luminex Screening kit as described in Chapter 2.20. PA: *P. aeruginosa*, Mac: Macrophages, Neut: Neutrophils. Results from 4 different supernatants obtained from four independent experimental repeats. Data analysed using one-way ANOVA. * = $p<0.05$.

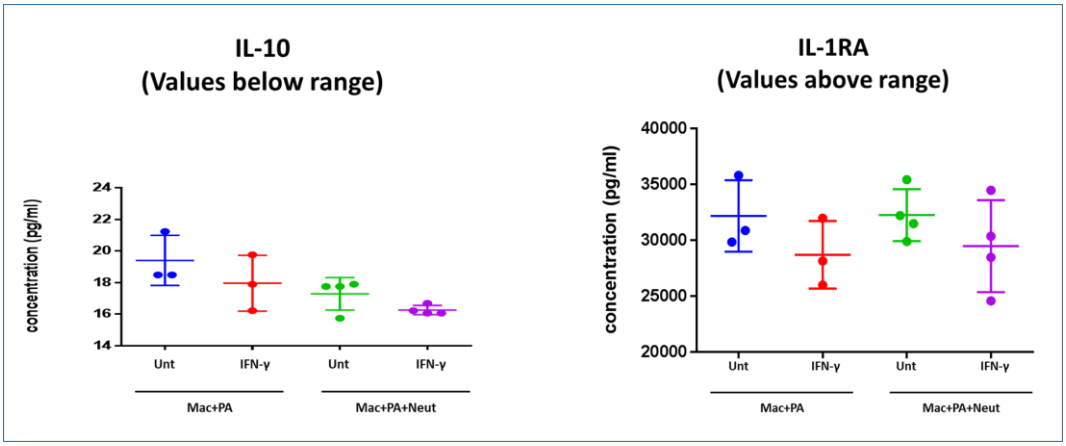


Figure 4.4: Effect of IFN-γ on cytokine production (IL-10 levels were below while IL-1RA levels were above the range of the standard of the cytokine assay).
Supernatants obtained from the experiment described above in Figure 4.3 were analysed for cytokines as described in Chapter 2.20. The levels of the anti-inflammatory cytokines (IL-10 and IL-1RA) could not be considered because IL-10 levels were below the standard of the assay while there were high amounts of IL-1RA which were above the standard of the assay. This figure is only to show the presence of these cytokines even though no conclusions can be made since they were out of the range of the standard of the assay. PA: *P. aeruginosa*, Mac: macrophages, Neut: neutrophils. Results from 4 different supernatants obtained from four independent experimental repeats.

4.3.4 Effect of IFN-γ on the ability of neutrophils to reduce PA growth

To determine if IFN-γ could have a direct effect on the ability of human neutrophils to kill PA, human neutrophils were pre-treated with IFN-γ for 30 min and infected with PA at MOI 5 as described in Chapter 2.21. The results showed no significant effect of IFN-γ on the ability of neutrophil cultures to control PA infection when assessed as total CFU (Figure 4.5, A) or percentage of bacteria remaining (Figure 4.5, B). The growth of PA was not directly affected by the presence of IFN-γ (Figure 4.5, A, compare blue and green data sets). These results suggest that, IFN-γ did not have a direct effect on neutrophil-mediated killing of PA.

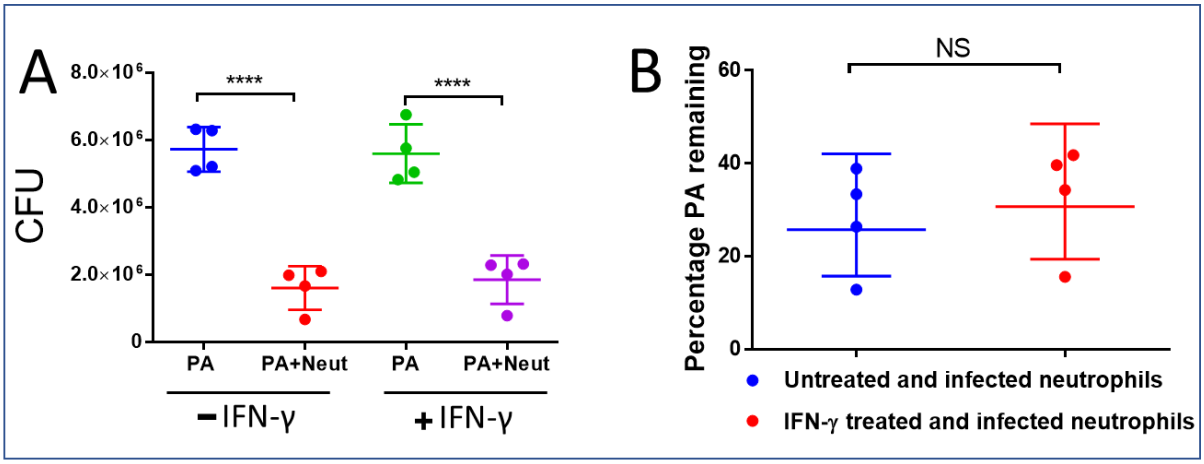


Figure 4.5: Effect of IFN-γ on the ability of human neutrophils to control PA.
Purified human neutrophils were either untreated or pre-treated for 30 min with IFN-γ and infected with PA at MOI 5, under opsonic conditions and incubated for a further 30 min at 37ºC at 8 rpm . Opsonised PA only and opsonised PA with IFN-γ cultures were used as controls. IFN-γ and serum concentrations were maintained throughout the experimental process. After 30 min cultures were processed for CFU analysis. **(A):** Effect of IFN-γ on the total bacterial CFU and **(B):** Percentage of bacteria remaining in infected neutrophils compared to bacteria only cultures. PA: *P. aeruginosa*, PMNs: neutrophils. Results from four independent repeats. Data analysed using one-way ANOVA **(A)** or Paired two-tailed t-test **(B)**. **** = p<0.0001, NS = Not significant.

4.3.5 Effect of IFN-γ with or without GM-CSF on the release of neutrophil elastase by neutrophils infected or uninfected with PA

To test if IFN-γ in the presence and absence of GM-CSF had a direct effect on the release of neutrophil elastase by uninfected and PA infected neutrophils, supernatants obtained from a modification of the experiment described in Chapter 2.21 were used. The modification of this experiment included incubating neutrophils either untreated or treated with only IFN-γ, or IFN-γ + GM-CSF, for 30 min at 37ºC at 8 rpm . These three sets of neutrophils were either left uninfected or infected with PA and further incubated for 30 min at 37ºC at 8 rpm . Supernatants were processed and were investigated for the release of neutrophil elastase following the procedure described in Chapter 2.19. The idea behind this experimental modification was to mimic the co-culture model where macrophages were grown in the presence of GM-CSF to see how the combination of these two cytokines (IFN-γ and GM-CSF) affected neutrophil control of PA. Also, the uninfected sets served as a control. As shown in

Figure 4.6, the release of neutrophil elastase was significantly increased in all cases by infections with PA (i.e. all the uninfected sets versus all the infected sets). However, there was no significant difference among the uninfected groups (i.e. untreated versus IFN-γ treated versus IFN-γ + GM-CSF treated) or among the infected groups (i.e. untreated versus IFN-γ treated versus IFN-γ + GM-CSF treated).

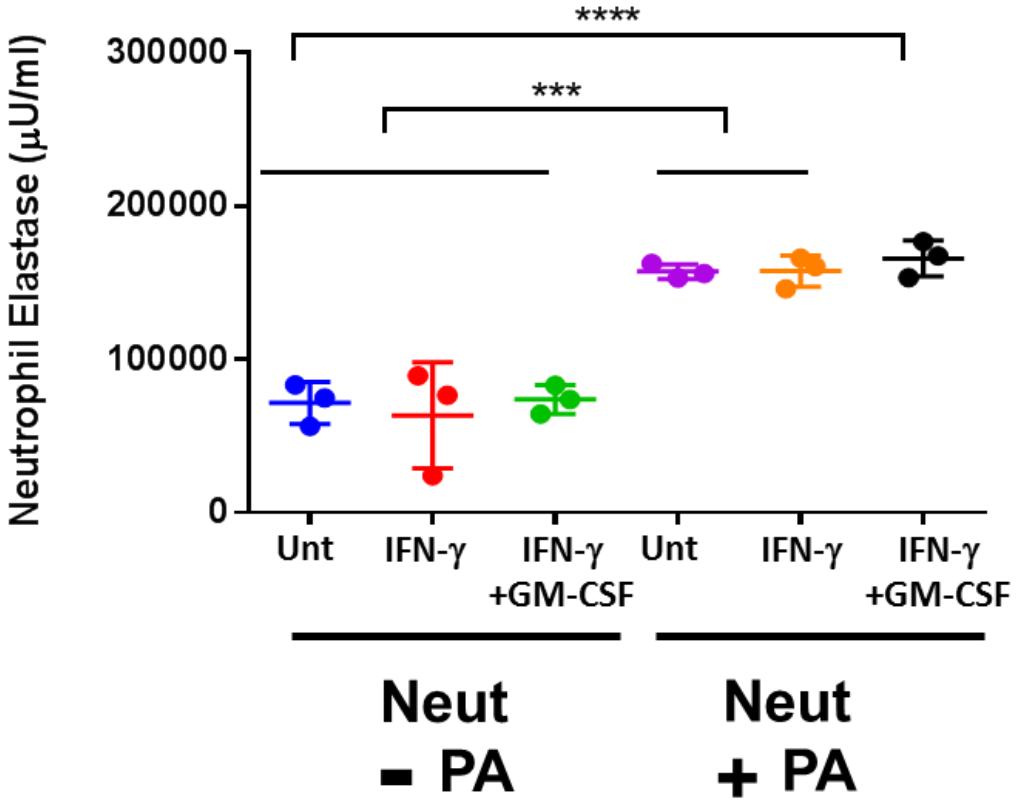


Figure 4.6: The release of neutrophil elastase is significantly increased by infecting neutrophils with PA.
Purified human neutrophils were either left untreated (Unt) or pre-treated for 30 min with IFN-γ or IFN-γ + GM-CSF and left uninfected or infected with PA at MOI 5 under opsonic conditions. IFN-γ, GM-CSF and serum concentrations were maintained throughout the experimental process. After 30 min samples were obtained for supernatants. Supernatants were tested for the presence of neutrophil elastase following the manufacturer's protocol.. PA: *P. aeruginosa*; Mac: macrophages; Neut: neutrophils. Results from three independent repeats. Data analysed using one-way ANOVA. *** = $p<0.001$, **** = $p<0.0001$.

4.3.6 IL-17A increased neutrophil-mediated killing of PA and induces morphological changes in PA-infected neutrophils

The MSc. project performed by Afrakoma Afriyie-Asante under my supervision tested the effect of IL-17A on neutrophil-mediated killing of PA. The procedure for this experiment is detailed in Chapter 2.21. Briefly, neutrophils were first primed by incubating for 30 min at 37ºC at 8 rpm in the presence or absence of IL-17A. These two sets of neutrophils were infected with PA under opsonic conditions (10% NHS), and we had conditions of opsonised PA only control and opsonised PA with IL-17A. These four sets were incubated for 30 min at 37ºC at 8 rpm and samples processed for CFU analysis (see Chapter 2.18). The results show that there was a significant reduction in PA CFU by the addition of neutrophils both in the presence and absence of IL-17A (Figure 4.7, A). In contrast to IFN-γ, there was a higher reduction in PA CFU when neutrophils were treated with IL-17A (Figure 4.7, C). Also, there was no significant effect on PA viability in the presence of IL-17A (i.e., PA-IL17A versus PA+IL-17A). These results indicated that IL-17A may have a direct effect on neutrophil-mediated killing of PA and that IL-17A did not have a direct cytotoxic effect on PA.

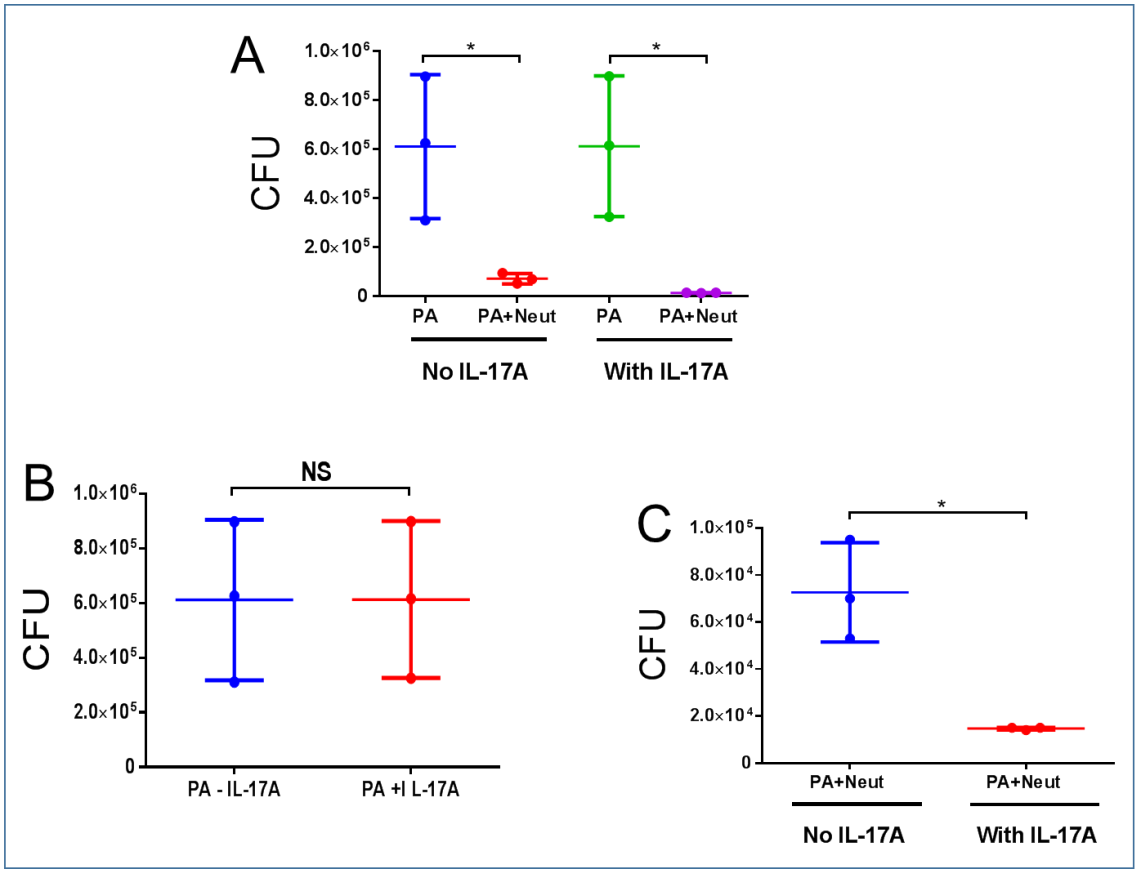


Figure 4.7: Increased reduction in PA CFU by neutrophils infected in the presence of IL-17A.
Purified human neutrophils were either left untreated or pre-treated for 30 min with IL-17A and infected with PA at MOI 5 under opsonic conditions and incubated for a further 30 min at 37ºC at 8 rpm . There were opsonised PA only and opsonised PA with IL-17A controls. IL-17A and serum concentrations were maintained throughout the experimental process. After the 30 min cultures were processed for CFU analysis. **(A)**: Effect of IL-17A on PA growth and on neutrophil-mediated killing of PA, **A is split into B and C**. **(B)**: The effect of IL-17A on PA growth. **(C):** The effect of neutrophil-mediated killing of PA. PA: *P. aeruginosa*, Neut: neutrophils. Results from three independent repeats. Data analysed using one-way ANOVA **(A)** or Paired two-tailed t-test **(B and C)**. * = $p<0.05$, NS = Not significant.

Furthermore, samples from these experiments were prepared for cytospins as described in Chapter 2.14. Samples were then stained with Hemacolor modified protocol (see Chapter 0) and examined under a bright field inverted microscope. The results show that there was no significant morphological changes in the neutrophils in the uninfected state with IL-17A treatment (

Figure 4.8), implying that IL-17A per se did not have a major influence on the neutrophil morphology.

Infection of the neutrophils with PA in the presence and absence of IL-17A changed the morphology of the neutrophils (Figure 4.9, A versus B). For clarity, Figure 4.9, A and B were magnified into Figure 4.10, A and B, which shows the differences in the morphology of infected neutrophils in the presence and absence of IL-17A.

The infected neutrophils without IL-17A treatment (Figure 4.10, A) showed some degree of phagocytosis of PA. There was also some degree of neutrophils clumping in this condition and some adherence of PA to the neutrophils. The characteristic segmented form of the neutrophil nuclei was also altered with some degree of cell damage (Figure 4.10, A). In contrast, infected neutrophils with IL-17A treatment (Figure 4.10, B) seemed to show more PA being phagocytosed by neutrophils. There was again some degree of alteration in the neutrophil nuclei with more neutrophils having disrupted cell membranes. More of the neutrophils appeared damaged which might be an indication of an elevated inflammatory response to PA. These features were evident in all three independent experimental repeats.

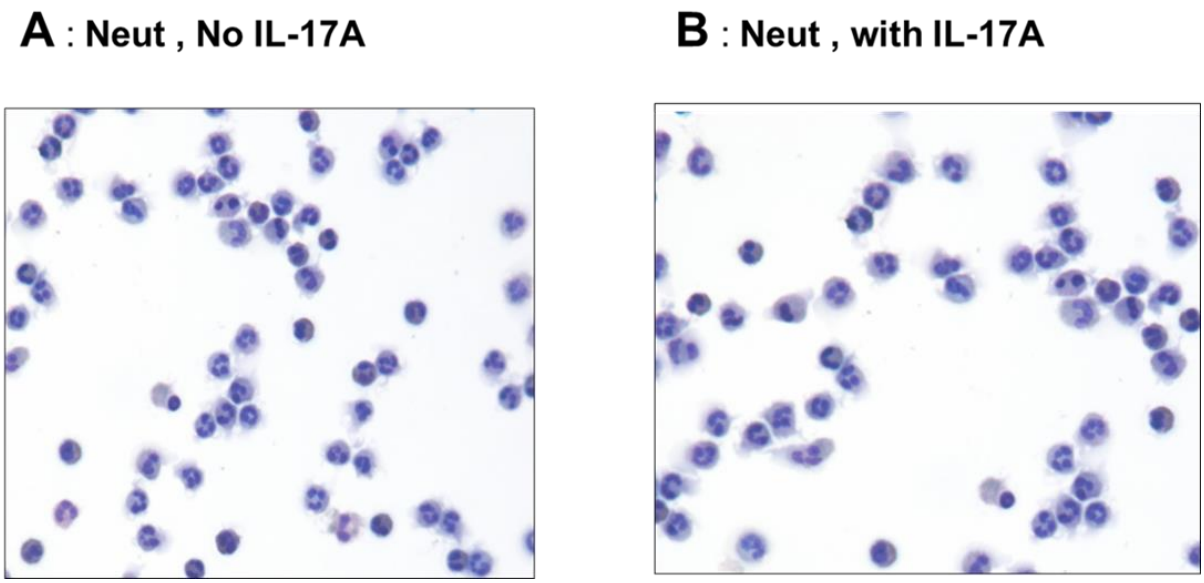


Figure 4.8: No obvious morphological changes in uninfected neutrophils with IL-17A treatment.
Purified human neutrophils were either untreated **(A)** or pre-treated for 30 min with IL-17A **(B)** at 37ºC at 8 rpm under opsonic conditions. The cells were fixed with 1% paraformaldehyde, cytospins prepared and stained with Hemacolor modified protocol. Slides were examined at X40 with a bright field inverted microscope.

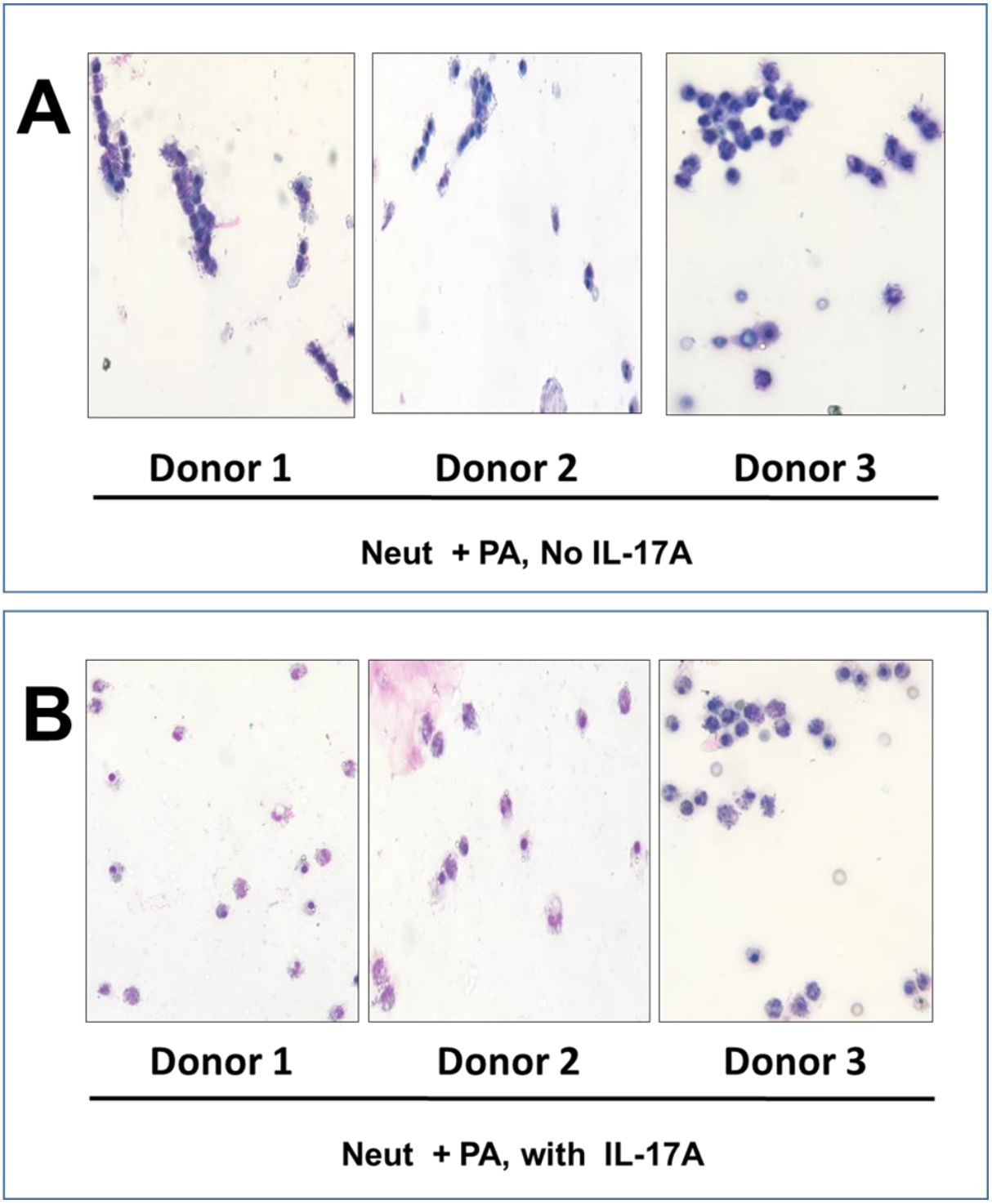


Figure 4.9: Morphological changes in infected neutrophils with or without IL-17A. Purified human neutrophils were either untreated or pre-treated for 30 min with IL-17A at 37ºC at 8 rpm and infected with PA at MOI 5 under opsonic conditions. Samples were incubated further for 30 min at 37ºC at 8 rpm. The cells were fixed with 1% paraformaldehyde and cytospins prepared and stained with Hemacolor modified protocol. Slides were examined at X40 with a bright field inverted microscope. **(A):** Represents infected neutrophils without IL-17A and **(B):** Represents infected neutrophils with IL-17A. **Figure 4.9** is electronically magnified into **Figure 4.10** for clarity. The images are from three independent experimental repeats.

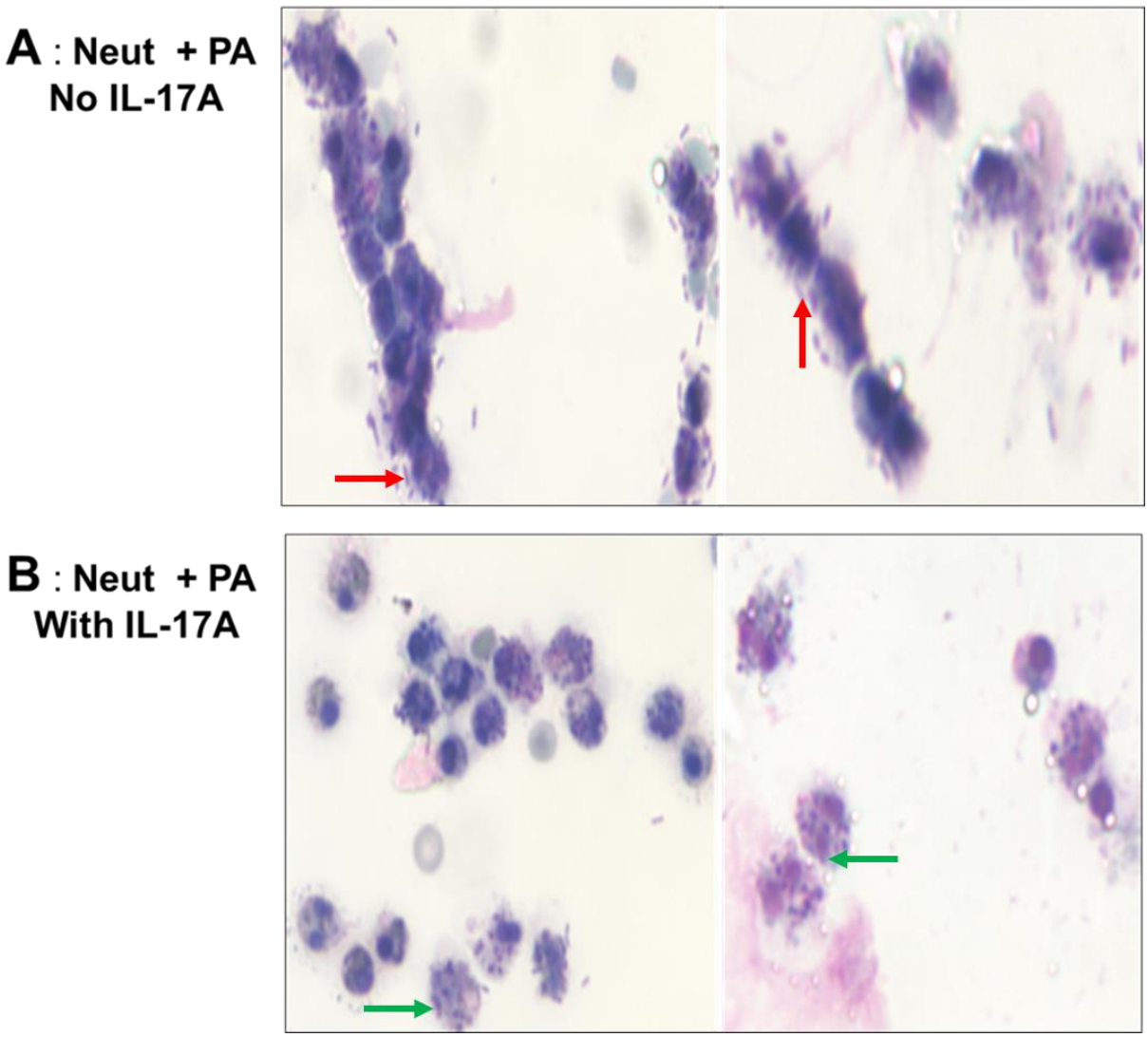


Figure 4.10: Electronically magnified view of the morphological changes in infected neutrophils with or without IL-17A.
Purified human neutrophils were either untreated or pre-treated for 30 min with IL-17A at 37ºC at 8 rpm and infected with PA at MOI 5 under opsonic conditions. Samples were incubated further for 30 min at 37ºC at 8 rpm. The cells were fixed with 1% paraformaldehyde and cytospins prepared and stained with Hemacolor modified protocol. Slides were examined at X40 with a bright field inverted microscope. displayed as infected neutrophils without IL-17A **(A)** is an electronic magnification of **Figure 4.9, A** above, representing infected neutrophils without IL-17A and **(B)** is an electronic magnification of **Figure 4.9, B** above, representing infected neutrophils with IL-17A**.** Red arrows point to adhered bacteria and green arrows point to phagocytosed bacteria.

4.3.7 Effect of IL-17A on the ability of macrophage/neutrophil co-cultures to clear PA infections

With the purpose of assessing the effect of IL-17A on the ability of macrophage/neutrophil co-cultures to clear PA, macrophages untreated or pre-treated with IL-17A for 24 h were infected with PA under opsonic conditions for 2 h at which time neutrophils were added with the macrophage: neutrophil ratio being 1:5, samples were obtained at 2 hpi for analysis of CFU and supernatants. Neutrophils were added 2 hpi and cultures incubated further for 30 min. Samples were obtained again 2.5 hpi for CFU analysis and supernatants (see Chapter 2.17 and Figure 2.2). Results from these experiments show that IL-17A did not have a significant effect on the ability of macrophages to clear PA infection 2 hpi as shown by the total CFU values (Figure 4.11 A, blue graphs). On the other hand, IL-17A had a marginal effect on promoting PA clearance by the macrophage/neutrophil co-cultures at 2.5 hpi as indicated by the total CFU values (Figure 4.11, A) and the percentage of remaining bacteria (Figure 4.11, B).

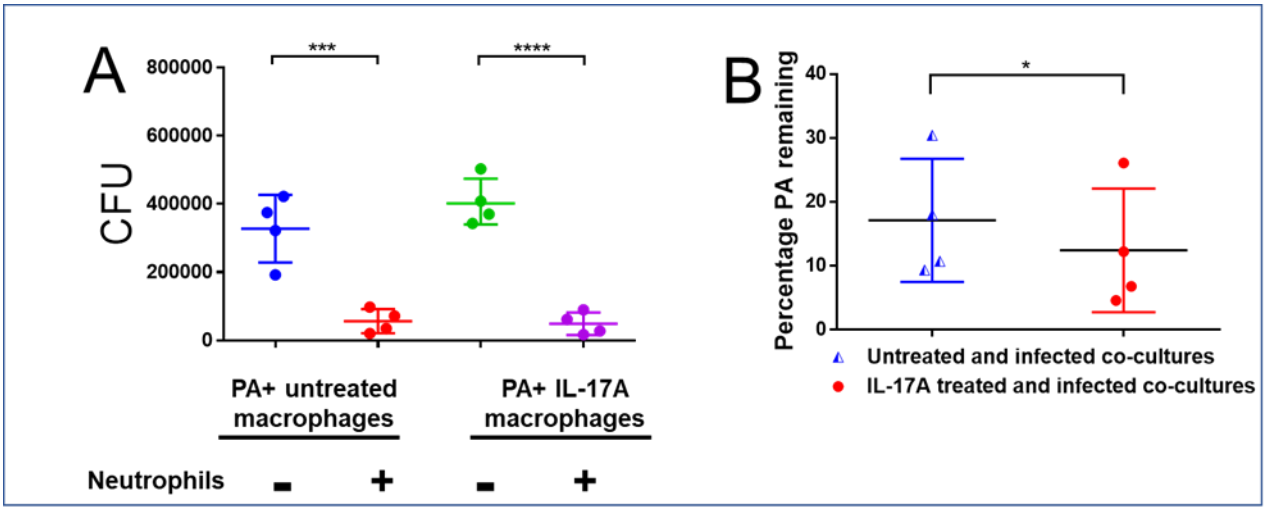


Figure 4.11: Effect of IL-17A on the ability of macrophage/neutrophil co-cultures to control PA growth.

Human macrophages (2 x10^5) generated in the presence of GM-CSF were left untreated or treated at day 6 with IL-17A. On the following day, macrophages were collected and infected with PA at MOI 1 in the presence of human serum. At 2 hpi, half of the culture was collected and processed for CFU quantification and preparation of supernatants and freshly purified human neutrophils were added (5 x10^5, macrophage: neutrophil ratio = 1:5). Co-cultures were incubated for 30 min and processed for CFU quantification and preparation of supernatants. IL-17A and serum concentrations were maintained throughout the experimental process.
(A): Effect of IL-17A on the ability of macrophages and macrophage/neutrophil co-cultures to control PA growth. **(B):** Effect of IL-17A on the percentage of PA remaining in the macrophage/neutrophil co-cultures (2.5 hpi) compared to the macrophage only cultures (2 hpi). PA: *P. aeruginosa*, Mac: macrophages, Neut: neutrophils. Results from four independent repeats. Data analysed using paired two-tailed t-test. * = $p<0.05$, *** = $p<0.001$, **** = $p<0.0001$, NS = Not significant.

4.3.8 Effect of IL-17A on the release of neutrophil elastase by macrophage/neutrophil co-cultures infected with PA

To assess the ability of IL-17A to modulate the release of neutrophil elastase by macrophage/neutrophil co-cultures infected with PA, supernatants obtained from the experiment described in Chapter 4.3.7, Figure 4.11 above were tested for the release of neutrophil elastase following the method described in Chapter 2.19. The results show that, there was no release of neutrophil elastase by infected macrophages only (2 hpi) either untreated or treated with IL-17A (Figure 4.12). However, infected macrophage/neutrophil co-cultures (2.5 hpi) released neutrophil elastase but the presence of IL-17A did not have a significant effect on the amount of neutrophil elastase released (Figure 4.12). Thus IL-17A did not influence the release of neutrophil elastase under our experimental conditions.

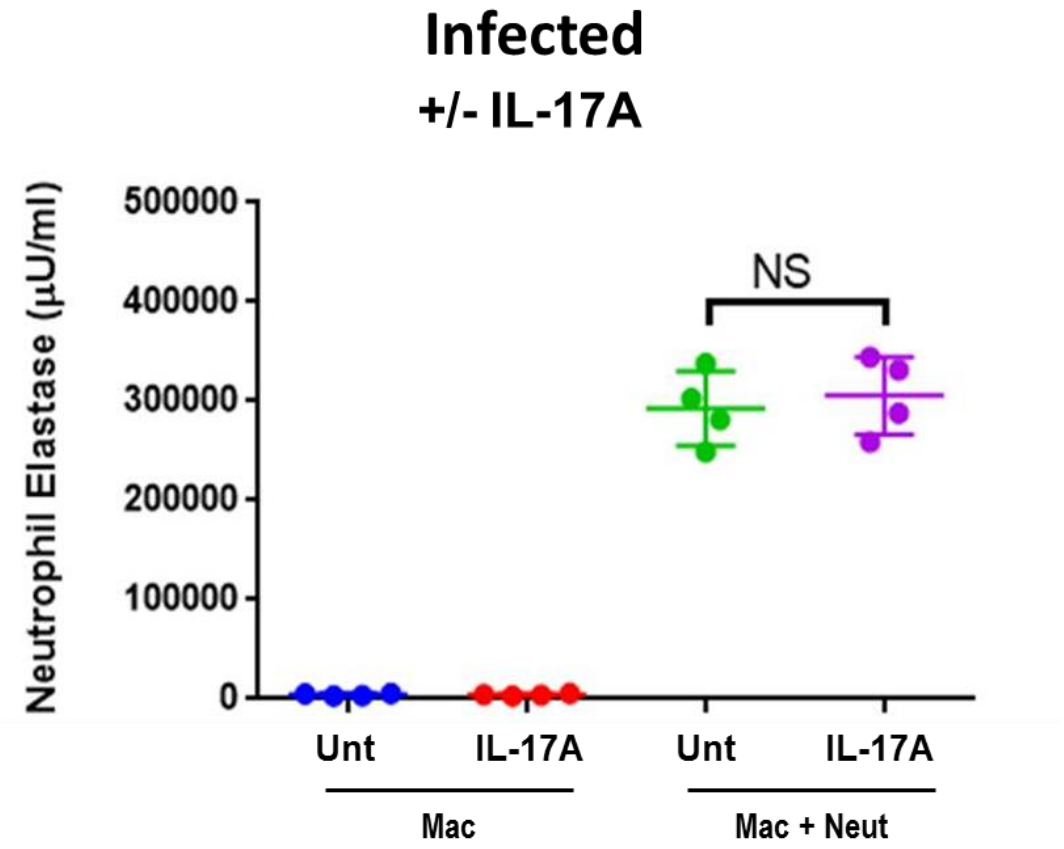


Figure 4.12: Effect of IL-17A on the ability of macrophage/neutrophil co-cultures to release neutrophil elastase.
Human macrophages (2 x10^5) generated in the presence of GM-CSF were left untreated (Unt.) or treated at day 6 with IL-17A. On the following day, macrophages were collected and infected with PA at MOI 1 in presence of human serum. At 2 hpi half of the culture was collected and processed for CFU quantification and preparation of supernatants and freshly purified human neutrophils were added (5 x10^5, macrophage: neutrophil ratio = 1:5). Co-cultures were incubated for 30 min and processed for CFU quantification and preparation of supernatants. IL-17A and serum concentrations were maintained throughout the experimental procedure. Supernatants from PA-infected macrophage only (2 hpi) and macrophage/neutrophil co-cultures (2.5 hpi) were tested for the presence of neutrophil elastase following the manufacturer's protocol as described in Chapter 2.19.. Mac: macrophages; Neut: neutrophils. Results from four independent repeats. Data analysed using one-way ANOVA. NS = Not significant.

4.3.9 Effect of IL-17A on cytokine production by macrophage/neutrophil co-cultures infected with PA

To assess the effect of IL-17A on the ability of infected human macrophage/neutrophil co-cultures to promote or hinder inflammation, supernatants from the experiment described above in Chapter 4.3.7, Figure 4.11 were tested for the presence of various pro-inflammatory and anti-inflammatory cytokines following the procedure described in Chapter 2.20. Precisely, supernatants from infected macrophages only (2 hpi) and macrophage/neutrophil co-cultures (2.5 hpi) were analysed for the presence of IL-1β, IL-1α, MIP-1α, MCP-1, IL-8, TNF-α, IL-10 and IL-1RA (Figure 4.13 and Figure 4.14).

The results show that, IL-17A did not have a significant effect on the production of most of the cytokines tested both in the case of infected macrophages only (2 hpi) and infected macrophage/neutrophil co-cultures (2.5 hpi). IL-8 produced by infected macrophages only (2 hpi) was reduced in both the untreated and IL-17A treated macrophage/neutrophil co-cultures (2.5 hpi) (Figure 4.13 IL-8 graph) most likely due to the dilution of supernatants at 2 hpi by the addition of neutrophils. There was no significant effect on IL-1β, IL-1α, MIP-1α, MCP-1 and TNF-α production (Figure 4.13). Meanwhile, the increase in IL-1 β with the addition of neutrophils to the macrophage cultures in untreated conditions was not observed in the IL-17A group in contrast with the effect seen in the IFN-γ group (note IL-1β graphs in Figure 4.3 versus Figure 4.13). These differences might be due to variations among the different donor groups used for these assays which led to a decision to compare the effect of these two cytokines (IFN-γ and IL-17A) side by side in the same donor groups (see Chapter 5).

IL-1RA levels were again above the range of the standard of the assay in all cases while IL-10 levels were below the range of the standard of the assay (Figure 4.14).

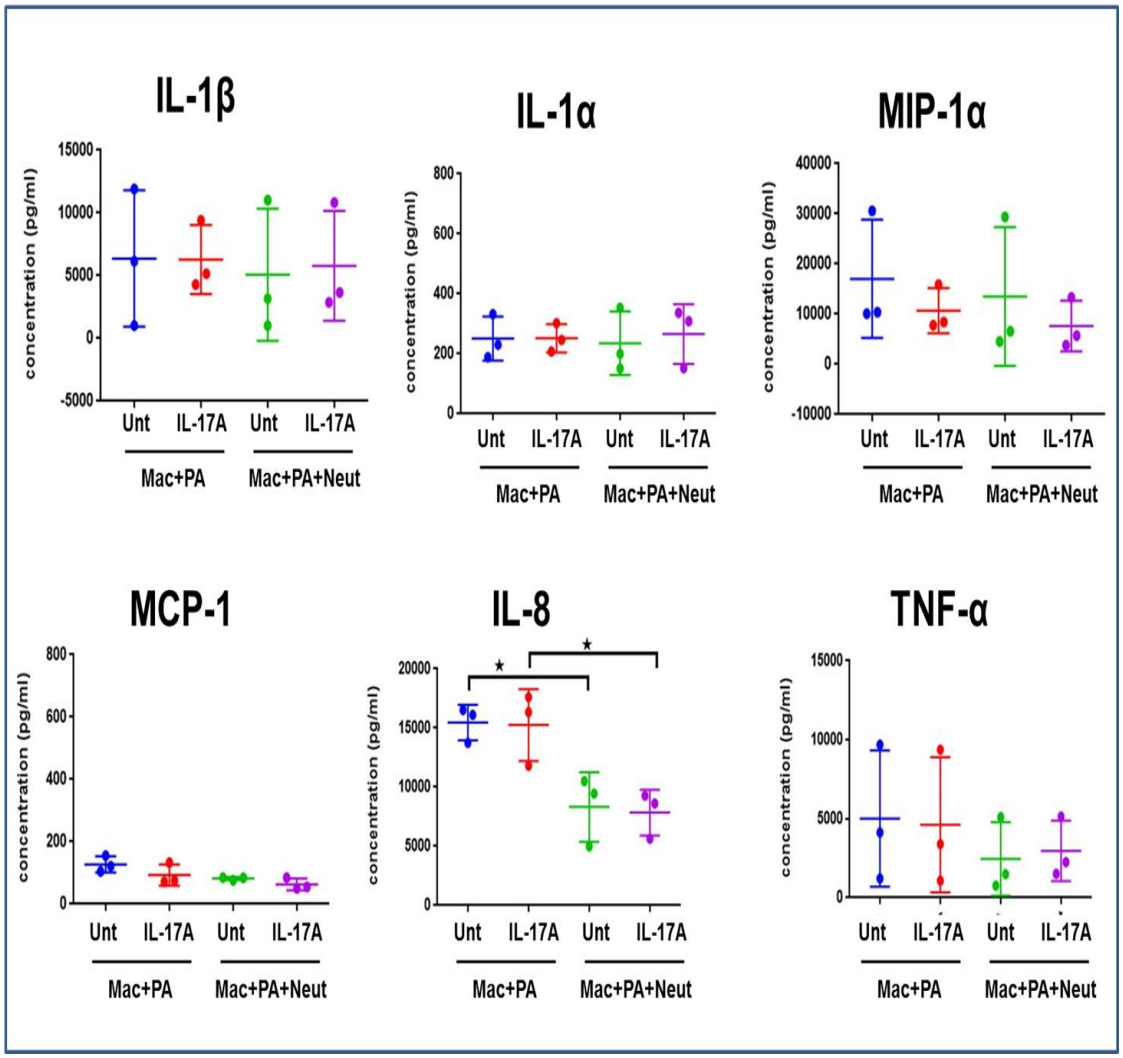


Figure 4.13: Effect of IL-17A on cytokine production by macrophage/neutrophil co-cultures infections of PA.
Human macrophages (2 x10^5) generated in the presence of GM-CSF were left untreated (Unt) or treated at day 6 with IL-17A. On the following day, macrophages were collected and infected with PA at MOI 1 under opsonic conditions. At 2 hpi half of the culture was collected and processed for CFU quantification and preparation of supernatants, and freshly purified human neutrophils were added (5 x 10^5, macrophage: neutrophil ratio = 1:5). Co-cultures were incubated for 30 min and processed for CFU quantification and preparation of supernatants. IL-17A and serum concentrations were maintained throughout the experimental process. Supernatants were analysed for cytokines following the manufacturer's protocol.. PA: *P. aeruginosa*, Mac: Macrophages, Neut: Neutrophils. Results from supernatants obtained from four independent experimental repeats. Data analysed using one-way ANOVA. * = $p<0.05$.

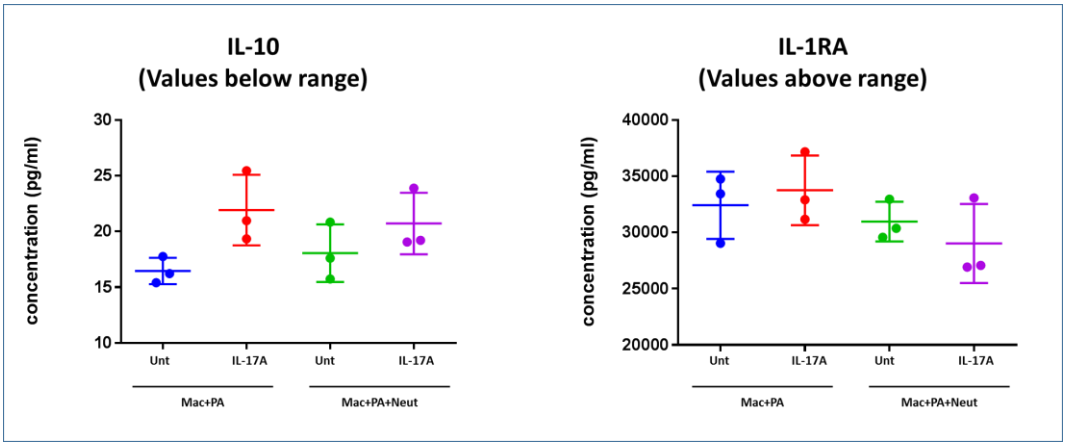


Figure 4.14: Effect of IL-17A on cytokine production by macrophage/neutrophil co-culture infections with PA (IL-10 levels were below while IL-1RA levels were above the range of the standard of the cytokine assay).
Supernatants obtained from the experiment described above in Figure 4.13 were analysed for cytokines. The levels of IL-10 and IL-1RA could not be considered because, IL-10 levels were below the limit of the standard of the assay while levels of IL-1RA were above the standard. This figure is only to demonstrate the presence of these cytokines even though no conclusions can be made since they were out of the range of the standards of the assay. PA: *P. aeruginosa*, Mac: macrophages, Neut: neutrophils. Results from 4 different supernatants obtained from four independent experimental repeats.

4.3.10 Effect of IL-17A on the ability of neutrophils to reduce PA growth

To determine if IL-17A could have an effect on the ability of human neutrophils to kill PA, human neutrophils were pre-treated with IL-17A for 30 min and infected with PA at MOI 5 under opsonic conditions as described in Chapter 2.21. In agreement with the co-culture results and the initial findings of the MSc. project by Afrakoma Afriyie-Asante which I supervised, IL-17A promoted bacteria clearance by neutrophils as shown by the total CFU values (Figure 4.15, A) and percentage of bacteria remaining (Figure 4.15, B). The growth of PA was not directly affected by the presence of IL-17A (Figure 4.15, A). These results further support a role for IL-17A in promoting bacteria killing probably through a direct action on human neutrophils.

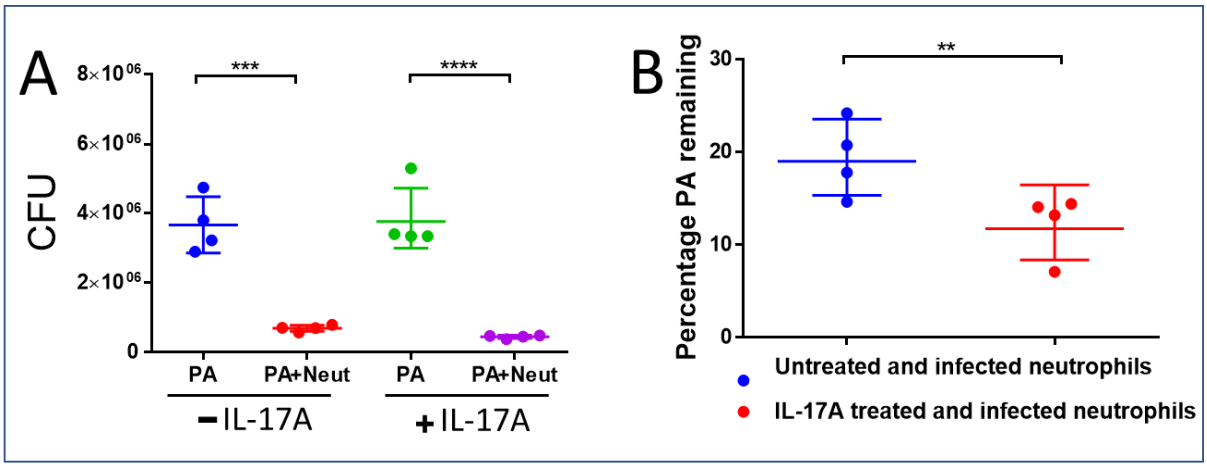


Figure 4.15: IL-17A has a direct effect on the ability of human neutrophils to clear PA. Purified human neutrophils were either left untreated or pre-treated for 30 min with IL-17A and infected with PA at MOI 5 under opsonic conditions. Cytokine and serum concentrations were maintained throughout the experimental process. After 30 min cultures were processed for CFU analysis. **(A):** The effect of IL-17A on PA only and on neutrophil-mediated killing of PA as measured by total CFU. **(B)**: The effect of IL-17A on neutrophil-mediated killing of PA as measured by the percentage of bacteria remaining in the infected neutrophil cultures compared to bacteria only cultures . PA: *P. aeruginosa*, Neut: neutrophils. Results from four independent repeats. Data analysed using one-way ANOVA **(A)** or Paired two-tailed t-test **(B)**.

4.3.11 Effect of IL-17A with or without GM-CSF on the release of neutrophil elastase by neutrophils infected or uninfected with PA

To test if IL-17A in the presence and absence of GM-CSF had a direct effect on modulating the release of neutrophil elastase by uninfected and PA infected neutrophils, supernatants obtained from an amendment of the experiment described in Chapter 2.21 were used. The amendment of this experiment included incubating neutrophils with either only IL-17A, or IL-17A + GM-CSF, for 30 min at 37ºC at 8 rpm. These three groups of neutrophils (untreated, IL-17A-treated and IL-17A+GM-CSF-treated) were either left uninfected or infected with PA and further incubated for 30 min at 37ºC, 8 rpm. Supernatants were processed as described in Chapter 2.18 and investigated for the release of neutrophil elastase following the procedure described in materials and methods (Chapter 2.19). The idea behind this experimental modification was to mimic the co-culture model where macrophages were grown in the presence of GM-CSF to see whether the combination of these two cytokines (IL-17A and GM-CSF) affected neutrophil control of PA. Also, the uninfected sets served as a control.

The results show that, the release of neutrophil elastase was significantly increased in all cases by infections with PA (i.e. all the uninfected groups versus all the infected groups) (Figure 4.16). However, there was no significant difference among the uninfected groups (i.e. untreated versus IL-17A treated versus IL-17A + GM-CSF treated) and also no differences among the infected groups (i.e. untreated versus IL-17A treated versus IL-17A + GM-CSF treated) (Figure 4.16).

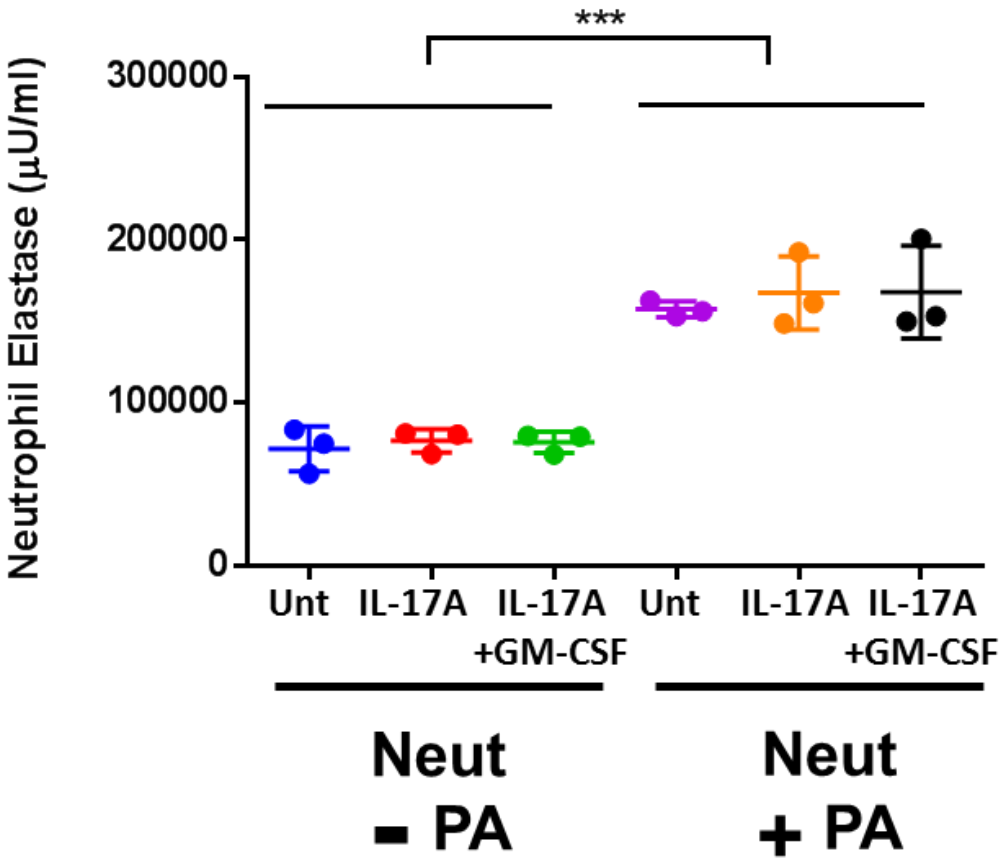


Figure 4.16: Effect of IL-17A with or without GM-CSF on the release of neutrophil elastase by infected and uninfected human neutrophils.
Purified human neutrophils were either untreated (Unt) or pre-treated for 30 min with IL-17A or IL-17A + GM-CSF and left uninfected or infected with PA at MOI 5 under opsonic conditions. IL-17A, GM-CSF and serum concentrations were maintained throughout the experimental process. After 30 min samples were obtained for supernatants. Supernatants were tested for the presence of neutrophil elastase following the manufacturer's protocol as described in materials and methods Chapter 2.19.. PA: *P. aeruginosa*; Mac: macrophages; Neut: neutrophils. Results from three independent repeats. Data analysed using one-way ANOVA. *** = $p<0.001$.

4.4 Discussion

Macrophages and neutrophils share special features which allow them to cross-talk for an effective immune response to invading pathogens (Silva, 2010b). Macrophages and neutrophils differentiate from a common myeloid progenitor cell (Iwasaki *et al.*, 2007) which is in agreement with the fact that both share a number of common characteristics. These characteristics include, phagocytosis (Nauseef, 2007) (although neutrophils are more phagocytic than macrophages (Silva *et al.*, 2012)), gene expression (such as PU.1 and SP1 genes) (Clarke *et al.*, 1998; Friedman, 2002), production of some cytokines (e.g. TNF-α and IL-1β) and chemokines (e.g. IL-8 and MIP-1α) (Cassatella, 1999; Kasama *et al.*, 2005; Tecchio *et al.*, 2014), expression of receptors on their surfaces specific for complement and immunoglobulins (such as Fc receptor I, Fc receptor II and Fc receptor III) (Dale *et al.*, 2008) and PRRs (Janeway, 1989). Macrophages and neutrophils also develop unique characteristics during their maturation process which give them the discrete specialised properties that complement their similarities (Friedman, 2002). Resident macrophages, many of which have an embryonic origin (Epelman *et al.*, 2014; Lavin *et al.*, 2015) and are capable of self-replication have long half-lives and are widely distributed in tissues to guard against invading microorganisms (Silva, 2010b). They are also less cytotoxic in their antimicrobial activity which is in agreement with their role as resident macrophages (Silva, 2010b). Indeed, tissue-resident macrophages are considered to have more of 'M2-like' phenotype and they play critical roles in tissue homeostasis (Davies *et al.*, 2013). The activation of M2-like phenotype involves the effect of mediators like M-CSF, TGF-β, glucocorticoids, IL-4 and IL-13 (Davies *et al.*, 2013). Thus similarities between 'M2-like' macrophages and resident macrophages might be due to the *in vivo* abundance of M-CSF and colony stimulating factor 1 receptor (CSF1R) (Davies *et al.*, 2013). On the other hand, neutrophils have a shorter life-span, are more cytotoxic in their antimicrobial activity which is why they are excluded from normal tissues and are stored in the bone marrow as resting cells and in the blood and only recruited when needed at infection sites (Silva, 2010b). Blood monocytes differentiate into

inflammatory macrophages during infection and in some instances give rise to resident tissue macrophages capable of self-replication (Silva, 2010b).

Macrophages and neutrophils cross-talk to promote defence against various pathogens (Silva, 2010b). Resident macrophages respond to invading pathogens and recruit neutrophils to infection sites by releasing a number of cytokines and chemokines to aid them in antimicrobial effector mechanisms. For instance, when macrophages and DCs encounter an invading pathogen, they secrete mediators such as IL-8, CXCL1/2/3, MCP-1 and MIP-1α (Cailhier *et al.*, 2005; De Filippo *et al.*, 2008), to attract neutrophils and monocytes to infection sites. The neutrophil response to the infection leads to the release of pro-inflammatory cytokines and chemokines which lead to the attraction of more macrophages and large numbers of neutrophils to the infection sites (Silva, 2010b; Sadik *et al.*, 2011; Tecchio *et al.*, 2014). This consequently amplifies the immune response to the invading pathogen (Silva, 2010b)..

The cooperation between macrophages and neutrophils can also serve as anti- inflammatory when macrophages take up apoptotic neutrophils (Silva, 2011). Some of the mechanisms that enhance the phagocytosis of apoptotic neutrophils by macrophages include the secretion of 'find me' signals (such as thrombospondin 1 and lysophosphatidylcholine) by apoptotic neutrophils which in turn attract macrophages (Munoz *et al.*, 2010). Interestingly, studies have shown the ability of macrophages to also secrete some 'find me' signals to attract apoptotic neutrophils for phagocytosis as it is reported that activated macrophages secrete the molecule MFG-8E (Milk fat globule epidermal growth factor 8, also known as lactadherin) to attract cells undergoing apoptosis, including neutrophils, for phagocytosis (Hanayama *et al.*, 2002). This molecule (MFG-8E) binds to phosphatidylserine (PS) on apoptotic neutrophil surfaces (Borisenko *et al.*, 2004). Other mediators involved in the resolution of inflammation include annexin-1 (Scannell *et al.*, 2007), maresin 1 (Serhan *et al.*, 2009), chemerin15 (Cash *et al.*, 2010), lipoxins, resolvins and protectins (Serhan *et al.*, 2008; Maderna *et al.*, 2009) and studies have shown the ability of macrophages and neutrophils to contribute in the generation of some of these pro-resolving factors (Levy *et al.*, 2001; Serhan *et al.*, 2008).

Although macrophages are the main scavenger cells taking up apoptotic cells and cell debris (Mosser *et al.*, 2008), neutrophils may back them up in a situation where the macrophages ability to scavenge is overwhelmed (Rydell-Tormanen *et al.*, 2006). Neutrophils also help in antigen cross presentation to T-cells by presenting antigens to macrophages and DCs through their uptake of infected neutrophils and also by directly presenting antigens to T-cells (Megiovanni *et al.*, 2006) with a consequent orchestration of the adaptive immune response. The idea of neutrophils directly presenting antigens to T-cells is quite fascinating as healthy neutrophils neither constitutively express MHC complexes nor T-cell costimulatory molecules (Radsak *et al.*, 2000). There is evidence to support the induction of neutrophils to express MHC complexes and costimulatory molecules and hence their ability to present antigens to T-cells (Fanger *et al.*, 1997; Radsak *et al.*, 2000). Radsak and colleagues (Radsak *et al.*, 2000) showed the induction of MHC class II and T-cell costimulatory molecules (CD80 and CD86) when healthy neutrophils were cultured with autologous serum in the presence of IFN-γ with or without GM-CSF (Radsak *et al.*, 2000). There is also a report of MHC class II positive neutrophils been able to present to T cells the staphylococcus enterotoxin E (SEE) super antigen in an MHC class II-dependent manner (Fanger *et al.*, 1997).

At sites of infection, macrophages increase neutrophil survival by releasing inflammatory mediators such as IL-1β, TNF-α, G-CSF, and GM-CSF (Lee *et al.*, 1993; Takano *et al.*, 2009) for an effective defence against the invading microbe. Once the infection is successfully controlled, macrophages phagocytose apoptotic neutrophils to avoid their lysis and the consequent tissue damage and to initiate resolution (Silva, 2010b). Macrophage phagocytosis of apoptotic neutrophils leads to production of anti-inflammatory cytokines such as IL-10 and TGF-β, which inhibit the pro-inflammatory response (Korns *et al.*, 2011). An effective cross-talk between macrophages and neutrophils allows for this effective use of powerful but dangerous antimicrobial systems to combat invading pathogens.

Previous studies have shown that macrophages modulate the immune response to PA infections without contributing directly to PA clearance, while neutrophils are key in clearing

PA infections (Lavoie *et al.*, 2011a; Mijares *et al.*, 2011b; Singh *et al.*, 2015). Macrophages infected with PA activate TLR-4 and TLR-5 by recognition of LPS and flagellin, respectively (Raoust *et al.*, 2009) and produce cytokines and chemokines such as KC, TNF-α and IL-6 (Raoust *et al.*, 2009). These chemokines cause neutrophil recruitment to infection sites (Raoust *et al.*, 2009). Also the T3SS of PA is shown to be recognised by macrophages with a consequent activation of caspase-1 and IL-1β production through the NLRC4 inflammasome (Lavoie *et al.*, 2011c). IL-1β can in turn activate the secretion of more neutrophil chemokines by airway epithelial cells (Le Berre *et al.*, 2011).

IFN-γ has an important role in activating macrophages (Hu *et al.*, 2008) and various researchers have used low concentrations of IFN-γ in priming cells for an enhanced immune response (Hu *et al.*, 2008) to pathogens. Furthermore, IFN-γ-activated macrophages infected with PA were found to have their cytokine profile altered to a more pro-inflammatory activity (Singh *et al.*, 2015). Also, macrophages have been reported to express receptors for IL17A (such as the high affinity IL-17RA–IL-17RC complex) (Cua *et al.*, 2010) which implies that priming of macrophages with IL-17A might have an effect on their response to PA infection.

The concentrations of cytokines used for the activation of macrophages and or neutrophils was selected based on published literature and previous work in our laboratory (i.e. IFN-γ=10ng/ml (Helseth *et al.*, 1989; Singh, 2012), IL-17A=15ng/ml (Afriyie-Asante, 2014)); the supplier information sheet stated the ED_{50} of IL-17A to be 1.5-7.5ng/ml based on its ability to neutralise IL-6 induced by IL-17 from cells of NIH-3T3 mouse embryonic fibroblast (Yao *et al.*, 1995a). We aimed to use an effective concentration of IL-17A in our assay by using twice the ED_{50} (i.e. 2x7.5 = 15ng/ml) in preliminary experiments performed by Afrakoma Afriyie-Asante which I supervised. This dose was found to be functioning in our assay readout (i.e. improved PA killing by neutrophils).

To the best of our knowledge, this is the first experimental work to investigate the cooperation of human macrophages and neutrophils in response to infections of PA. However, the

cooperation of macrophages and neutrophils in response to other organisms (including intracellular and extracellular pathogens as well as fungi) have been studied. Although macrophages are considered the main effector phagocytes for intracellular pathogens (Pamer, 2008), their cooperation with neutrophils have been reported to be essential in the immune response to these pathogens. For instance, neutrophil depletion affected early innate immune response in infections of several intracellular pathogens including *L. pneumophila* (Tam *et al.*, 2008), *S. dublin* (Fierer, 2001), *B. pseudomallei* (Easton *et al.*, 2007), *C. trachomatis* (Barteneva *et al.*, 1996), *L. monocytogenes* (Zhu *et al.*, 2009), *F. tularensis* (Sjostedt *et al.*, 1994), *S. enterica serovars Typhimurium* (Seiler *et al.*, 2000) among others. This indicates an important role for neutrophils in the early immune response to these intracellular pathogens. Also the study of Martineau and his colleagues (Martineau *et al.*, 2007) showed a negative correlation between the risk of being infected with *M. tuberculosis* and neutrophil numbers in the peripheral blood using groups of individuals in contact with tuberculosis. These authors also reported the presence of antimicrobial activity against *M. tuberculosis* and *M. bovis* in healthy donor whole blood cultures and this anti-microbial activity was decreased when they used CD15 antibodies to selectively deplete neutrophils from the whole blood (Martineau *et al.*, 2007).

The possible mechanisms for the collaboration between macrophages and neutrophils in the immune response against these pathogens could be either effector functions of neutrophils or their immunomodulatory abilities (Pedrosa *et al.*, 2000; Appelberg, 2007; Easton *et al.*, 2007). For instance, neutrophils have been reported to have the ability of increasing the antimicrobial capabilities of macrophages by providing the macrophages with neutrophil antimicrobial granule molecules (Silva *et al.*, 1989; Byrd *et al.*, 1991; Sharma *et al.*, 2000). This idea of macrophage and neutrophil cooperation whereby neutrophils transfer their powerful antimicrobial granule molecules to infected macrophages was initially proposed by Silva and colleagues (Silva *et al.*, 1989) using mycobacteria-infected murine models (Silva *et al.*, 1989). They showed that at various infection sites, macrophages take up neutrophils and that these

macrophages were positive for the neutrophil granule molecule lactoferrin, especially the mycobacteria infected macrophages. This same study also reported that neutrophil granules were transferred to macrophage cultures infected with *M. microti and M. avium* which led to an increased microbicidal properties of these macrophages (Silva *et al.*, 1989).

Other researchers also showed this mechanism of macrophage and neutrophil teamwork in *L. pneumophila* (Byrd *et al.*, 1991) and again *M. tuberculosis* (Miyakawa *et al.*, 1996). Furthermore, the neutrophil granule molecule myeloperoxidase (MPO) and the Defensin Human Neutrophil Peptide-1(HNP-1) have been proposed to have a role in defence against *M. tuberculosis and M. avium* infections (Ogata *et al.*, 1992; Miyakawa *et al.*, 1996; Sharma *et al.*, 2000). Although some studies have demonstrated the direct antimicrobial effect of purified neutrophil granule molecules against various intracellular microorganisms *in vitro* (Ogata *et al.*, 1992; Miyakawa *et al.*, 1996; Sharma *et al.*, 2000; Martineau *et al.*, 2007), there are also reports of macrophages taking up these granule molecules with a subsequent increase in the macrophages antimicrobial properties (Byrd *et al.*, 1991; Sharma *et al.*, 2000).

In a study by Tan and his colleagues (Tan *et al.*, 2006) using human macrophage and neutrophil cultures, the authors showed that there was a transfer of the antimicrobial molecule HNP-1 from neutrophils that had been taken up my *M. tuberculosis* infected macrophages. They also showed that HNP-1 was located at the specific sites where *M. tuberculosis* reside in the macrophages. The presence of this peptide in the infected macrophages correlated positively with bacteria killing (Tan *et al.*, 2006). There was also a decrease in mycobacteria viability in the presence of only purified neutrophil granules (Tan *et al.*, 2006). This further confirms the previous reports (discussed above) from murine models. Thus the ability of macrophages to phagocytose neutrophils does not only serve as an anti-inflammatory process but also increases the microbicidal properties of macrophages. This implies that the macrophages do not only phagocytose apoptotic neutrophils but could also phagocytose non-apoptotic ones expressing the “eat-me” signals to increase their microbicidal activity (Dias-Baruffi *et al.*, 2003) . Indeed, the expression of phosphatidylserine (PS) which is one of the

“eat-me” signals on apoptotic neutrophils surfaces (Ravichandran, 2010) have also been shown to be expressed on activated neutrophils independent of apoptosis (Dias-Baruffi *et al.*, 2003; Stowell *et al.*, 2007; Soehnlein, 2009). Neutrophils could also have a direct effect on intracellular pathogens existing outside their host cells as is the case during transmission of intracellular pathogens from one cell to another cell or in the case of pathogens that reside extracellularly and only multiply intracellularly (Casadevall *et al.*, 1999) .

Another cooperation between macrophages and neutrophils is neutrophils ability to transfer pathogens to macrophages for elimination. Intracellular bacteria was found inside neutrophils in *in vivo* models of infections with *Salmonella* (Rydstrom *et al.*, 2007), *Mycobacteria* (Silva *et al.*, 1989; Kisich *et al.*, 2002; Abadie *et al.*, 2005), *L. pneumophila* (Davis *et al.*, 1983) and *L. monocytogenes* (Marco *et al.*, 1997), these neutrophils could transfer some intracellular pathogens they have phagocytosed already to macrophages. This can happen when the macrophages phagocytose the neutrophils and then eliminate the pathogens within the neutrophils (Kisich *et al.*, 2002; Rydstrom *et al.*, 2007).

There are also reports on macrophage and neutrophil collaboration against fungi (such as *Candida albicans (Villar et al., 2008)* and *Aspergillus fumigatus (Segal, 2007)*), and protozoa (such as *Leishmania major (Ribeiro-Gomes et al., 2004)* as well as *Trypanosoma cruzi (Lima et al., 1985))*. The study of Lima and colleague reported an increase in macrophage antimicrobial capacity against *T. cruzi* upon acquiring the neutrophil granule molecule lactoferrin (Lima *et al.*, 1985) . Others also reported an enhance macrophage antimicrobial property against *C. albicans* (Marodi *et al.*, 1998) upon attaining the neutrophil granule molecule MPO, and also against *H. capsulatum* when macrophages expressed the neutrophil granule molecule HNP-1 (Couto *et al.*, 1994).

Likewise, the collaboration between macrophages and neutrophils was also reported against extracellular pathogens through secretion of chemokines by macrophages to attract neutrophils to infection sites, direct phagocytosis and killing of extracellular bacteria and

transfer of neutrophil granules to enhance the microbicidal abilities of macrophages (Silva, 2010b).

Even though neutrophils are the main effector cells for controlling extracellular pathogens (Mayadas *et al.*, 2014) and can eliminate these bacteria either through phagocytosis, the release of their potent antimicrobial granule molecules, or by NETosis (Mayadas *et al.*, 2014) as described earlier in Chapter 1, neutrophils cooperation with macrophage has been shown to enhance the initial control of some extracellular pathogens (Thomas *et al.*, 2000). For instance, *in vitro* infections of macrophages with *S. pyogenes* caused macrophages to secrete CXCL8 (a neutrophil chemoattractant) and CCL2/3 (chemoattractants for monocytes) (Veckman *et al.*, 2003). Also, there are reports of increased susceptibility of mice to *S. aureus* (Verdrengh *et al.*, 2000) and *S. pyogenes* (Goldmann *et al.*, 2004) upon macrophage depletion.

Other researchers have also reported that, cultured macrophages have an increased antimicrobial ability against phagocytosed PA *(Mathy-Hartert et al., 1996)* and *E. coli* (Lincoln *et al.*, 1995) upon the acquisition of neutrophils granule molecule MPO.

All these reports discussed above show how imperative and unequivocal the cooperation between macrophages and neutrophils is in dealing with invading pathogens and therefore the possibility of activation of macrophages having an effect on neutrophils function.

Interestingly, our findings show that IFN-γ treatment of macrophages did not improve PA clearance (Figure 4.1). This finding is consistent with the findings of Singh and colleagues where they reported that upon infections with PA, IFN-γ activation of macrophages increased their production of MCP-1 and TNF-α but decreased IL-10 production, whereas the activation of macrophages with GM-CSF and IFN-γ increased their production of IL-6 and even more decreased IL-10 production, even though there was no increase in PA clearance or macrophage survival. Thus, human macrophages activated with IFN-γ with or without GM-

CSF were found to be more pro-inflammatory based on their cytokine profiles but still could not control PA growth or survive infection (Singh *et al.*, 2015).

It is also consistent with the work of Murphey and colleagues where IFN-γ was administered to mice after caecal ligation and punture (CLP), their data showed a more pro-inflammatory response with IFN-γ but no significant effect with PA clearance or mice survival (Murphey *et al.*, 2004). IFN-γ activation of macrophages produces classical activated macrophages also termed as M1-macrophages (Martinez *et al.*, 2014). The idea of macrophage activation was introduced by Mackaness during his experiment on secondary infections of Bacillus Calmette-Guerin (BCG) and *Listeria* which caused an increased antimicrobial capacity of macrophages (Mackaness, 1962). Later studies associated this increased macrophage response to the concept of Th-1 and IFN-γ production (Nathan *et al.*, 1983). Other researchers later proposed the alternative activated macrophage [induced by IL-4 and IL-13 (M2 activation, as mention above] which gave completely different response from the IFN-γ activated macrophages (Stein *et al.*, 1992; Doyle *et al.*, 1994) and this was associated with Th-2 responses. Current studies have expanded the knowledge on M1 and M2 macrophages and shown their link to the Th-1 and Th-2 responses respectively (Mills *et al.*, 2000). Although M1 and M2 macrophages are not mutually exclusive, their generation depend on the type of infection and the host milieu with stimuli such as IFN-γ with LPS or TNF-α inducing M1 polarisation, while IL-4, IL-13 and IL-10 inducing M2 polarization (Mantovani *et al.*, 2004) and currently GM-CSF and M-CSF for M1 and M2 polarisation respectively (Verreck *et al.*, 2004). M2 macrophages are more anti- inflammatory mostly involved in tissue repair and maintaining homeostasis while M1 macrophages are more pro-inflammatory (Hamilton, 2008).

Indeed, IFN-γ is known to be the chief cytokine on macrophage activation (Goldberg *et al.*, 1990) with its effect on increasing macrophage oxidative burst (Nathan *et al.*, 1984), pro-inflammatory cytokine production (Muhl *et al.*, 2003), MHC I and MHC II expression (Steeg *et al.*, 1982; King *et al.*, 1983), expression of Fc receptors (Guyre *et al.*, 1983) and macrophage antiviral activity (Vogel *et al.*, 1986).

IFN-γ activation of macrophages is known to be potent in controlling intracellular bacteria and these macrophages are mostly beneficial in acute infections as described in infections with *L. monocytogenes* (Shaughnessy *et al.*, 2007) , *M. tuberculosis* (Chacon-Salinas *et al.*, 2005). *S. typhimurium* and *S. typhi* (Benoit *et al.*, 2008). Benoit and partners (Pfeffer *et al.*, 1993) have also shown that IFN-γ and TNF-α (classical M1 cytokines) deficient mice died from infections with *L. monocytogenes*. However contribution of IFN-γ-activated macrophages to control of extracellular bacteria is still controversial and specifically to PA is mostly immunomodulatory (Goldmann *et al.*, 2004; Murphey *et al.*, 2004; Singh, 2012). Thus our study showing no effect on PA clearance with IFN-γ activation of macrophages was in line with other previous findings but contrasted with the study of Speert and Thorson who reported that macrophages treated with IFN-γ had a reduced ability to control PA infections under both opsonic and non-opsonic conditions (Speert *et al.*, 1991). This was attributed to a weakened Fc receptor mediated phagocytosis by the IFN-γ treated macrophages compared to untreated (Speert *et al.*, 1991). This difference with our findings might be due to differences in experimental conditions as they treated macrophages with IFN-γ from day 0 of monocyte culture and added more IFN-γ to cultures only on day 2 and day 4, but they washed the macrophages to get rid of any IFN-γ prior to infection and did not maintain IFN-γ in the infection process, while we added IFN-γ on day 6 of culture of the monocyte-derived macrophages, harvested on Day 7 and maintained cytokine concentrations throughout the experimental procedure. Considering cytokine degradation (Wright *et al.*, 1986), it is necessary to maintain the cytokine concentrations over time. Meanwhile, Speert and Thorson in the same study (Speert *et al.*, 1991) also reported an increase in superoxide anions which will be consistent with pro-inflammatory state.

Our findings also showed higher total CFU and percentage of remaining CFU in the IFN-γ treated macrophage/neutrophil co-cultures when compared to the untreated controls (Figure 4.1). Reason(s) for this observation was unclear. To the best of our knowledge, this work is the first to model macrophage/neutrophil co-cultures infections with PA and the effect of

inflammatory mediators on this model, hence there were no literature in agreement or in contrast to this finding of IFN-γ dampening macrophages and neutrophils co-cultures ability to control PA infections. It may however be due to a cumulative minor dampened effect of IFN-γ on both the macrophages and neutrophils which became obvious in the macrophage/neutrophil co-cultures. More significantly, since IFN-γ did not affect the ability of neutrophils to kill PA (Figure 4.5), these findings could be caused by IFN-γ-treated macrophages reducing neutrophil activity when in co-culture. More work will be needed to decipher the mechanisms behind this. In the meantime, studies have also shown that IFN-γ has some anti-inflammatory properties through it induction of Interleukin 1-receptor antagonist (IL-1RA), Interleukin 18 binding protein (IL-18BP), down regulation of the production of pro-inflammatory cytokines, induction of apoptosis and suppressors of cytokine signalling (SOCS) (Muhl *et al.*, 2003). Heiko and his collaborator (Muhl *et al.*, 2003) provide a detailed review on the anti-inflammatory properties of IFN-γ. Thus IFN-γ may exhibit its pro-inflammatory or anti-inflammatory properties depending on the infection conditions. It is therefore possible that, in the macrophage/neutrophil co-culture infection model in the presence of IFN-γ, IFN-γ may control the neutrophilic response by exerting some of its anti-inflammatory properties (such as decreasing pro-inflammatory cytokines production) as was seen in further studies detailed in Chapter 5.

Furthermore, IFN-γ did not affect neutrophil only mediated killing of PA except in one of the donors where there was a trend towards increased bacterial CFU in the presence of IFN-γ (Figure 4.5, B). Treatment of human neutrophils with IFN-γ has been described to enhance their ability to kill a number of microbial infections Such as *Legionellla pneumophila*, *Enterococcus faecalis*, *Mycobacterium fortuitum* and *Brucella abortus* (Blanchard *et al.*, 1989; Onyeji *et al.*, 1999; Ellis *et al.*, 2004), but the effect of human IFN-γ treatment on human neutrophils ability to clear PA is still unclear. In a study by Silvia and her colleagues, they used two strains of PA which differed in their ability to produce metalloproteases. They infected burned mice with these strains in two scenarios, firstly IFN-γ pre-treatment was followed by

infection and secondly, IFN-γ pre-treatment followed by infection with continued injection of therapeutic doses of IFN-γ throughout the infection. They observed a significant mice survival in the second scenario with only one of the strains; CF1M (which produced less than 1µg/ml of the PA endoproteases namely: alkaline protease and elastase) (Pierangeli *et al.*, 1993). The reasons for the difference in the findings of Silvia and her colleagues and the findings in this study may be the difference in infection models, experimental set up and strains of bacteria used. Besides, Silvia and her colleagues have also shown that there was no significant difference in both strains of PA in the case of pre-treatment with IFN-γ except with continues injection of therapeutic doses of IFN-γ before they could see an effect in only one of the strains. As they could not see any effect with the other strain, these findings suggest that IFN-γ may be able to enhance bacteria clearance only in certain strains of PA. Hence it may be worth testing other strains of PA using our co-culture model in future studies.

Likewise, there was no direct effect of IFN-γ on PA viability in our study. This finding contrasted with the study of Wu and colleagues which showed an enhanced PA virulence in the presence of IFN-γ. This they attributed to a direct binding of IFN-γ to the PA outer membrane protein (oprF), which resulted in the expression of PA-I Lectin as a virulence determinant (Wu *et al.*, 2005).

On the other hand, IL- 17A increased PA clearance in the macrophage/neutrophil co-cultures which was also true for the neutrophils only infections with PA (Figure 4.11 and Figure 4.15). Since IL-17A did not affect PA clearance by macrophages, this finding implies that IL-17A might have a direct potentiating effect on neutrophil-mediated killing of PA. This finding is in agreement with a previous study showing increased killing of *S. pneumoniae* by IL-17A-treated human neutrophils (Lu *et al.*, 2008).

IL-17A has been found to promote the control of extracellular bacterial infections (Yao *et al.*, 1995b). This increase in PA clearance in the presence of IL-17A might be due to the ability of IL-17A to recruit more neutrophils to the PA infection site or more significantly, the IL-17A

increasing neutrophil function. Studies have demonstrated that, IL-17A increases neutrophil numbers in bronchoalveolar space of PA infected lungs (Ye *et al.*, 2001). A study by Taylor and colleagues showed that IL-17A increased reactive oxygen species production by neutrophils infected with *A. fumigatus* (Taylor *et al.*, 2014a).

Again the presence of IL-17A is also shown to increase the release of neutrophil potent antimicrobial granule molecules such as MPO, elastase (Hoshino *et al.*, 1999), lipocalin and β defensins (Nakae *et al.*, 2002) which will enhance neutrophil killing ability. Furthermore, there are other reports of IL-17A increasing neutrophil antimicrobial capacity by the release of nitric oxide (Fierro *et al.*, 1999) and matrix metalloproteinase 9 (Prause *et al.*, 2009).

The importance of the receptor for IL-17A is demonstrated in the fact that IL-17RA deficient mice exhibit less neutrophil recruitment and reduced PA clearance in the airway (Hoshino *et al.*, 1999). As mentioned above, the IL-17A receptor is a heterodimer consisting of IL-17RA and IL-17RC (Gaffen, 2009b) subunits. Both human and murine neutrophils lack the IL-17RC subunit but express only the IL-17RA and the adaptor Act-1 (Pelletier *et al.*, 2010; Taylor *et al.*, 2014a) under unstimulated conditions. However, expression of IL-17RC could be induced by stimulation with either Dectin-2 or a combination of IL-6 with IL-23 but not the individual cytokines alone (Taylor *et al.*, 2014b). IL-17RC expression could also be enhanced further by stimulating neutrophils with hyphal extract from *A. fumigatus* and this is dependent on Dectin-2 (Taylor *et al.*, 2014a). Thus, it is possible that IL-17RC expression was augmented under our experimental conditions with a consequent increase in PA clearance by neutrophils activated through engagement of the heteromeric IL-17RA-IL-17RC complex which has higher affinity for interacting with IL-17A than just the monomeric IL-17RA (Cua *et al.*, 2010).

The microscopic examinations of neutrophils with/without IL-17A in the absence of infection showed that IL-17A did not have any obvious effect on neutrophil morphology. Interestingly, internalisation of PA by the infected neutrophils was obvious in both the presence and absence of IL-17A but more bacteria seemed to be internalised in the presence of IL-17A (Figure 4.10, B) while more bacteria were adhered but not internalised in the absence of IL-17A (Figure

4.10, A). This implies that, although the neutrophils could phagocytose PA, IL-17A might be aiding neutrophils ability to phagocytose PA. The fact that there seemed to be more bacteria being phagocytosed by the neutrophils in the presence of IL-17A (Figure 4.10, B) compared to without IL-17A (Figure 4.10, A) might explain why we observed better bacteria killing in IL-17A treated neutrophils. Thus, IL-17A might be important in promoting effective neutrophil clearance of PA infections.

On the other hand, more neutrophils had disintegrated nuclear content in the presence of IL17A which implies that more of the neutrophils were damaged in the course of clearing PA. This might have a negative consequence of increasing cytotoxicity and inflammation. Thus although IL-17A could improve PA clearance, there is also a possibility of increasing cytotoxicity and inflammation if not carefully controlled, hence the beneficial effect of IL-17A might be a compromise and will need to be controlled and considered prudently. Further work is required to extend these observations to the macrophage/neutrophil co-culture infections of PA and determine the mechanism(s) responsible. Future work involving microscopic examination of stained macrophages, neutrophils and bacteria in the presence and absence of either IFN-γ or IL-17A might give an indication of which of the cells is internalising less or more bacteria in the presence or absence of either cytokine treatment in the co-culture (see Chapter 5 for details).

It is also possible that our observation of an increase in PA clearance in the presence of IL-17A in the macrophage/neutrophil co-culture is not only a direct effect on the neutrophil-mediated killing of PA but also an increase in the macrophage activity when in co-culture with the neutrophils in the presence of IL-17A. As mentioned earlier, neutrophils can clear pathogens in various ways including phagocytosis and inside the phagosomes, neutrophils release their potent antimicrobial granule molecules such as defensins, cathepsins, lysozyme and lactoferrin (Borregaard *et al.*, 1990; Hager *et al.*, 2010). These antibacterial proteins either acts on intracellular or extracellular pathogens when released into the phagosomes or the extracellular environment respectively (Kolaczkowska *et al.*, 2013). Also, activated neutrophils can clear extracellular pathogens by releasing NETs (Kolaczkowska *et al.*, 2013). As

mentioned above, there have been reports of neutrophils transferring their microbicidal activity to macrophages by the transfer of their granule molecules (Byrd *et al.*, 1991; Sharma *et al.*, 2000). So the macrophages might have gained extra microbicidal capacity by ingesting neutrophils in the co-culture in the presence of IL-17A.

Surprisingly, our data showed that, although neutrophil elastase was released upon infections with PA in both the macrophage/neutrophil co-cultures and the neutrophils only cultures (Figure 3.16, Figure 4.2 and Figure 4.12), there was no difference in neutrophil elastase release in the presence and absence of either IFN-γ or IL-17A. The specificity of the neutrophil elastase assay was proven by the lack of neutrophil elastase detection under conditions where neutrophils were absent (Figure 3.16, Figure 4.2 and Figure 4.12). It was also clear that infections with PA increased the release of neutrophil elastase (Figure 4.6 and Figure 4.16). Neutrophil elastase is one of the neutrophil proteases released upon neutrophil defence activity. The activity of neutrophil elastase is tightly regulated by protease inhibitors during normal body functions (Travis *et al.*, 1983) but this is not the case at inflammatory sites as there is an imbalance between high levels of neutrophil elastase and the endogenous protease inhibitors (Morrison *et al.*, 1990; Kawabata *et al.*, 2002). Thus the unregulated proteolytic activity of this enzyme can lead to tissue damage. It is therefore not surprising that neutrophil elastase has been implicated in the pathophysiology of a number of conditions relating to degradation of extracellular matrix proteins including lung injury (Havemann *et al.*, 1984; Janoff, 1985). Hence, based on previous reports of IFN-γ and IL-17A correlating with better and worse lung function respectively in CF, we had expected that IL-17A will induce more neutrophil elastase activity in supernatants compared to IFN-γ, but this was not the case. Lee and colleagues (Lee *et al.*, 1981) have shown elevated levels of neutrophil elastase in bronchoalveolar lavage of patients suffering from respiratory distress syndrome using 23 adult and 55 non adult patients. In contrast, Idell and colleagues (Idell *et al.*, 1985) did not detect neutrophil elastase in bronchoalveolar lavage but both studies (Lee *et al.*, 1981; Idell *et al.*, 1985) showed elevated levels of neutrophil elastase complexed with α1-protease inhibitor.

Idell and colleagues (Idell *et al.*, 1985) also showed a strong positive correlation between these complexes and lung function. Other researchers have also shown increased levels of neutrophil elastase activity in acute lung injury and in adult patients suffering from respiratory distress syndrome (Donnelly *et al.*, 1995; Tonz *et al.*, 1995). The contrast between these findings and ours might be due to the difference in experimental models (*in vivo* versus *in vitro*) as there may be differences in the neutrophil numbers *in vivo*. Also, measuring fluid from the bronchoalveolar lavage has an added advantage of measuring neutrophil elastase released directly in local lung space (Kawabata *et al.*, 2002).

To explore other possible mechanisms behind the positive and negative correlation between IFN-γ and IL-17A respectively with lung function in CF, supernatants from the PA infected macrophage/neutrophil co-cultures in the presence and absence of either IFN-γ or IL-17A were tested for the presence of cytokines. A combination of cytokines and chemokines (including IL-1β, IL-1α, IL-1RA, TNF-α, MIP-1α, MCP-1, IL-8 and IL-10) were selected and tested in the supernatants based on their important roles in the immune response to pathogens and specifically to PA. For instance, IL-1β is a pro-inflammatory cytokine known for its importance in early immune response to PA (Skerrett *et al.*, 2007; Wangdi *et al.*, 2010). IL-1β is produced as pro-IL-1β which needs to be cleaved to the active IL-1β by caspase-1 through the activation of the inflammasome (Li *et al.*, 1995). Studies have shown that both caspase-1 and the IL-1 receptor (IL-1R) signalling are necessary for recruitment of neutrophils to infection sites *in vivo* (Wangdi *et al.*, 2010; Mijares *et al.*, 2011a). Indeed it is shown that, production of IL-1β by macrophages stimulates the production of neutrophil chemokines by epithelial cells in the airway (Mijares *et al.*, 2011a). The study of Wangdi and colleagues (Wangdi *et al.*, 2010) used anakinra which corresponds to IL-1RA to demonstrate the importance of IL-1R signalling as a requirement for early recruitment of neutrophils and the control of PA infection in transgenic animal models.

Also, production of MCP-1 in response to PA infection induces the migration of macrophage and phagocytosis in both *in vivo* and *in vitro* studies (Amano *et al.*, 2004) which eventually

increases macrophage ability to phagocytose apoptotic neutrophils and initiate resolution and repair. The study of Kannan and colleagues (Kannan *et al.*, 2009) reported that PA infections of mice deficient in MCP-1 resulted in a minor decrease in the clearance of PA but also resulted in increased tissue injury of the lung.

MIP-1-α can also aid in the recruitment of neutrophils indirectly and contribute to high neutrophil numbers in corneal infections of PA with a consequent tissue damage (Kernacki *et al.*, 2001). It also recruits other immune cells and induces the production of other cytokines and chemokines (such as TNF-α, IL-6 and IL-1 α) from macrophages (Cook, 1996; Xue *et al.*, 2007). IL-8 is also known for the recruitment and activation of neutrophils and other immune cells (Oppenheim *et al.*, 1991; Mukaida, 2003).

Production of TNF-α serves a dual purpose of both pro-inflammatory and anti–inflammatory functions. There are reports of TNF-α supporting a modest increase in airway bacteria clearance (Gosselin *et al.*, 1995; Sadikot *et al.*, 2006a). However, TNF-α could also induce IL-10 and Mucin 1 (Muc1) which are anti-inflammatory, through the interaction of TNF-α with Tumour necrosis factor receptor 1 (TNFR1) (Daftarian *et al.*, 1996; Choi *et al.*, 2011). Muc1 is known to inhibit TLR signalling [101] and also stimulates apoptosis of neutrophils (Choi *et al.*, 2011). It is also reported that Muc 1 mutant animals have increased neutrophils numbers in their airways and a minor increase in their ability to clear bacterial infections (Choi *et al.*, 2011). IL-10 is also an anti-inflammatory cytokine and its production is shown to peak at later time points of PA infections during acute lung infection when pro-inflammatory cytokine levels start decreasing (Choi *et al.*, 2011; Wolbeling *et al.*, 2011). The role of IL-10 in PA control is still inconclusive but studies have shown that, early recruitment of neutrophils is essential for clearing PA infections and that it is detrimental when the amount of IL-10 is higher than that of pro-inflammatory cytokines during early PA infections (Sun *et al.*, 2009). But then again, it is also detrimental when there is an over exuberant and uncontrolled immune response due to continues high amounts of pro-inflammatory cytokines and IL-10 deficiency (Spight *et al.*,

2005). Thus there is the need for a balance between both pro-inflammatory and anti-inflammatory cytokines for an effective immune response to PA.

Our data showed that, even in the absence of either IFN-γ or IL-17A treatment, infected macrophages could produce multiple cytokines including pro-inflammatory cytokines (such as IL-1β, IL-1α and TNF-α), chemokines (such as MIP-1α, MCP-1 and IL-8), and anti-inflammatory cytokines (such as IL-10 and IL-1RA). This finding is consistent with the work of Singh (Singh, 2012) where PA infected macrophages were induced by PA to produce a number of cytokines and chemokines as early as 2 hpi although cytokine production peaked at 4hpi. These cytokines and chemokines produced by macrophages could modulate neutrophil function when in co-culture with the macrophages. For instance, IL-8 can increase neutrophil recruitment to the PA infection site. IL-1β and IL-1α are pro-inflammatory and could increase the neutrophilic inflammatory response. However IL-1RA could block the binding of the IL-1 cytokines (such as IL-1β and IL-1 α) to the IL-1R, thereby blocking their signalling and eventually reducing the inflammatory state. The anti-inflammatory cytokine IL-10 could also regulate the inflammatory process during infection.

Our data also show a significant increase in IL-8 in the presence of IFN-γ in the infected macrophages which became significantly reduced in the infected macrophage/neutrophil co-cultures (Figure 4.3). The study of Singh (Singh, 2012) also reported an increased in IL-8 production by GM-CSF macrophages treated with IFN-γ compared with GM-CSF macrophages in the absence of IFN-γ, but this was only for the uninfected conditions and the effect was not reflected in the PA infected conditions. The increase in IL-8 in the presence of IFN-γ in the infected macrophages in our study is in agreement with the work of Gerene and colleagues (Denning *et al.*, 1998) who reported an increased expression of IL-8 in response to PA pyocyanin.

IL-8 is a dominant neutrophil chemoattractant protein secreted by a number of cell types such as T cells, neutrophils and monocytes (Oppenheim *et al.*, 1991; Mukaida, 2003). Thus the

increase in IL-8 in the presence of IFN-γ by the infected macrophages might imply an enhanced neutrophil recruitment under these conditions. However, once the neutrophils were added to the macrophages as seen in the macrophage/neutrophil co-cultures (Figure 4.3), IL-8 was significantly reduced which might be due to the macrophages modifying their effect when in co-culture with the neutrophils to control the neutrophilic inflammatory response or perhaps just due to the dilution of cultures (2 hpi) by the addition of the neutrophils to the infected macrophages before further incubation (2.5 hpi).

Contrary to the effect seen with IFN-γ, IL-17A did not affect the release of IL-8 in the infected macrophages. However, there was a reduction of IL-8 in the infected macrophage/neutrophil co-cultures compared to infected macrophages only in both untreated and IL-17A treated conditions (Figure 4.13). It is possible that the decrease in IL-8 in the macrophage/neutrophil co-cultures compared to macrophages only in the IL-17A treatment groups is also due to the fact that, both the macrophages and bacteria were diluted (2 hpi) by the addition of the neutrophils before further incubation (2.5 hpi).

Furthermore, there was an increase in the pro-inflammatory cytokine IL-1β when neutrophils were added to the macrophage cultures (untreated conditions) and a significant decrease in IL-1β in the presence of IFN-γ in the macrophage/neutrophil co-culture (Figure 4.3). This suggests a potential anti-inflammatory ability of IFN-γ in this model. Meanwhile, the increase in IL-1β upon neutrophil addition to the macrophage cultures in untreated conditions were not observed in the IL-17A group [see Figure 4.3, note IL-1β graph (blue versus green graph) compared to IL-1β graph in Figure 4.13 (blue versus green graph)]. This might be due to differences in the immunological responses mounted by the different donor groups, which led to a decision to compare the effect of these two cytokines side by side in the same donor groups before any conclusions could be drawn. The finding of the side by side comparison of these two cytokines in the same donor groups is detailed in Chapter 5.

4.5 Summary and conclusions

This Chapter investigates the effect of Th-1 (IFN-γ) and Th-17 (IL-17A) inflammation on a classical *in vitro* acute infection model of PA and a possible cross-talk between human macrophages and neutrophils during such infections. For this, monocyte derived macrophages were pre-treated with either IFN- γ or IL-17A for 24 h prior to infections. Treated and untreated human macrophages were infected with PA at MOI 1 and incubated for 2 h. Half of the samples were obtained 2 hpi for analysis of CFU and supernatants and freshly isolated neutrophils were added at this time. Samples were further incubated for 30 min (2.5 hpi) and samples were collected for analysis of CFU and supernatants. From the results obtained, the following conclusions could be drawn:

1. IFN-γ does not promote PA clearance by macrophage/neutrophil co-cultures, if anything, it reduces clearance and it does not have a significant direct effect on neutrophil-mediated killing of PA.
2. IL-17A marginally promotes PA clearance in the macrophage/neutrophil co-cultures perhaps through a direct effect on neutrophils but may increase cytotoxicity and inflammation if not properly controlled.
3. IFN-γ and IL-17A do not affect production of neutrophil elastase both in infected neutrophils and macrophage/neutrophil co-cultures.
4. Infected macrophages produce cytokines and chemokines which could modulate neutrophil function when in co-culture with the macrophages.
5. IL-8 production by infected macrophages was significantly increased in the presence of IFN-γ.
6. IL-1β was increased with neutrophil addition to the macrophage infections in some assays but reduced in the presence of IFN-γ.
7. There was a trend towards an increase in IL-1α production when neutrophils were added to the macrophage infections which also had a tendency to reduce in the presence of IFN-γ.

8. Production of MCP-1 and TNF-α by infected macrophages also had a tendency to increase in the presence of IFN-γ and the trend was maintained in the macrophage/neutrophil co-cultures.
9. MIP-1α production was not clearly affected by IFN-γ in macrophages and macrophage/neutrophil co-cultures.
10. There was no significant effect of IL-17A on the cytokines tested both in the macrophages only and the macrophage/neutrophil co-culture infections.

These results support the suitability of this infection model to investigate human anti-microbial immunity and how it is influenced by inflammatory conditions. Hence, this model was exploited for further experiments detailed in Chapter 5. Chapter 5 describes the use of the macrophage/neutrophil co-culture model to compare the effect of IFN-γ alongside IL-17A on the ability of macrophages and/or neutrophils to promote inflammation during PA infection within the same donor groups and the possible mechanisms involved in these interactions.

Chapter 5

Mechanism of differential interleukin-1β production by macrophage/neutrophil co-cultures infected with *Pseudomonas aeruginosa*

Chapter 5: Mechanism of differential interleukin-1β production by macrophage/neutrophil co-cultures infected with *Pseudomonas aeruginosa* (PA)

5.1 Introduction

IL-1β is a powerful pro-inflammatory cytokine essential for an effective immune response against invading pathogens (Dinarello, 1996). The gene expression for IL-1β is regulated by NF-κB mediated signalling (Al Moussawi *et al.*, 2014) but IL-1β is typically produced as an inactive precursor (pro-IL-1β) in response to pathogen-associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) (Lopez-Castejon *et al.*, 2011). Pro-IL-1β is cleaved to the active IL-1β by caspase-1 (Wilson *et al.*, 1994), which is also activated through the formation of inflammasomes (Martinon *et al.*, 2002). The inflammasomes as mentioned earlier in Chapter 1, are a group of scaffolding and signalling cascades involved in the detection of pathogens and stimuli (including pathogenic and physiological) with a consequent processing of the inactive cytokines pro-IL-1β and pro-IL-18 into their active forms IL-1β and IL-18, respectively, and the induction of an inflammatory form of cell death called pyroptosis (Latz *et al.*, 2013; Sharma *et al.*, 2016). The inflammasomes are made up of a sensor molecule, an adaptor called ASC [apoptosis-associated speck-like protein containing a CARD (caspase activation and recruitment domain)] and the inactive pro-caspase-1 (Sharma *et al.*, 2016). There are various types of inflammasome sensors including the nucleotide binding domain (NOD)–like receptors (NLRs), absent in melanoma 2 (AIM2)–like receptors (ALRs), and pyrin (Sharma *et al.*, 2016).

The majority of the inflammasomes contain a member of the NLR family as their inflammasome sensor molecule. Most of the NLR proteins except NLRP1 have a 3-domain structure, with either a pyrin domain (for most of the NLRPs) or CARD domain (for NLRC4) at their amino-terminal death-fold domain, a NACHT/NOD nucleotide-binding domain at the centre and a carboxyl-terminal region containing the LRRs (Ting *et al.*, 2008). Members of the NLR family iinclude the NLRP1 (NOD, leucine-rich repeats (LRR), and pyrin domain-

containing 1), NLRP3 (NOD, LRR, and pyrin domain-containing 3), NLRP6 (NOD, LRR, and pyrin domain-containing 6), NLRP7 (NOD, LRR, and pyrin domain-containing 7), the NLRP12 (NOD, LRR, and pyrin domain-containing 12) and the NLRC4 (NOD, LRR and CARD-containing 4); NLRC4 is also called IPAF (Latz *et al.*, 2013). In the pyrin-containing NLRs, the amino-terminal death-fold domain interacts with the adaptor ASC which acts as a bridge between the NLR sensor and pro-caspase-1.The NACHT domain has an ATPase activity and is also involved in protein oligomerization while the LRR domain has regulatory functions (Latz *et al.*, 2013). NLRP1 has an additional domain called function-to-find domain (FIIND) and a CARD region at the C-terminus (Latz *et al.*, 2013). Although the recruitment of ASC is avoidable for both NLRC4 and NLRP1 because of the presence of CARD, recruitment of ASC significantly increases inflammasome complex formation and the activation of IL-1β (Proell *et al.*, 2013).The exact mechanism of ASC recruitment by NLRC4 and mouse NLRP1B is unclear (Latz *et al.*, 2013).

Other inflammasomes such as the AIM2 and IFN-γ-inducible protein 16 (IFI16) have a PYHIN (pyrin and HIN domain-containing protein) (Latz *et al.*, 2013). AIM2 has a pyrin domain for ASC recruitment as well as a HIN domain for binding to DNA while IFI16 contain a pyrin domain for ASC recruitment and two HIN domains for binding to DNA (Latz *et al.*, 2013). Retinoic acid-inducible gene I (RIG-I) protein is also implicated in inflammasome complex formation with the recruitment of ASC and caspase-1 perhaps through its CARD (Poeck *et al.*, 2010).

The inflammasomes can be triggered by a number of pathological and physiological stimuli; for instance NLRP1 can be activated by the recognition of bacterial peptidoglycan compound muramyl dipeptide while the murine NLRP1B recognises toxins (Boyden *et al.*, 2006). NLRP3 is activated by intracellular damage caused by pathogens (e.g. bacterial toxins) or sterile stimuli such as crystalline material (Horvath *et al.*, 2011) while NLRC4 responds to microbial ligands (Abdelaziz *et al.*, 2010) such as bacterial flagellin, pilli or the T3SS that are recognised through NLR family apoptosis inhibitory proteins (NAIPs). NAIPs bind NLRC4 and as

mentioned above, NLRC4 may form an inflammasome complex with or without the recruitment of the adaptor ASC (Latz *et al.*, 2013). RIG-I and the PYHIN proteins are activated by recognition of nucleic acids (Poeck *et al.*, 2010). Specifically, AIM2 recognises dsDNA via its HIN200 domain. Pyrin recognises modifications in Rho GTPases caused by bacterial toxins (Latz *et al.*, 2013). A detailed review of the several inflammasome activators is given by Horvath and colleagues (Horvath *et al.*, 2011).

Activation of the inflammasome is crucial for the removal of damaged cells as well as pathogenic substances (Sharma *et al.*, 2016). When a specific stimulus is detected by an inflammasome sensor molecule, the sensor becomes activated and recruits ASC to form distinct foci in the activated cell. ASC is encoded by PYCARD and it connects caspase-1 to the inflammasomes sensors (Sharma *et al.*, 2016). ASC has a pyrin domain and a CARD. The inflammasome sensor interacts with ASC through the pyrin domain (Lamkanfi *et al.*, 2014) which causes the assembly of ASC into a speck (Vanaja *et al.*, 2015). The recruited ASC uses its CARD to subsequently recruit units of pro-caspase-1 which are brought very close to one another and this closeness causes autolytic cleavage of pro-caspase-1 to generate active caspase-1. The active caspase-1 then proteolytically cleaves the pro-cytokines of IL-1β and IL-18 to their active forms and can also induce pyroptosis (Sharma *et al.*, 2016). As mentioned above, studies have shown that NLRC4 does not interact with its activators directly but through the NAIPs which recognises the ligands before inducing NLRC4 activation (Sharma *et al.*, 2016). Four types of NAIPs are found in mice including NAIP1, NAIP2, NAIP5 and NAIP6. NAIP1 senses bacterial needle proteins, NAIP2 senses bacterial inner rod proteins while NAIP5 and NAIP6 sense flagellin (Endrizzi *et al.*, 2000; Sharma *et al.*, 2016). However, only one NAIP is known in humans which senses flagellin as well as needle proteins (Endrizzi *et al.*, 2000; Yang *et al.*, 2013; Kortmann *et al.*, 2015). In addition to T3SS and flagellin, PA has a number of components that can activate the inflammasome in varying degrees including its LPS and pilus (Cohen *et al.*, 2013). Thus one of the possible mechanisms of IL-1β processing in our assays is caspase-1-mediated activation on IL-1β production (Figure 5.1).

There are alternative ways of IL-1β processing besides the activation of caspase-1 through inflammasomes. Experiments involving the use of murine models deficient in inflammasome components supported an alternative IL-1β processing mechanism independent of the inflammasome activation (Cheng *et al.*, 2008). For instance, IL-1β, but not caspase-1, has been shown to be very important in the immune response to *C. trachomatis* (Cheng *et al.*, 2008). Furthermore, the importance of IL-1β in the immune defence against disseminated candidiasis (Lu *et al.*, 2000; Greten *et al.*, 2007) has been reported. However, a normal resistance to disseminated candidiasis was observed in mice deficient in caspase-1 (Mencacci *et al.*, 2000) which suggests an alternative mechanism of IL-1β processing. Other studies have shown that serine proteases derived from neutrophils such as proteinase-3, elastase, and cathepsin-G can process pro- IL-1β to the active IL-1β (Dinarello, 1996; Coeshott *et al.*, 1999). Proteinase-3 can also process pro-IL-18 into active IL-18 (Sugawara *et al.*, 2001).

The study of Greten and colleagues confirmed the activation of pro-IL-1β to active IL-1β independent of the inflammasome (Greten *et al.*, 2007) using a mouse model. Greten and colleagues (Greten *et al.*, 2007) also showed that an increased processing of pro-IL-1β in macrophages depended on caspase-1 and this was inhibited by NF-κB-dependent gene products, while the processing of pro-IL-1β in neutrophils was independent of caspase-1 but depended on serine proteases which were also inhibited by NF-κB-dependent gene products. This phenomenon could explain the previous observations in the *Candida* model in that, as neutrophils are the major cells in the inflammatory infiltrates in disseminated candidiasis (Sugawara *et al.*, 2001), the neutrophil proteinase-3 is likely to be involved in IL-1β processing instead of caspase-1 (Mencacci *et al.*, 2000). During arthritis, the inflammatory infiltrate is composed of neutrophils and macrophages but during the acute stages, the neutrophils are the most abundant in the inflammatory infiltrates and only a negligible role of caspase-1 for IL-1β processing was observed with neutrophil serine proteases such as proteinase-3 playing a major role (Joosten *et al.*, 2009). However, caspase-1 was shown to have a major role in IL-1β processing during the chronic stage of arthritis when macrophages dominate the

inflammatory infiltrate (Joosten *et al.*, 2009). Interestingly, studies have identified another mechanism of pro-IL-1β processing besides neutrophil serine proteases such as a protease derived from *Candida albicans* (Beausejour *et al.*, 1998). Thus neutrophil proteases and perhaps other proteases from pathogens might be involved in processing IL-1β. Therefore, it was important to investigate the effect of inhibiting neutrophil-derived protease such as neutrophil elastase on the production of IL-1β in our assays (Figure 5.1)

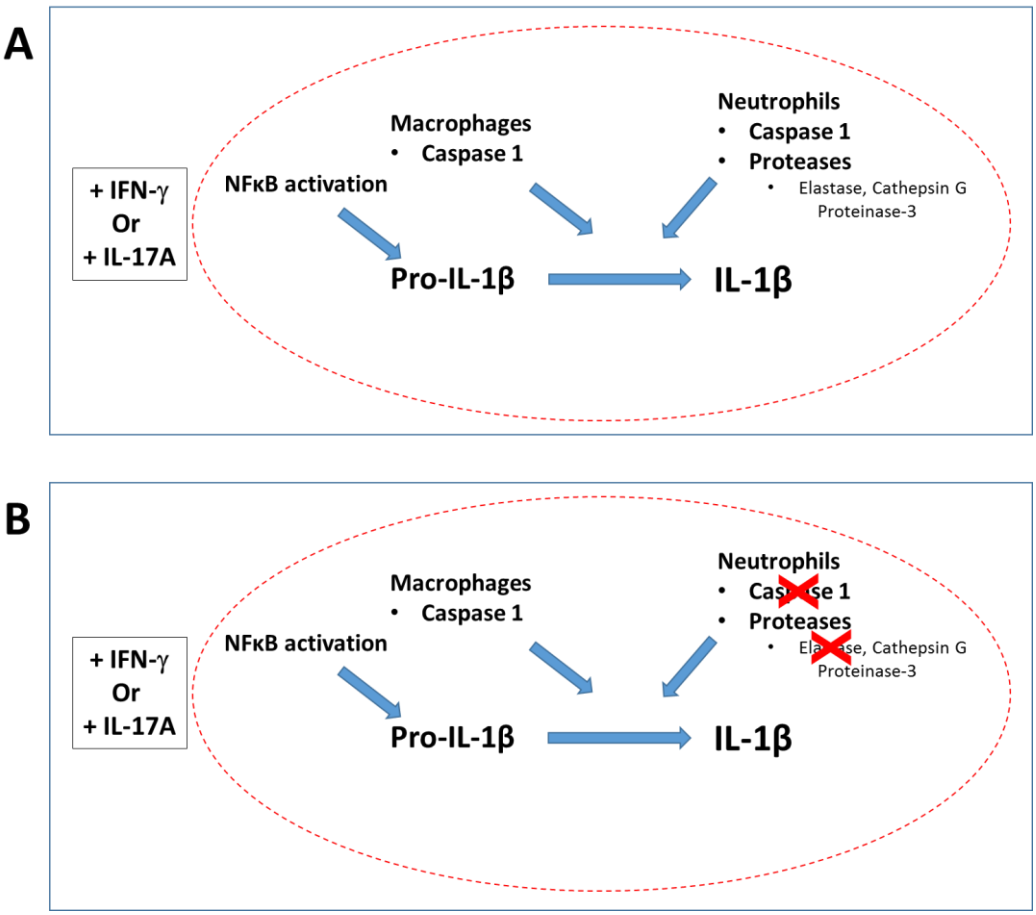


Figure 5.1: Testing the potential mechanism of differential interleukin-1β production by macrophage/neutrophil co-cultures infected with PA in the presence of either IFN-γ or IL-17A treatment.

The effect of inflammatory mediators such as IFN-γ and IL-17A on the ability of macrophage/neutrophil co-cultures to respond to PA infection (CFU and cytokine production especially IL-1β) was tested. **(A)** IL-1β is produced as an inactive form (pro-IL-1β), through NF-κB activation which needs to be processed. Pro-IL-1β could be processed by either caspase-1 through inflammasome activation (from our assay conditions, caspase-1 could be derived from macrophages or neutrophils) or neutrophil derived proteases (such as neutrophil elastase, cathepsin G or proteinase-3). **(B)** To test for the possible mechanisms involved in IL-1β processing in our assays, we used inhibitors to either block caspase-1 activity or neutrophil elastase activity.

As discussed earlier in Chapter 4, we observed a potential anti-inflammatory property of IFN-γ since there was less IL-1β production by IFN-γ treated macrophage/neutrophil co-cultures compared to IL-17A treated macrophage/neutrophil co-cultures. Also, there was an increase in the production of IL-1β when neutrophils were added to the infected macrophages even in the untreated conditions (i.e. no pre-treatment of macrophages with cytokines). However, this increase in IL-1β after addition of neutrophils was only seen in the infection assays with and without IFN-γ but not in the infection assays with and without IL-17A. One reason for this could be the fact that we used different donor groups for the different assays and since different individuals have different immunological responses, there might be some inadvertent bias in the donor groups used for these two assay sets. Therefore, it was considered necessary to compare the effect of these two cytokines (IFN-γ and IL-17A) side by side within the same donor group so as to avoid any accidental bias in donor selection.

This Chapter therefore focused on determining the effect of IFN-γ and IL-17A on the ability of either macrophages, neutrophils or the macrophage/neutrophil co-cultures within the same donor groups to produce cytokines and also determine the possible mechanism(s) involved. For this purpose, infection assays were performed with and without pre-treatment with either IFN-γ or IL-17A. Supernatants from these assays were analysed for cytokines and chemokines as well as lactate dehydrogenase (LDH). Macrophage/neutrophil infection assays were also performed in the presence and absence of inhibitors (including a pan-caspase inhibitor; Z-VAD and a neutrophil elastase inhibitor; EIII) and supernatants were analysed for cytokines, chemokines, LDH and neutrophil elastase. These inhibitors were included at the time of neutrophil addition to investigate the effect of the inhibitors on the contribution of neutrophils to infection outcome. In some instances a reporter of caspase 1 activation (FAM-YVAD-FMK, which labels active caspase-1) was used to investigate inflammasome activation during infection.

5.2 Materials and methods

5.2.1 Effect of IFN-γ and IL-17A on macrophage/neutrophil co-cultures or neutrophils cultures uninfected or infected with PA

To assess the effect of IFN-γ and IL-17A on the macrophage/neutrophil co-cultures as well as neutrophils only infections with PA, the procedure for the macrophage/neutrophil co-culture infections of PA described in Chapter 2.17 and Figure 2.2 was followed but with some modifications. PA inoculum was prepared as described in Chapter 2.6. The appropriate inoculum of PA was opsonised by incubation at 37^{o}C at 8 rpm in the presence of 10% NHS in HBSS complete medium for 20 min. Monocyte-derived macrophages prepared as described in Chapter 2.11 were left untreated or pre-treated with either IFN-γ or IL-17A for 24 h prior infection with PA. Untreated or cytokine-treated macrophages (2 x 10^5 macrophages in HBSS complete medium) were infected with opsonised PA at MOI of 1, alongside vials containing opsonised PA in HBSS complete medium were also prepared. Samples were incubated at 37^{o}C at 8 rpm for 2 h. Then half of the samples were collected for preparation of supernatants which was frozen for further analysis. Purified neutrophils (5 x 10^5 in HBSS complete medium, ratio of macrophage: neutrophil 1:5) were then added accordingly at 2 hpi: this will give “PA with neutrophils” and “PA with macrophages and neutrophils” conditions. Samples were incubated for a further 60 min at 37^{o}C at 8 rpm after which supernatants were again prepared and frozen for further analysis. This process is summarised in Figure 5.2. There were also uninfected conditions of both the macrophage/neutrophil co-cultures as well as the neutrophils only in the presence and absence of either IFN-γ or IL-17A which were also processed as described above (Chapter 5.2.1) except that opsonised PA was replaced with just HBSS complete medium with 10% NHS to serve as the baseline control.

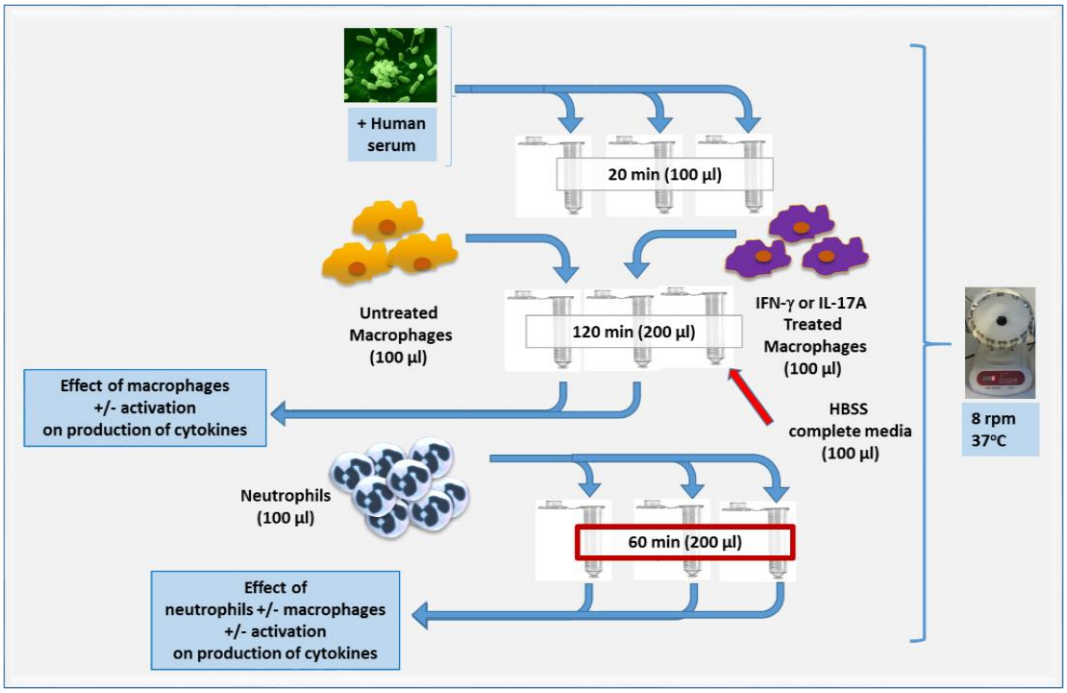


Figure 5.2: Effect of IFN-γ or IL-17A on cytokine production by macrophage/neutrophil co-cultures and neutrophils only cultures infected with PA. To test the effect of inflammatory mediators such as IFN-γ and IL-17A on the ability of macrophage/neutrophil co-cultures as well as neutrophils only to produce cytokines in response to PA infection, macrophages were either left untreated or pre-treated with the cytokine of interest for 24 h prior to the infection assay. PA from -80°C stock was streaked onto LB agar plates and incubated at 37°C overnight. A single colony was picked from the plate the next day into 5 ml X-Vivo 15 medium and incubated again overnight at 37°C, 200/220 rpm. The next day, 1% inoculum of OD_{600nm} = 1 (final OD_{600nm} = 0.01) was prepared and incubated for 3 h at 37°C, 200/220 rpm to obtain a log-phase PA culture. PA was then washed and appropriate inoculum made which was pre-opsonised with 10% NHS. PA was incubated with treated or untreated macrophages or with HBSS complete medium for 2 h and samples obtained 2 hpi for the preparation of supernatants. Freshly prepared neutrophils were added accordingly and incubated further for 60 min after which samples were again processed for supernatants. Serum, IFN-γ or IL-17A concentrations were maintained throughout the experiment.

5.2.2 Effect of a pan-caspase inhibitor (Z-VAD) and a neutrophil elastase inhibitor III (EIII) on PA viability

To assess the possible mechanism(s) involved in the processing of IL-1β, inhibitors such as Z-VAD (a pan-caspase inhibitor) and EIII (a neutrophil elastase inhibitor) were employed in the macrophage/neutrophil co-culture infection assays. These inhibitors needed to be initially tested for their effect on PA viability before being used in the infection assays. For this, PA inoculum was prepared as described in Chapter 2.6. The amount of PA inoculum usually employed in the macrophage/neutrophil co-culture infection assays ($1x10^6$/ml) was used. PA was opsonised by incubation at 37^{o}C at 8 rpm in the presence of 10% NHS in HBSS complete medium for 20 min. Pre-opsonised PA (100µl) was incubated for 30 min at 37^{o}C at 8 rpm in the presence and absence of either of the inhibitors before samples were obtained for CFU. The specific inhibitors used were Z-VAD-FMK (V116-2MG, Sigma-Aldrich) and ELASTASE INHIBITOR III (EIII, 324745-5MG, CALBIOCHEM). Both inhibitors were reconstituted in dimethyl sulfoxide (DMSO) following the manufacturer's instructions. Also, both inhibitors were used at a final working concentration of 20 µM based on published literature and manufacture's information as described earlier for Z-VAD by D'Agnillo and colleagues (D'Agnillo *et al.*, 2013) and for EIII: The inhibition constant (K_i) indicated by the manufacturer was 10 µM, the K_i concentration is that which gives 50% of maximum inhibition, thus 20 µM was used to aim for maximal inhibition.

5.2.3 Analysis of the effect of inhibitors (Z-VAD and EIII) on macrophage/neutrophil co-culture infections with PA.

To determine the possible mechanism(s) involved in the increased production of IL-1β by infected macrophage/neutrophil co-cultures upon addition of neutrophils, inhibitors (i.e. Z-VAD and EIII) were incorporated into the macrophage/neutrophil co-culture infection assay at the time neutrophils were added. For this PA inoculum was prepared as described in Chapter 2.6. The appropriate inoculum of PA was opsonised by incubation at 37^{o}C at 8 rpm in the presence of 10% NHS in HBSS complete medium for 20 min. Monocyte-derived macrophages prepared as described in Chapter 2.11 (2×10^5 macrophages in HBSS complete medium) were infected with PA at MOI of 1 under opsonic conditions. Infected macrophages were incubated at 37^{o}C at 8 rpm for 2 h. Then half of the samples were removed and processed for CFU quantification and collection of supernatants. Purified neutrophils (5×10^5 in HBSS complete medium, ratio of macrophage: neutrophil 1:5) were then included accordingly at this time point with or without the addition of either of the inhibitors. Both inhibitors were used at a final working concentration of 20 μM as explained above in Chapter 5.2.2. Samples were incubated further for 60 min at 37^{o}C at 8 rpm after which cultures were processed for CFU quantification and collection of supernatants. The procedure is summarised in Figure 5.3

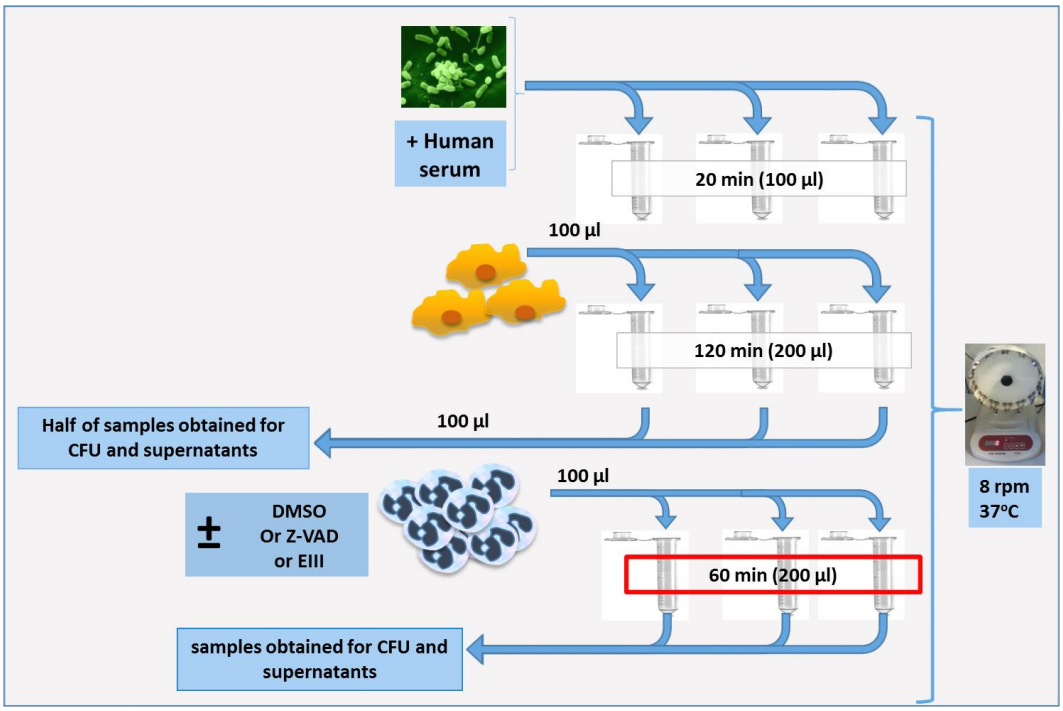


Figure 5.3: Macrophage/neutrophil co-culture infections of PA to test the effect of inhibitors (Z-VAD and EIII) on cytokine production and CFU.
PA was incubated initially with human macrophages for 2 h in the presence of serum and half of the samples obtained 2 hpi were processed for the preparation of supernatants and CFU quantification. Freshly purified human neutrophils were added (5 x10^5, macrophage: neutrophil ratio 1:5) at 2 hpi in the presence and absence of either DMSO, Z-VAD or EIII. Human macrophage/neutrophil co-culture infections were incubated further for 60 min and processed for supernatants and CFU quantification.

5.2.4 Cytokine quantification from supernatants

Supernatants were analysed for cytokines and chemokines following the manufacture's protocol as described earlier in Chapter 2.20.

5.2.5 Neutrophil elastase assay

Supernatants from the co-culture infection assay with or without inhibitors were analysed for neutrophil elastase following the manufacture's protocol as described earlier in Chapter 2.19.

5.2.6 Lactate dehydrogenase (LDH) assay

Supernatants from the infection assays above (Chapter 5.2.3) were tested for the release of lactate dehydrogenase (LDH) using an LDH cytotoxicity detection kit (catalogue number 11644793001, Roche) and following the manufacturers' protocol but with volume adjustments. An LDH Positive control (catalogue number ab150446, Abcam) of known concentration of LDH (100µg/ml) was also used alongside the test samples. In brief, equal volumes of test samples and reaction mixture were mixed and incubated for 30 min in the dark before reading. In this assay, 25µl of test supernatants, LDH positive control (diluted 1 in 12.5 with HBSS complete medium with 10% NHS as recommended by the manufacturer e.g., 2µl positive control + 23µl medium) or HBSS complete medium with 10% NHS only was added to wells accordingly in a 96 well microplate (Coster). Then 25µl of the reaction mixture was added to each well and samples incubated for 30 min at room temperature protected from light. Each condition was performed in triplicate wells. The plate was read using a microplate reader (Multiskan FC, Thermo Scientific) at 450nm and wavelength correction at 600nm. Corrected optical densities (ODs) were taken for both the positive control and the test samples and used in estimating the amount of LDH in the test samples. LDH in test sample was estimated as = {(average OD of test sample / (Average OD of positive control X dilution factor (i.e.12.5)} X [LDH of positive control (i.e. 100µg/ml)]. Data was analysed with Microsoft Excel 2013 version and GraphPad Prism 6

5.2.7 Analysis of caspase-1 activation in macrophages uninfected or infected with PA in the presence of IFN-γ or IL-17A

To investigate active caspase-1 in infected and uninfected macrophages treated with IFN-γ or IL-17A, PA inoculum was prepared as described in Chapter 2.6 and desired concentrations made. PA at MOI of 1 was opsonised by incubation at 37^{o}C at 8 rpm in the presence of 10% NHS in HBSS complete medium for 20 min and monocyte-derived macrophages pre-treated with IFN-γ or IL-17A for 24 h prior infections with PA were infected accordingly. There were also samples of uninfected macrophages where HBSS complete medium were added to make the volumes equal to that of the infected conditions. Samples were incubated at 37^{o}C at 8 rpm for 2 h. At 2 hpi, samples were then incubated with FAM-FLICA (FAM-YVAD-FMK (655), catalogue number: 98, Immunochemistry Technologies) following the manufacturers' protocol as briefly described below:

The stock of FAM-FLICA was initially reconstituted in 50 µl of DMSO, then further diluted 1:5 with PBS and this diluted FAM-FLICA was used at a working concentration of 1:30. After 2 hpi, 7 µl of diluted FAM-FLICA was added to conditions to be labelled. Samples were incubated for 45 min in the dark after which 100 µl of the culture was placed on a poly-D-lysine (P0899, Sigma-Aldrich) coated 12mm round glass coverslips (Academy NPC12, Smith Scientific Limited) inserted in 24 well tissue culture plates (Coster). These samples were incubated for 45 min at 4^{o}C or on ice ensuring samples were centred with the aid of a centre bubble. Plates were centrifuged for 10 min at 250g at 4^{o}C, maximum acceleration and deceleration. Supernatants were then removed, and cells were washed with 500 µl of 1 X apoptosis wash buffer (provided with the FAM-FLICA kit). The wash buffer was taken off gently and the wash repeated twice. After the last wash, 200 µl of 1X apoptosis wash buffer was added to each well and 1µl of either Hoechst stock (200 µg/ml, part of the FAM-FLICA kit content) was added to conditions to be counterstained with Hoechst,

while1 µl of Propidium Iodide (PI: 250 µg/ml, part of the FAM-FLICA kit content) was added to conditions to be counterstained with PI as per the manufacturer's recommendations (0.5% v/v

for both Hoechst and PI). Samples were swirled gently and incubated for 5 min at room temperature before washing again twice as described above. In some instances, samples were counterstained with both Rhodamine phalloidin (Invitrogen R415: Stock concentration of 1mg/ml and at a final working concentration of 1µg/ml; thus 1:1000) as well as Hoechst.

Finally 450 µl of apoptosis wash buffer and 50 µl of manufacturer's fixative (1 in 10 dilution of fixative) was added to each coverslip, mixed and incubated for 15 min at 4^{o}C. Fixative was taken off and samples washed twice again as described above.

The coverslips containing the cells were removed from the 24 well tissue culture plates with the aid of a bent needle and forceps. Slides were blotted on a clean tissue and air dried before mounting with vectashield (Vector Laboratories Inc., H-1400). Slides were left to solidify in the dark and kept at 4^{o}C protected from light until ready to analyse using a 710 confocal microscope (Zeiss). Confocal images were analysed using Zen 2.3 software or Volocity software.

5.2.8 Analysis of caspase-1 activation in macrophages/neutrophil co-cultures infected with PA in the presence and absence of IFN-γ or IL-17A

To observe active caspase-1 during the macrophage/neutrophil co-culture infections with or without IFN-γ or lL-17A treatment, the procedure for the macrophage/neutrophil co-culture infections with PA in the presence and absence of IFN-γ or lL-17A Chapter 2.17 and Figure 2.2 was followed but with some modifications. Macrophage cultures were incubated for 2 h before neutrophil addition with a further 30 min incubation. Then FAM-FLICA (FAM-YVAD-FMK (655), catalogue number: 98, Immunochemistry Technologies) was added to a final dilution 1:30 (7 µl in 200 µl) and the co-cultures were further incubated for 45 min protected from light. Samples were processed further as above in Chapter 5.2.7.

5.3 Results

5.3.1 Analysis of the effect of IFN-γ and IL-17A on cytokine production by macrophages and/or neutrophils infected with PA

To assess the effect of IFN-γ and IL-17A on the ability of macrophage/neutrophil co-cultures as well as neutrophils only to produce cytokines in response to PA infection, macrophages untreated or pre-treated with IL-17A or IFN-γ for 24 h were either left uninfected or infected with PA under opsonic conditions for 2 h. Samples were collected 2 hpi for analysis of CFU and preparation of supernatants. Neutrophils were also added 2 hpi with macrophage: neutrophil ratio 1:5. The assay was continued as described previously (Chapter 5.2.1 and Figure 5.2). On this occasion "neutrophils only" infections were also included to determine the ability of these cells to produce cytokines under the experimental conditions. The uninfected macrophage/neutrophil co-culture conditions were obtained by replacing opsonised PA with HBSS complete medium with 10% NHS. The infected neutrophil only conditions were obtained by the addition of 100 µl of HBSS complete medium to opsonised PA instead of macrophages in the presence of 10% NHS, with or without IFN-γ or IL-17A mimicking the infected macrophage/neutrophil co-culture model. Cultures were incubated for an hour after the addition of neutrophils and samples processed for supernatants.

Supernatant samples from infected and uninfected macrophages only (2 hpi), infected and uninfected macrophage/neutrophil co-cultures (3 hpi) as well as infected neutrophils only (3 hpi) were analysed for IL-1β, IL-1α, MCP-1, MIP-1α, TNF-α, IL-6, and IL-8 as described in Chapter 2.20. These cytokines were selected based on the results obtained with samples collected 30 min after addition of neutrophils (see Figure 4.3 and Figure 4.13).

Hence, the data collected derive from samples taken 2 hpi: uninfected and infected macrophages only samples (i.e., Mac and Mac+PA samples); samples taken 3 hpi: uninfected macrophage/neutrophil co-culture, infected macrophage/neutrophil co-culture and infected neutrophil only samples (i.e., Mac+Neut, Mac+PA+Net, and PA+Neut samples).

The results showed that IL-1β and IL-1α production by the macrophages and macrophage/neutrophil co-cultures depended on infection with PA as there were only negligible amounts of both cytokines produced under uninfected conditions (Figure 5.4).

IL-1β production by infected cultures was increased with the addition of neutrophil and further incubation for one hour (to 3 hpi final). This was in spite of the fact that 3 hpi samples had been diluted 1 in 2 (i.e. since 100 µl was removed at 2 hpi and neutrophils in 100 µl were added at that point; refer to Figure 5.2). The increase in IL-1β was statistically significant for untreated conditions and IL-17A-treatment conditions but not for the IFN-γ-treated conditions (Figure 5.4, IL-1β graph). In agreement with previous observations, there was a highly statistically significant reduction in IL-1β production by macrophage/neutrophil co-cultures in the presence of IFN-γ when compared to IL-17A-treated cultures (Figure 5.4, IL-1β graph).

Likewise, IL-1α production by infected cultures was significantly increased with the addition of neutrophils and further incubation for one hour (to 3 hpi final). The increase in IL-1α production was significant for untreated conditions and IL-17A-treatment conditions but not significant for the IFN-γ treated conditions (Figure 5.4, IL-1α graph). There was also a significant reduction in IL-1α production by IFN-γ treated compared to IL-17A-treated macrophage/neutrophil co-cultures (Figure 5.4, IL-1α graph). Thus neutrophils promote the release of IL-1β and IL-1α in macrophage/neutrophil co-cultures and IFN-γ appears to have a potential anti-inflammatory role by preventing the increase of the pro-inflammatory cytokines IL-1β and IL-1α.

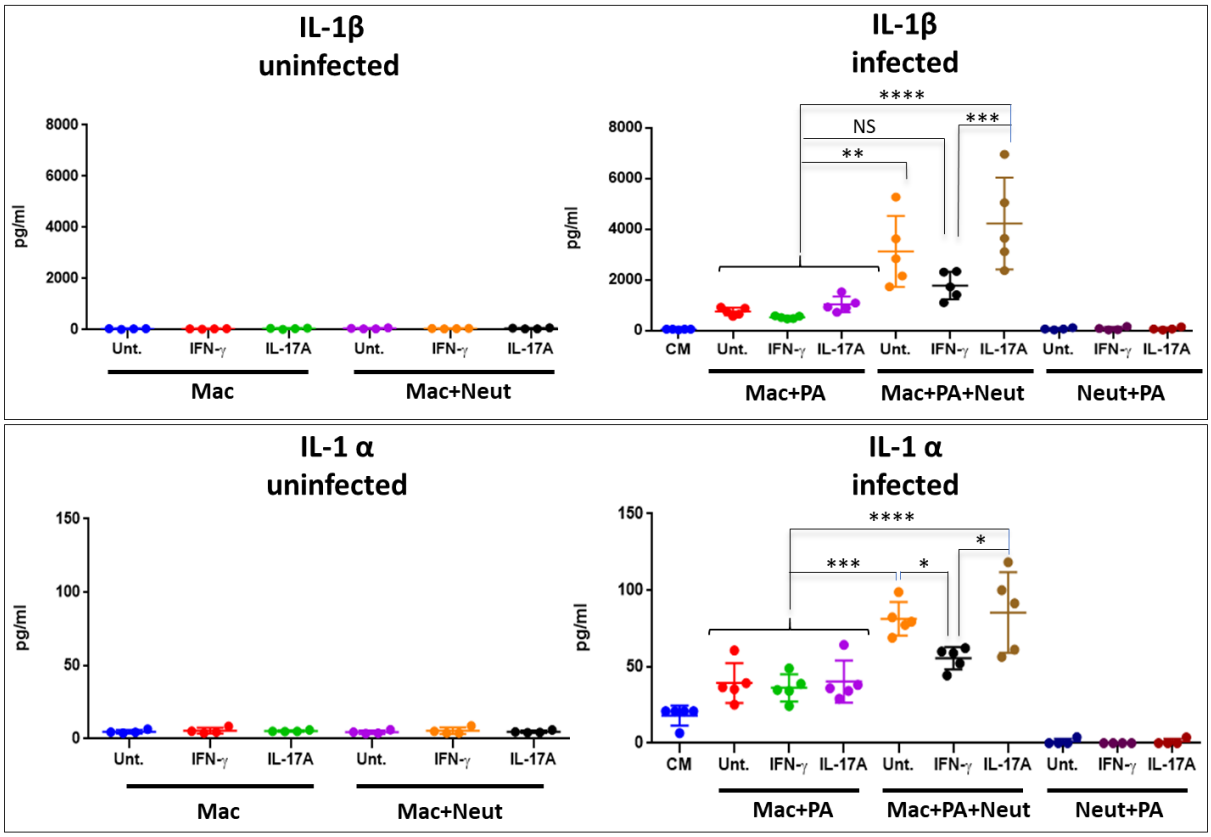


Figure 5.4: Effect of IFN-γ and IL-17A on IL-1β and IL-1α production by macrophages and or neutrophils uninfected or infected with PA.
Human macrophages (2 x10^5) generated in the presence of GM-CSF were left untreated or treated at day 6 with IFN-γ or IL-17A. On the following day, macrophages were collected and either uninfected or infected with PA at MOI 1 in the presence of human serum. At 2 hpi, half of the culture was collected and processed for supernatants and freshly purified human neutrophils were added (5 x10^5, macrophage: neutrophil ratio 1:5). Co-cultures were incubated further for 60 min and processed for supernatants. There were also conditions of neutrophils only infections with PA. IFN-γ, IL-17A and serum concentrations were maintained throughout the experimental process. Supernatants were analysed for cytokines following the manufacturer's protocol**.** PA: *P. aeruginosa*, Mac: macrophages, Neut: Neutrophils, CM: complete medium (Hanks balanced salt solution with Ca^{2+} and Mg^{2+} with 5% Human Serum Albumin with 10% Normal Human Serum)**.** Results from four independent experimental repeats. Data analysed using one-way ANOVA. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001

The results also showed that IFN-γ significantly increased MCP-1 production by uninfected macrophages and the trend was maintained in uninfected macrophage/neutrophil co-cultures (Figure 5.5, MCP-1 uninfected graph). Similarly, IFN-γ increased MCP-1 production in the infected macrophage cultures and the trend was maintained in the infected

macrophage/neutrophil co-cultures (Figure 5.5, MCP-1 infected graph) although the differences were less obvious. This may be due to the dilution of samples by the addition of the neutrophils as explained above.

MIP-1α was not significantly affected by either IFN-γ or IL-17A treatment both in uninfected and infected conditions (Figure 5.5, MIP-1α uninfected and infected graphs). Since there was no effect seen on MCP-1 and MIP-1α production with the addition of neutrophils to the macrophages and both cytokines were absent in the neutrophils only conditions (Figure 5.5), this implies that the macrophages may be the chief source of MCP-1 and MIP-1α under our experimental conditions.

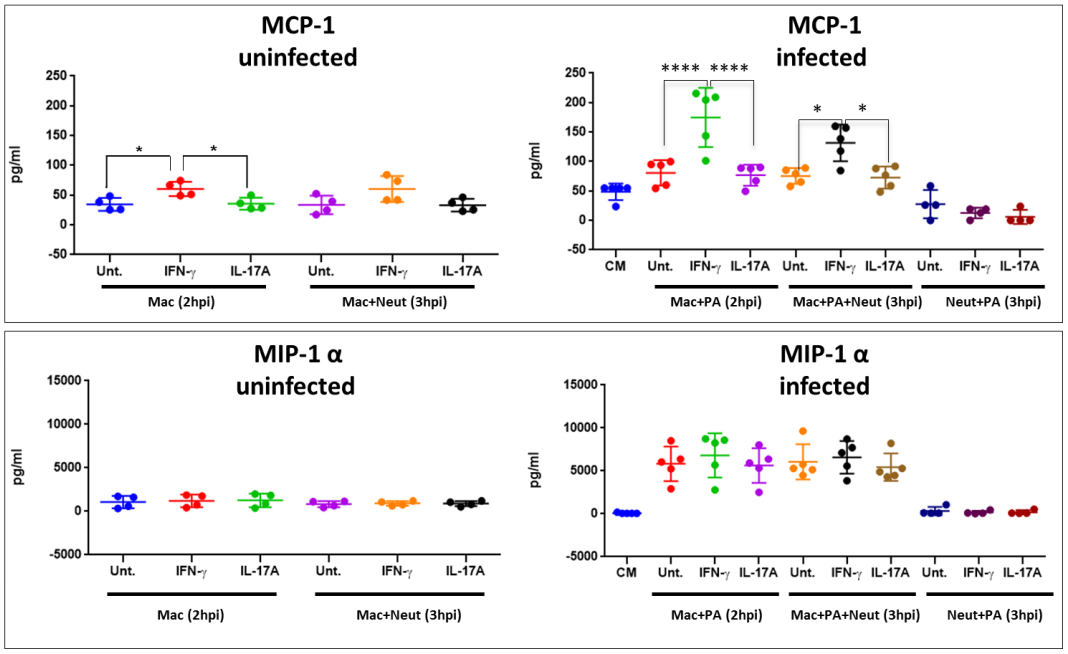


Figure 5.5: Effect of IFN-γ and IL-17A on MCP-1 and MIP-1α production by macrophages and or neutrophils uninfected or infected with PA.
Human macrophages (2 x10^5) generated in the presence of GM-CSF were left untreated or treated at day 6 with IFN-γ or IL-17A. On the following day, macrophages were collected and either uninfected or infected with PA at MOI 1 in the presence of human serum. At 2 hpi, half of the culture was collected and processed for supernatants and freshly purified human neutrophils were added (5 x10^5, macrophage: neutrophil ratio 1:5). Co-cultures were incubated further for 60 min and processed for supernatants. There were also conditions of neutrophils only infections with PA. IFN-γ, IL-17A and serum concentrations were maintained throughout the experimental process. Supernatants were analysed for cytokines following the manufacturer's protocol**.** PA: *P. aeruginosa*, Mac: macrophages, Neut: Neutrophils, CM: complete medium**.** Results from four independent experimental repeats. Data analysed using one-way ANOVA. * = $p<0.05$, **** = $p<0.0001$

The production of TNF-α and IL-6 by the macrophages and macrophage/neutrophil co-cultures depend on the infection with PA (Figure 5.6). IFN-γ increased TNF-α production in the infected macrophages in some donors and the trend was maintained in the macrophage/neutrophil co-cultures (Figure 5.6, TNF-α infected graph), there was no effect on TNF-α levels by the addition of neutrophils and no TNF-α was detected in the supernatants from infected neutrophils only (Figure 5.6, TNF-α infected graph). This implies that the macrophages were the main source of TNF-α under our experimental conditions. Similarly, there was no significant effect on IL-6 production in both infected macrophages and macrophage/neutrophil co-cultures by either IFN-γ or IL-17A treatment and infected neutrophils did not produce IL-6 (Figure 5.6, IL-6 infected graph).

In general, for all the cytokines analysed, IL-17A either did not affect or slightly increase cytokine production by either the macrophages or macrophage/neutrophil co-cultures (Figure 5.4, Figure 5.5 and Figure 5.6). Also, neutrophils alone did not produce any of the cytokines tested under our experimental conditions (IL-1 α, IL-1 β, TNF- α, IL-6, MCP-1 and MIP-1α as shown in Figure 5.4, Figure 5.5 and Figure 5.6) except IL-8, and the IL-8 production was neither affected significantly by IFN-γ or IL-17A treatment (Figure 5.7). IFN-γ reduced IL-1-β and IL-1-α but promoted MCP-1 production.

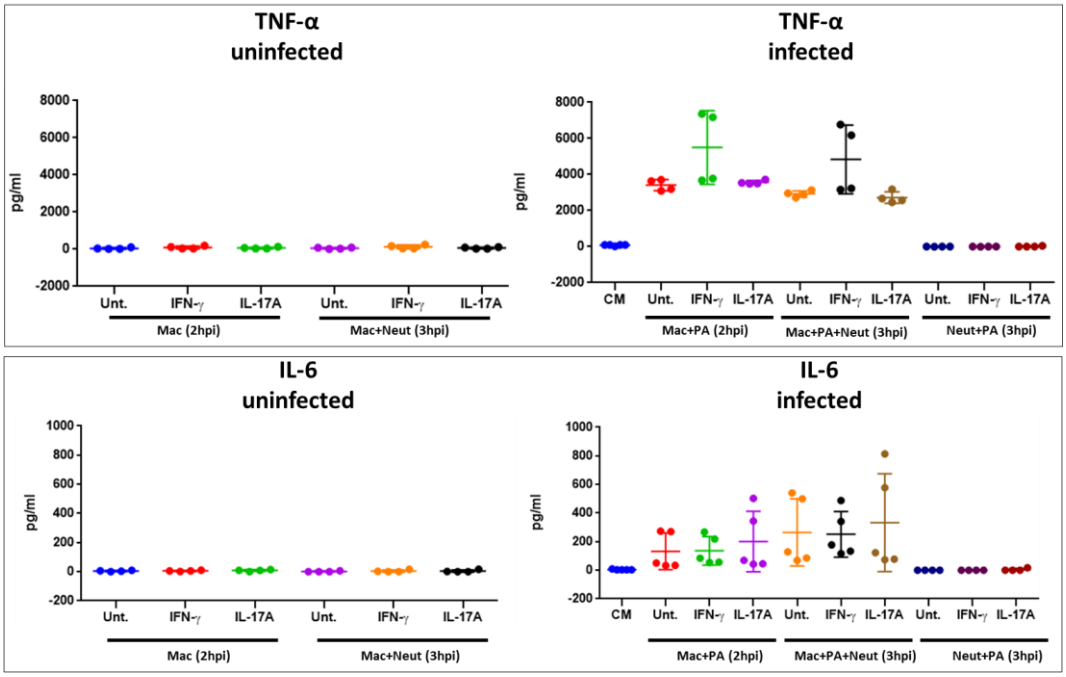


Figure 5.6: Effect of IFN-γ and IL-17A on TNF-α and IL-6 production by macrophages and or neutrophils uninfected or infected with PA.
Human macrophages (2 x10^5) generated in the presence of GM-CSF were left untreated or treated at day 6 with IFN-γ or IL-17A. On the following day, macrophages were collected and either uninfected or infected with PA at MOI 1 in the presence of human serum. At 2 hpi, half of the culture was collected and processed for supernatants and freshly purified human neutrophils were added (5 x10^5, macrophage: neutrophil ratio 1:5). Co-cultures were incubated further for 60 min and processed for supernatants. There were also conditions of neutrophils only infections with PA. IFN-γ, IL-17A and serum concentrations were maintained throughout the experimental process. Supernatants were analysed for cytokines following the manufacturer's protocol. PA: *P. aeruginosa*, Mac: macrophages, Neut: Neutrophils, CM: complete medium**.** Results from four independent experimental repeats. Data analysed using one-way ANOVA.

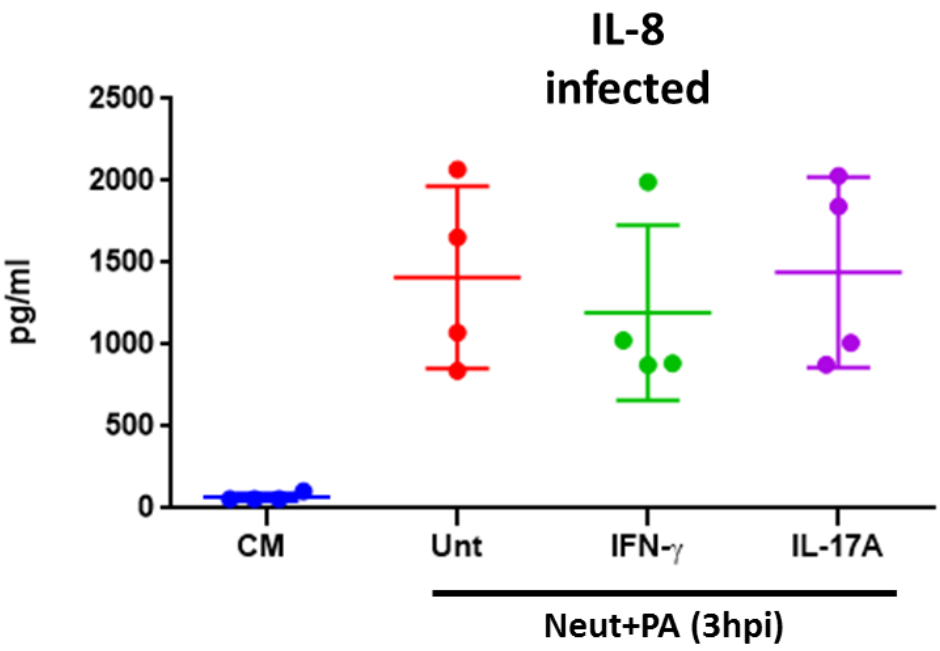


Figure 5.7: IL-8 production by neutrophils infected with PA is not significantly affected by IFN-γ or IL-17A treatment.

100 µl of opsonised PA was incubated with just HBSS complete medium instead of macrophages in the presence of 10% NHS with or without IFN-γ or IL-17A mimicking the macrophage/neutrophil co-culture model. Samples were incubated for 2 h before half of the culture was collected and processed for supernatants and 100 µl of neutrophils was added. This was further incubated for an hour and samples processed for supernatants. This was done alongside the macrophage/neutrophil co-cultures uninfected or infected with PA. IFN-γ, IL-17A and serum concentrations were maintained throughout the experimental process. Supernatants were analysed for cytokines following the manufacturer's protocol. PA: *P. aeruginosa*, Neut: Neutrophils, CM: complete medium. Results from four independent repeats. Results from four independent experimental repeats. Data analysed using one-way ANOVA.

5.3.2 Effect of IFN-γ and IL-17A on LDH release by macrophage/neutrophil co-cultures infected or uninfected with PA

To test for cell death during infection, the release of lactate dehydrogenase (LDH) into the supernatants by macrophage/neutrophil co-cultures uninfected or infected with PA as well as by neutrophils infected with PA, was analysed as described in Chapter 5.2.6.

The results showed that there was no LDH release by bacteria only control conditions both with and without IFN-γ or IL-17A (Figure 5.8, A). However, LDH was released by infected macrophages (2 hpi, Figure 5.8, A), infected neutrophils (3 hpi, Figure 5.8, B) and infected macrophage/neutrophil co-cultures (3 hpi, Figure 5.8, B). Presence of IL-17A or IFN-γ did not affect LDH levels. Addition of neutrophils increased LDH release by the cultures but this was not affected by the presence of IFN-γ or IL-17A (Figure 5.8, A versus B).

Due to insufficient samples, the release of LDH in uninfected versus infected samples could only be compared for one of the donors of which the mean of triplicate repeats within the same experiment is shown in Figure 5.9. No difference between uninfected and infected conditions in both the macrophages, neutrophils and macrophage/neutrophil co-cultures with or without IFN-γ or IL-17A treatment (Figure 5.9) was observed suggesting that PA infection did not cause cell death at these times post-infection.

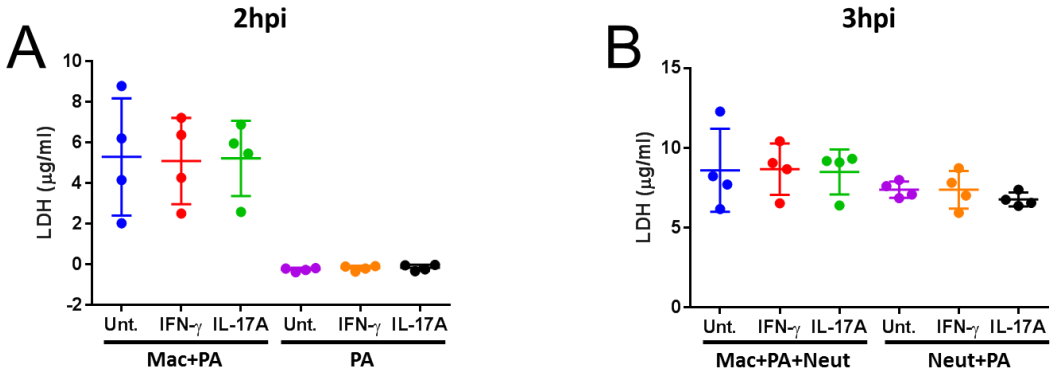


Figure 5.8: LDH release was not significantly affected by IFN-γ or IL-17A treatment in either macrophages or macrophage/neutrophil co-cultures infected with PA.
Human macrophages (2 x10^5) generated in the presence of GM-CSF were left untreated or treated at day 6 with IFN-γ or IL-17A. On the following day, macrophages were collected and infected with PA at MOI 1 in the presence of human serum. At 2 hpi, half of the culture was collected and processed for supernatants and freshly purified human neutrophils were added (5 x10^5, macrophage: neutrophil ratio 1:5). Co-cultures were incubated further for 60 min and processed for supernatants. There were also conditions of neutrophils only infections with PA. IFN-γ, IL-17A and serum concentrations were maintained throughout the experimental process. Supernatants from the 2 hpi and 3 hpi were tested for the presence of LDH following the manufacturer's protocol as described in Chapter 5.2.6. **(A):** shows LDH released by infected macrophages in the presence and absence of either IFN-γ or IL-17A treatment and the LDH released by PA only conditions in the presence and absence of either IFN-γ or IL-17A treatment. **(B):** shows LDH released by infected macrophage/neutrophil co-cultures or neutrophil only cultures in the presence and absence of either IFN-γ or IL-17A treatment. PA: *P. aeruginosa,* Mac: macrophages, Neut: Neutrophils. Results from 4 different supernatants obtained from four independent experimental repeats. Data analysed using one-way ANOVA.

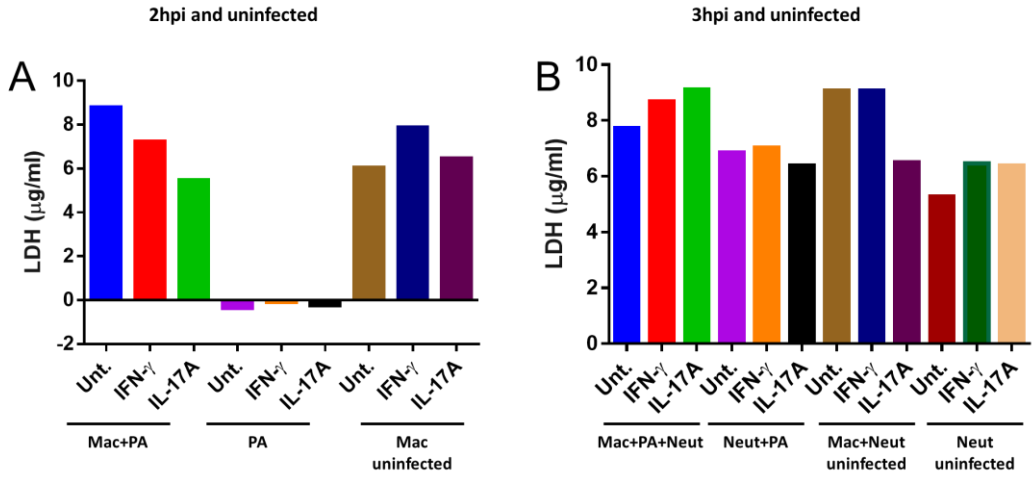


Figure 5.9: LDH release was not significantly affected by IFN-γ or IL-17A treatment and no difference in cultures uninfected or infected with PA.
Human macrophages (2 x10^5) generated in the presence of GM-CSF were left untreated or treated at day 6 with IFN-γ or IL-17A. On the following day, macrophages were collected and either uninfected or infected with PA at MOI 1 in the presence of human serum. At 2 hpi, half of the culture was collected and processed for supernatants and freshly purified human neutrophils were added accordingly (5 x10^5, macrophage: neutrophil ratio 1:5). Co-cultures were incubated further for 60 min and processed for supernatants. There were also conditions of neutrophils only infections with PA. IFN-γ, IL-17A and serum concentrations were maintained throughout the experimental process. Supernatants from the 2 hpi and 3 hpi were tested for the presence of LDH following the manufacturer's protocol as described in Chapter 5.2.6. **(A)**: shows LDH released by infected and uninfected macrophages as well as PA only in the presence and absence of either IFN-γ or IL-17A treatment. **(B):** shows LDH released by uninfected and infected macrophage/neutrophil co-cultures as well as uninfected and infected neutrophils both in the presence and absence of either IFN-γ or IL-17A treatment. PA: *P. aeruginosa*, Mac: Macrophages, Neut: Neutrophils. Results from the mean of triplicate repeats within a single experimental supernatant.

5.3.3 Effect of inhibitors (Z-VAD and EIII) on PA viability

Before employing the inhibitors Z-VAD and EIII in the infection model, it was necessary to initially test their effect on PA viability. For this, pre-opsonised PA was incubated with the inhibitors as described in Chapter 5.2.2 and samples analysed for CFU. The results showed no significant effect of the inhibitors on PA viability. The effect of DMSO (1 in 1000 dilution: final concentration) was also tested as a baseline control as the inhibitors were initially reconstituted with DMSO before further dilutions with HBSS complete medium, and the final working concentration of DMSO in the test samples was 1 in 1000. DMSO at this concentration did not have any significant effect on PA viability.

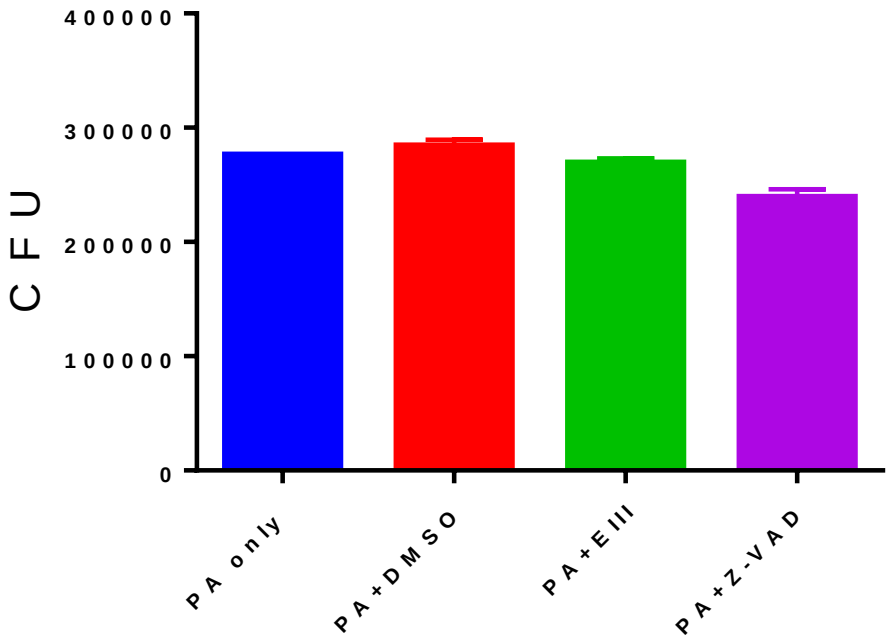


Figure 5.10: The inhibitors (Z-VAD and EIII) at 20μM final concentration had no significant effect on PA viability.
Pre-opsonised PA was incubated with either DMSO (1 in 1000 final concentration) or Z-VAD or EIII (both at 20μM final concentration) for 30 min at 37°C at 8 rpm in the presence of 10% NHS. After 30 min cultures were processed for CFU analysis. This figure shows the inhibitors had no effect on PA viability. PA: *P. aeruginosa*. Results from duplicates of the same experiment.

5.3.4 Effect of inhibitors Z-VAD and EIII on the ability of macrophage/neutrophil co-cultures to control PA growth

To determine the effect of Z-VAD (pan-caspase inhibitor) and EIII (elastase inhibitor) on the ability of human macrophage/neutrophil co-cultures to control the growth of PA, inhibitors were incorporated at the time of adding neutrophils to the macrophage/neutrophil co-culture infection assay as detailed in Chapter 5.2.3. The effect of DMSO (1 in 1000 diluted final concentration) was also tested as a control. The results showed that the ability of the macrophages/neutrophil co-cultures to control PA growth was not affected by DMSO at this concentration (Figure 5.11). There was a trend towards an increase in the number of CFU in the macrophage/neutrophil co-cultures in the presence of EIII and Z-VAD although this was more obvious in the Z-VAD-treated samples (Figure 5.11, B). None of these differences reached statistical significance.

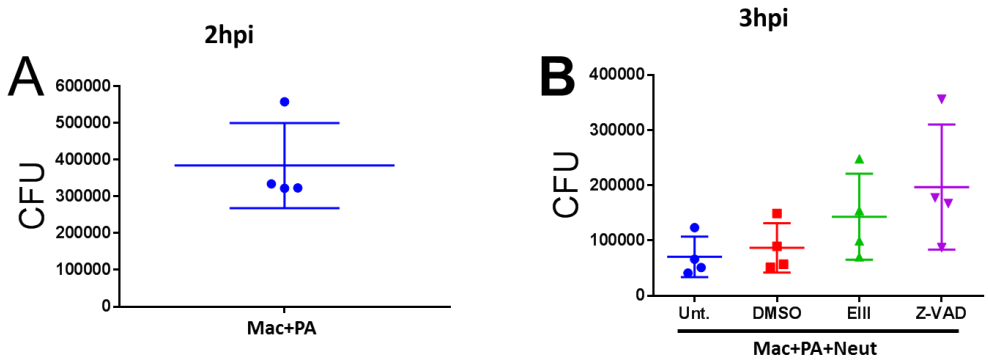


Figure 5.11: Effect of Z-VAD and EIII on the ability of macrophage/neutrophil co-cultures to control PA growth

PA was pre-opsonised with 10% NHS. Human macrophages (2 x10^5) generated in the presence of GM-CSF were infected with PA at MOI 1 in the presence of human serum for 2 h and samples obtained 2 hpi for CFU and supernatants. Freshly prepared neutrophils (5 x10^5, macrophage: neutrophil ratio 1:5) were added accordingly and either Z-VAD or EIII both at a 20μM final concentration or DMSO (1 in 1000 final concentration) were added at the same time as the neutrophils (2 hpi) and incubated further for 60 min. Samples were then processed again for CFU and supernatants at 3 hpi. **(A):** Represents the PA CFU in the presence of macrophages at 2 hpi. **(B):** Represents PA CFU at 3 hpi in the presence of macrophages and neutrophils and in the absence and presence of either of the inhibitors (Z-VAD or EIII) or DMSO. PA: *P. aeruginosa*, Mac: Macrophages, Neut: Neutrophils. Results from four independent experimental repeats.

5.3.5 Mechanism of enhanced IL-1β production in infected macrophage/neutrophil co-cultures compared to infected macrophages only cultures.

To determine the possible mechanism(s) involved in the increased amounts of IL-1β observed in the infected macrophage/neutrophil co-cultures compared to infected macrophages, the effect of the pan-caspase inhibitor Z-VAD and the neutrophil elastase inhibitor EIII on the ability of infected human macrophage/neutrophil co-cultures to promote IL-1β production was investigated alongside the production of IL-1α and TNF-α. For this, supernatants from infected macrophages only (2 hpi) and macrophage/neutrophil co-cultures incubated in the absence or presence of Z-VAD or EIII (3 hpi) were analysed for the presence of IL-1β, IL-1α, and TNF-α (Figure 5.12).

The results showed that there was a significant increase in the amount of IL-1β produced in the infected macrophage/neutrophil co-cultures (3 hpi) compared to infected macrophages only (2 hpi) in the cases of untreated-, DMSO- and EIII-treated conditions (Figure 5.12, IL-1β graph). However, the production of IL-1β was drastically reduced in the Z-VAD-treated conditions (Figure 5.12, IL-1β graph), implying that this increase in IL-1β upon neutrophil addition was depended on caspase activity.

There was a tendency to increased amounts of IL-1α in the infected macrophage/neutrophil co-cultures (3 hpi) compared to infected macrophages only (2 hpi) which reached statistical significance only for the EIII-treated conditions (Figure 5.12, IL-1α graph). Thus IL-1α production was not affected by caspase activity under our experimental conditions.

TNF-α production by the macrophage/neutrophil co-cultures was not affected by either Z-VAD or EIII (Figure 5.12, TNF-α graph).

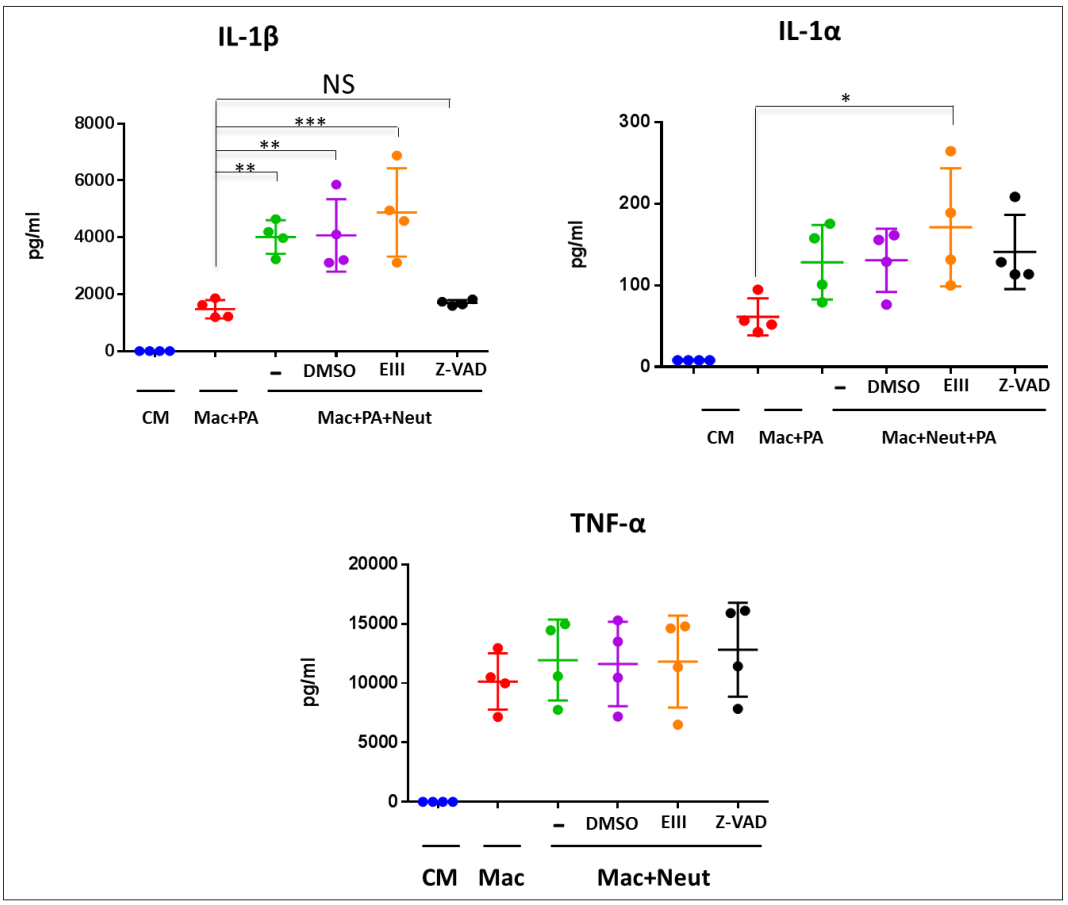


Figure 5.12: The increased IL-1β production by the infected macrophage/neutrophil co-cultures compared to infected macrophages only cultures is dependent on Caspase activity.

Human macrophages (2 x10^5) generated in the presence of GM-CSF were infected with PA at MOI 1 in the presence of human serum for 2 h and samples obtained at 2 hpi were processed for supernatants. Freshly prepared neutrophils (5 x10^5, macrophage: neutrophil ratio 1:5) were added accordingly and either Z-VAD or EIII both at a 20μM final concentration or DMSO (1 in 1000 final concentration) were added at the same time of adding the neutrophils (2 hpi) and incubated further for 60 min. Samples (3 hpi) were then processed for supernatants.. PA: *P. aeruginosa*, Mac: Macrophages, Neut: Neutrophils. Results from 4 different supernatants obtained from four independent experimental repeats. Data analysed using one-way ANOVA. NS = Not significant, * = p<0.05, ** = p<0.01, *** = p<0.001.

5.3.6 Effect of Z-VAD and EIII on neutrophil elastase release by macrophage/neutrophil co-cultures infected with PA

To investigate the ability of Z-VAD and EIII to modulate the release of neutrophil elastase by macrophage/neutrophil co-cultures infected with PA, supernatants obtained 3 hpi from the experiment described above in Chapter 5.2.3 were tested for the release of neutrophil elastase following the procedure described in Chapter 2.19. As shown in Figure 5.13, the release of neutrophil elastase by infected macrophage/neutrophil co-cultures was undetectable in the presence of the neutrophil elastase inhibitor (EIII). There was no difference in the amount of neutrophil elastase released by either untreated compared to DMSO or Z-VAD treated samples (Figure 5.13). Thus, the effectiveness of EIII as a neutrophil-elastase inhibitor was confirmed by this assay.

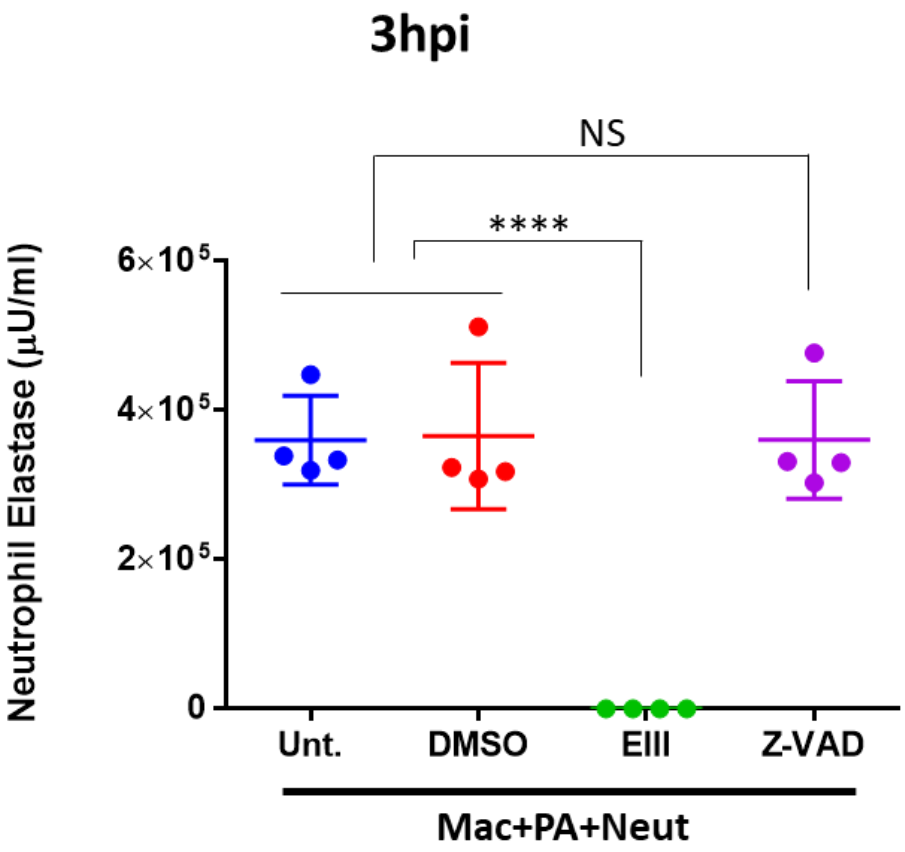


Figure 5.13: Effect of Z-VAD and EIII on the release of neutrophil elastase by macrophage/neutrophil co-cultures infected with PA.
Human macrophages (2 x10^5) generated in the presence of GM-CSF were infected with PA at MOI 1 in the presence of human serum for 2 h and half of the samples collected at 2 hpi. Freshly prepared neutrophils (5 x10^5, macrophage: neutrophil ratio 1:5) were added accordingly and either Z-VAD or EIII both at a 20µM final concentration or DMSO (1 in 1000 final concentration) were added at the same time as the neutrophils (2 hpi) and incubated further for 60 min. Samples were then processed for supernatants at 3 hpi. Supernatants obtained at 3 hpi were analysed for the release of neutrophil elastase. PA: *P. aeruginosa*, Mac: Macrophages, Neut: Neutrophils. Results from 4 different supernatants obtained from four independent experimental repeats. Data analysed using one-way ANOVA. **** = $p<0.0001$, NS = Not significant.

5.3.7 Effect of inhibitors (Z-VAD and EIII) on the release of LDH by macrophage/neutrophil co-cultures infected with PA

To test the effect of the inhibitors (Z-VAD and EIII) on LDH release by macrophage/neutrophil co-cultures infected with PA, supernatants from the experiments described above in Chapter 5.2.1 were analysed for the release of LDH following the manufacture's procedure as described in Chapter 5.2.6.

The results showed no significant increase in LDH release by infected macrophage/neutrophil co-cultures under untreated, DMSO and EIII-treated conditions (Figure 5.14, B). However, the release of LDH was significantly increased in the Z-VAD-treated conditions compared to untreated and DMSO-treated conditions (Figure 5.14, B).

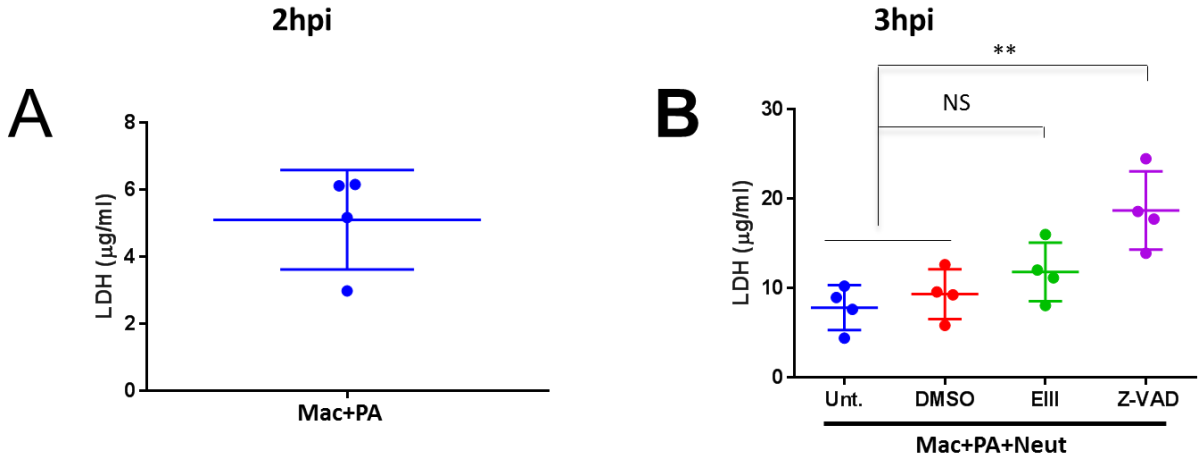


Figure 5.14: Effect of Z-VAD and EIII on LDH release by macrophages/neutrophil co-cultures infected with PA.
Human macrophages (2 x10^5) generated in the presence of GM-CSF were infected with PA at MOI 1 in the presence of human serum for 2 h and half of the samples taken off 2 hpi. Freshly isolated neutrophils (5 x10^5, macrophage: neutrophil ratio 1:5) were added and either Z-VAD or EIII both at 20 µM final concentration or DMSO (1 in 1000) were added at the same time of adding the neutrophils (2 hpi) and incubated further for 60 min. Samples were then processed for supernatants at 3 hpi. Supernatants obtained 3 hpi were analysed for the release of LDH. PA: *P. aeruginosa*, Mac: Macrophages, Neut: Neutrophils. Results from 4 different supernatants obtained from four independent experimental repeats. Data analysed using one-way ANOVA. ** = $p<0.01$, NS = Not significant.

5.3.8 Mechanism of differential IL-1β production by IFN-γ versus IL-17A treated cultures

As part of the investigations into the mechanisms involved in the increased IL-1β production upon addition of neutrophils which was curtailed by IFN-γ treatment (Figure 5.4). Macrophages only infections: uninfected or infected with PA with IFN-γ or IL-17A treatment were performed as described in Chapter 2.17, Figure 2.2. The samples were labelled for active caspase-1 using FAM-FLICA and processed for confocal microscopy as detailed in Chapter 5.2.7.

The results showed that there was active Caspase-1 in infected macrophages treated with either IFN-γ (Figure 5.15, A and A1) or IL-17A (Figure 5.16, A and A1). However, the presence of active caspase-1 (positivity for FAM-FLICA: green) was dependent on infection with PA as there were no active caspase-1 labelling in the uninfected IFN-γ- (Figure 5.15, C) and IL-17A (Figure 5.16, C)-treated macrophages. Meanwhile, there was no obvious difference in the amount of active caspase-1 present in the IFN-γ- versus IL-17A-treated macrophages (Figure 5.15 versus Figure 5.16).

Infections of macrophage/neutrophil co-cultures with PA with either IFN-γ or IL-17A treatment were also performed as described in Chapter 5.2.8. These samples were also labelled for active caspase-1 with FAM-FLICA only after addition of neutrophils as a means to identify the cell responsible for the increase in IL-1β production upon addition of neutrophils. Samples were also counterstained with both Rhodamine phalloidin (RP) and Hoechst as detailed in Chapter 5.2.7

The results showed that active caspase-1 was present in the infected macrophage/neutrophil co-cultures treated with either IFN-γ (Figure 5.17, A and A3) or IL-17A (Figure 5.17, B and B3). However, it appeared that there were more caspase-1 negative cells in the IL-17A-treated macrophage/neutrophil co-cultures compared to IFN-γ-treated macrophage/neutrophil co-cultures (Figure 5.17, B and B4) versus (Figure 5.17, A and A4), although it must also be noted that more of the IFN-γ treated cells adhered to the poly-lysine coated slides compared to IL-

17A-treated cells and hence, more cells were observed per field in the IFN-γ treated conditions.

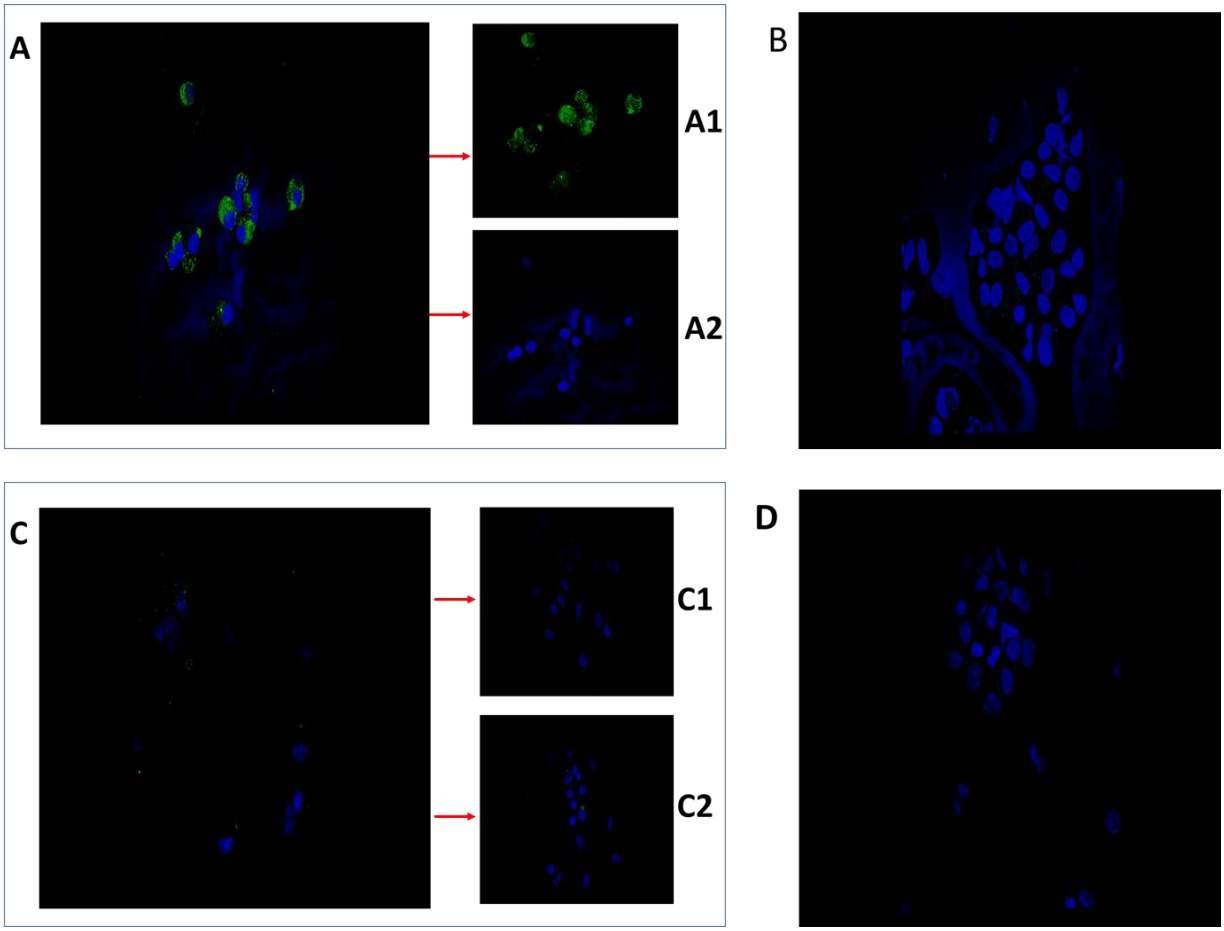


Figure 5.15: Confocal microscopy examination of caspase 1 activation in macrophages either uninfected or infected with PA in the presence of IFN-γ.
Human macrophages (2 x10^5) generated in the presence of GM-CSF were treated at day 6 with IFN-γ. On the following day, macrophages were collected and either left uninfected or infected with PA at MOI 1 in the presence of human serum for 2 h. 2 hpi samples were either left unlabelled or labelled with FAM-FLICA and incubated for 45min in the dark. 100 µl of sample from each condition was placed on a poly-lysine coated coverslip, mounted and observed under a 710 confocal microscope. Caspase-1 stained with FAM-FLICA **(green)** and nuclei stained with Hoechst **(blue)**. **(A):** Represents IFN-γ treated macrophages infected with PA and labelled with FAM-FLICA and Hoechst, **A** has been split into the individual single labels for FAM-FLICA **(A1)** and Hoechst **(A2). (B):** Represents IFN-γ treated macrophages infected with PA and labelled with only Hoechst showing just the nuclei stained. **(C):** Represents IFN-γ treated macrophages uninfected and labelled with FAM-FLICA and Hoechst, **C** has been split into **C1 and C2** and it shows that both have only the nuclei stained with Hoechst and that active Caspase-1 was not present**. (D):** Represents IFN-γ treated macrophages uninfected and labelled with only Hoechst showing just the nuclei stained. Images analysed with Volocity (X64 magnification) and the same settings were applied in processing all images.

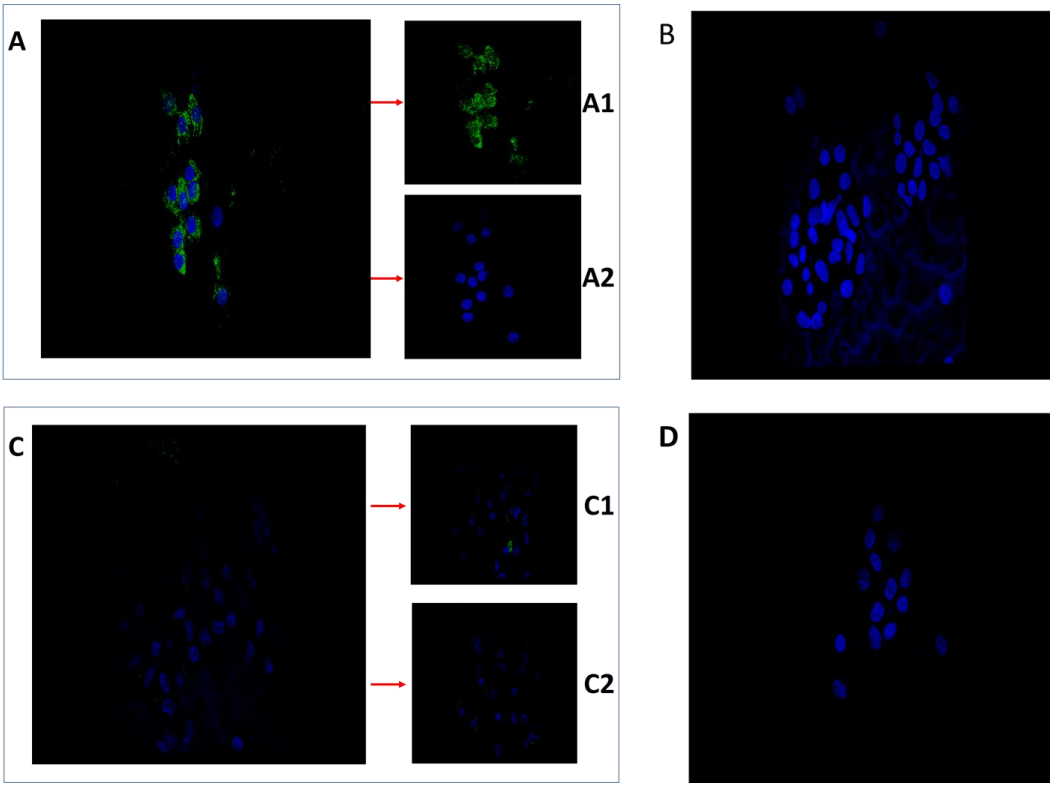
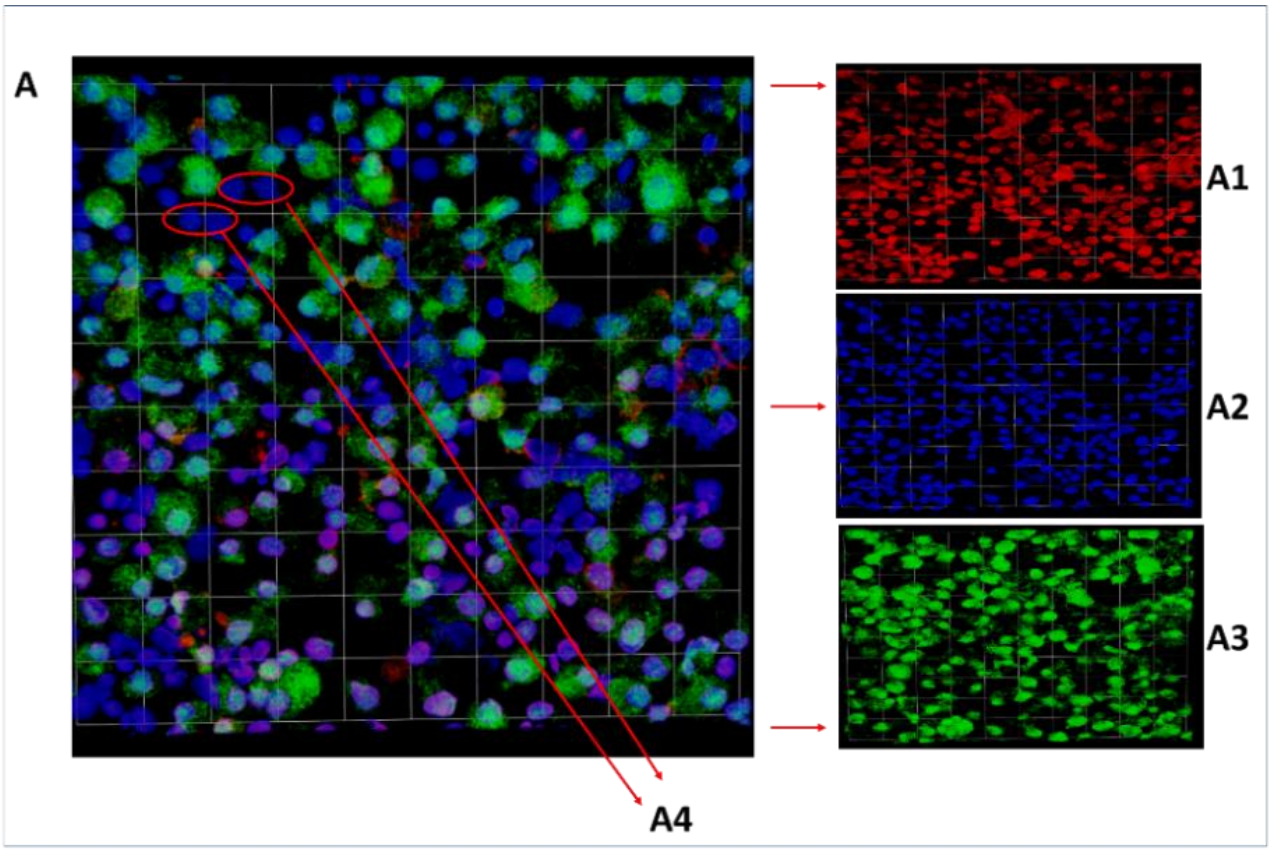


Figure 5.16: Confocal microscopy examination of caspase-1 activation in macrophages either uninfected or infected with PA with IL-17A treatment

Human macrophages (2 x10^5) generated in the presence of GM-CSF were treated at day 6 with IL-17A. On the following day, macrophages were collected and either left uninfected or infected with PA at MOI 1 in the presence of human serum for 2 h. 2 hpi samples were either left unlabelled or labelled with FAM-FLICA and incubated for 45min in the dark. 100 µl of sample from each condition was placed on a poly-lysine coated coverslip, mounted and observed under a confocal microscope. Caspase-1 stained with FAM-FLICA **(green)** and nuclei stained with Hoechst **(blue)**. **(A):** Represents IL-17A-treated macrophages infected with PA and labelled with FAM-FLICA and Hoechst, **A** has been split into the individual single labels for FAM-FLICA **(A1)** and Hoechst **(A2). (B):** Represents IL-17A-treated macrophages infected with PA and labelled with only Hoechst showing just the nuclei stained. **(C):** Represents IL-17A treated macrophages uninfected and labelled with FAM-FLICA and Hoechst, **C** has been split into **(C1 and C2)** and it can be seen that both have only the nuclei stained with Hoechst and that active caspase-1 was not present**. (D):** Represents IL-17A treated macrophages uninfected and labelled with only Hoechst showing just the nuclei stained. Images analysed with Volocity (X64 magnification) and the same settings were applied in processing all images.


A
A1
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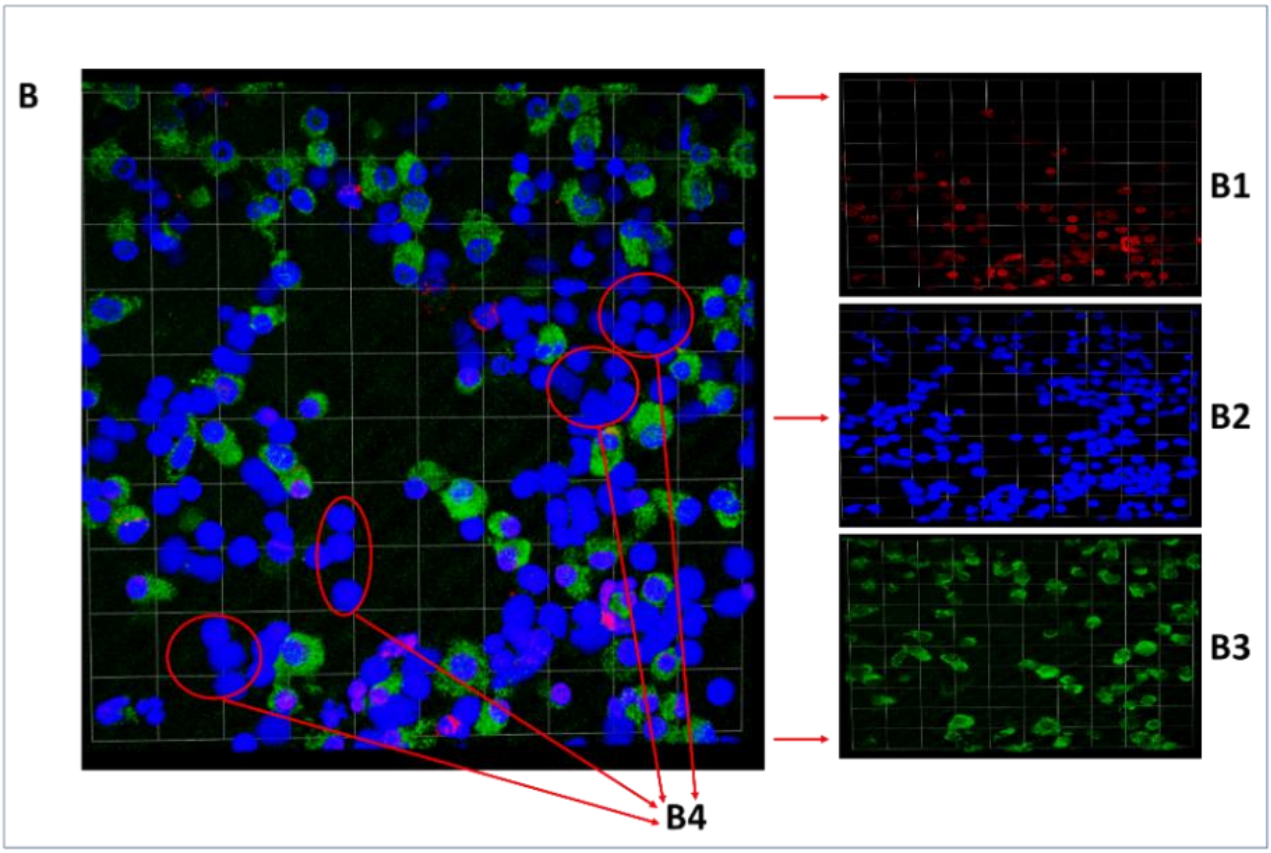

B
B1
B2
B3
B4

Figure 5.17: Confocal microscopy examination of caspase-1 activation in macrophage/neutrophil co-cultures infected with PA with either IFN-γ or IL-17A treatment.
Human macrophages (2 x10^5) generated in the presence of GM-CSF were treated at day 6 with either IFN-γ or IL-17A. On the following day, macrophages were collected and infected with PA at MOI 1 in the presence of human serum for 2 h. Freshly purified neutrophils (5 x10^5, macrophage: neutrophil ratio 1:5) were added accordingly and further incubated for 30 min. 2.5 hpi samples were either left unlabelled or labelled with FAM-FLICA and incubated for 45 min in the dark. 100 µl of sample from each condition was placed on a poly-lysine coated coverslip, mounted and observed under a confocal microscope. Actin was stained with Rhodamine phalloidin **(red)**, nuclei stained with Hoechst **(blue)**, caspase-1 stained with FAM-FLICA **(green)**. **(A):** Represents IFN-γ-treated macrophages/neutrophil co-cultures infected with PA and labelled with FAM-FLICA, Rhodamine-phalloidin and Hoechst. **A** has been split into the individual single labels for Rhodamine phalloidin **(A1),** Hoechst **(A2)** and FAM-FLICA **(A3)** in addition to a representation of cells which are negative for Caspase-1 **(A4).**
(B): Represents IL-17A-treated macrophages infected with PA and labelled with FAM-FLICA, Rhodamine-phalloidin and Hoechst. **B** has been split into the individual single labels for Rhodamine phalloidin **(B1),** Hoechst **(B2)** and FAM-FLICA **(B3)** as well as a representation of cells which are negative for Caspase-1 **(B4).** Images analysed with Zen 2.3 software and the same settings were applied in processing all images.

5.4 Discussion

This Chapter provides a comparison on the effect of IFN-γ and IL-17A on either macrophages or neutrophils or macrophage/neutrophil co-cultures upon infection with PA and some preliminary insights into potential mechanisms involved.

The initial observation of a possible anti-inflammatory property of IFN-γ by its ability to reduce the production of the pro-inflammatory cytokine IL-1β (detailed in Chapter 4.3.3 and Figure 4.3) was confirmed by testing the effect of both IFN-γ and IL-17A side by side within the same donor groups on the production of the cytokines IL-1β, IL-1α, MCP-1, MIP-1α, TNF-α, IL-6 and IL-8. These cytokines were selected due to their significant roles in the immune defence against invading pathogens and precisely against PA as already discussed in detail in Chapter 4. Our study showed that IL-1α and IL-1β were consistently increased whenever neutrophils were added to the infected macrophage cultures. The reason for the increased IL-1β and IL-1α secretions with neutrophils addition to the infected macrophage cultures might be as a result of neutrophils orchestrating the inflammatory response to PA infections by the release of cytokines, chemokines and neutrophil proteases (Pham, 2008). As mentioned earlier, neutrophil derived proteases (such as elastase, cathepsin G and proteinase 3) are capable of processing both pro-IL-1β and pro-IL-1α to the active forms (Afonina *et al.*, 2015) . Thus, neutrophils in our infection model might release their proteases to aid in the processing of IL-1β and IL-1α. Although our data showed that there was no difference in neutrophil elastase tested under all conditions, other neutrophil proteases might be involved in this process and hence future studies involving the test for other neutrophil proteases might be more informative.

The work of Patankar and colleagues reported enhanced levels of IL-1β in the presence of neutrophils in mouse models of acute PA infections (Patankar *et al.*, 2015). Patankar and colleagues also demonstrated that the production of IL-1β was dependent on neutrophils but independent of the inflammasome adaptor ASC (Patankar *et al.*, 2015). Karmakar and colleagues also reported increased IL-1β levels mediated by neutrophils in a mouse model of

corneal infections with PA (Karmakar *et al.*, 2012). This group showed as well that the production of IL-1β was independent on NLRC4 and ASC but rather reliant on serine proteases in both mouse and human neutrophils infected with PA (Karmakar *et al.*, 2012). The involvement of proteases in the processing of IL-1β has been reported by a number of researchers (Dinarello, 1996; Coeshott *et al.*, 1999) as detailed earlier in Chapter 5.1 and is associated with an increased inflammatory response (Afonina *et al.*, 2015). However, our data demonstrated that the increase in IL-1β production by neutrophil addition to the infected macrophage cultures was dependent on caspase-1 and not neutrophil elastase (Figure 5.12). This is consistent with the finding that there were constant levels of neutrophil elastase released under all conditions with or without IFN-γ or IL-17A treatment regardless of the fact that the levels of IL-1β produced were significantly decreased with IFN-γ compared to IL-17A treatment.

It was clear that IL-17A either did not affect or slightly increase cytokine production by either the macrophages or macrophage/neutrophil co-cultures as values with IL-17A treatment tended to tally with untreated conditions or were slightly more than r untreated conditions suggesting that IL-17A does not significantly affect cytokine production by macrophages and macrophage/neutrophil co-cultures in response to infections with PA. The treatment of macrophages with IFN-γ led to reduced production of IL-1α and IL-1β compared to untreated co-cultures although these were only statistically significant for IL-1α, but the reduction in IL-1β was more evident and statistically significant when comparing IL-17A and IFN-γ-treated cultures (Figure 5.4). IFN-γ also increased MCP-1 production (Figure 5.5) and promoted increase TNF-α in some of the donors in the macrophage cultures (Figure 5.6).

This study is the first to demonstrate the ability of IFN-γ to dampen the positive effect of neutrophils on IL-1β and IL-1α release by macrophages/neutrophils co-cultures. This suggests a potential anti-inflammatory property of IFN-γ under the assay conditions. This finding could be due to the combined effect of reduced production of IL-1β by the macrophages (Figure 5.4, infected IL-1β graph, 2 hpi) in the presence of this cytokine and reduced neutrophil activity

when in co-culture with IFN-γ-treated macrophages as suggested by their reduced killing capacity under these conditions (Figure 4.1). IFN-γ-treated macrophages could decrease neutrophils life span as a means to control inflammation (Nandi *et al.*, 2011). This would be in agreement with IFN-γ increasing the production of MCP-1 by macrophages as MCP-1 promotes monocyte recruitment which is considered a hallmark of the resolution of inflammation (Soehnlein *et al.*, 2010).

Indeed, the anti-inflammatory property of IFN-γ has been demonstrated by a number of studies. IFN-γ is generally known for its pro-inflammatory activity but some studies have shown some intriguing anti-inflammatory properties of IFN-γ. For instance, previous studies have shown the ability of IFN-γ to suppress IL-1β production from synovial fluid macrophages in patients suffering from rheumatoid arthritis (Ruschen *et al.*, 1989). Similarly, the work of Mayer-Barber and colleagues (Mayer-Barber *et al.*, 2011b) shows the ability of IFN-γ to significantly inhibit IL1β production in mice infected with *M. tuberculosis* (Mayer-Barber *et al.*, 2011b). Furthermore, the production of IL-1β induced by silica was significantly dampened by IFN-γ (Sihvola *et al.*, 1989).

Indeed, the modulation of IL-1 and IL-18 has been targeted for therapy based on their involvement in inflammatory diseases (Dinarello, 1996; Gracie *et al.*, 2003). IFN-γ has been shown to exert its anti-inflammatory properties by a number of mechanisms including the release of cytokine antagonists such as IL-1RA (Dinarello, 1996) and IL-18BP (Dinarello, 2000). Both IL-1RA and IL-18BP are secreted natural occurring inhibitors specific for IL-1 and IL-18 respectively (Muhl *et al.*, 2003). Indeed, abundant IL-1RA was detected in our assays although no conclusions could be drawn because the values were above the standard of the assay (Figure 4.4).

IFN-γ is reported to increase the release of IL-1RA in patients suffering from renal cell carcinoma who are undergoing IFN-γ therapy (Tilg *et al.*, 1993). Other studies have also reported an enhanced IL-1RA release in human chondrocytes treated with IFN-γ (Henrotin *et al.*, 2000), as well as in neutrophils (McDonald *et al.*, 1998) and in airway epithelial cells

(Levine *et al.*, 1997). Similarly, IFN-γ is shown to induce the production of IL-18BP in PBMC (Corbaz *et al.*, 2002), monocytes (Veenstra *et al.*, 2002) and hepatocytes (Hurgin *et al.*, 2002). Another mechanism by which IFN-γ exerts its anti-inflammatory properties is by inducing the expression of suppressors of cytokine signalling (SOCS) as well as the induction of apoptosis (Muhl *et al.*, 2003).

Furthermore, our data showed that the neutrophils alone did not produce any of the cytokines tested under our experimental conditions except for IL-8, and the IL-8 production was not affected significantly by IFN-γ or IL-17A. The lack of IL-1β production by PA-infected neutrophils in this study was quite unexpected as IL-1β production by human neutrophils in response to infections with PA has been previously reported (Karmakar *et al.*, 2012). Correspondingly, human neutrophils have been shown to express the NLRC4 inflammasome which is upregulated by lipopolysaccharide (Bakele *et al.*, 2014) and so we expected an increase in IL-1β production by neutrophils in response to PA infections through the activation of the NLRC4 inflammasome. Meanwhile, our data showed that isolated neutrophils were effective in controlling PA growth and also produced IL-8 which indicates that they could sense and respond to the presence of the bacterial infection. Our findings may be due to low cytokine production per cell basis observed in neutrophils (Nathan, 2006) which could be compensated for *in vivo* by the large numbers of neutrophils recruited to infection sites making them important inflammatory mediators (Mantovani *et al.*, 2011).

Based on the initial findings of less PA clearance and less production of IL-1α and IL-1β in the presence of IFN-γ by the macrophage/neutrophil co-cultures coupled with the marginal increase in PA clearance and higher production of IL-1α and IL-1β in the presence of IL-17A by the macrophage/neutrophil co-cultures, we proposedcell death in the presence of IFN-γ as one of the possible mechanisms behind this. Hence we tested for LDH in our assay supernatants but surprisingly, there was no difference in LDH production under all infected conditions of either IFN-γ or IL-17A treatment (Figure 5.8). The difference in LDH released between uninfected and infected cultures was tested only in one of the donors due to inadequate samples and this showed no difference in LDH released from uninfected versus

infected conditions (Figure 5.9). LDH is a cytoplasmic enzyme which is soluble and present in all cells; the damage of cell plasma membrane causes the release of LDH into the extracellular space (Chan *et al.*, 2013). The assay principle is based on the ability of LDH to first convert nicotinamide adenine dinucleotide (NAD+) to reduced nicotinamide adenine dinucleotide (NADH) by catalysing the oxidation of lactate to pyruvate and then the transfer of hydrogen ions from NADH by a catalyst to a tetrazolium salt: INT which becomes reduced into a formazan. The amount of LDH released is directly proportional to the amount of coloured formazan measured and hence also directly proportional to the amount of cell damage (Chan *et al.*, 2013). Our data showing no difference in LDH release among all conditions as all the values were within the variability range, was perhaps due to the timing of supernatant collection and the low bacteria MOI used. Meanwhile, the finding is consistent with the work of Singh (Singh, 2012) in which differences in LDH between uninfected and infected macrophages with PA at MOI 1 were observed only after 2 hpi. However, another study by Sutterwala and colleagues (Sutterwala *et al.*, 2007a) shows a drastic macrophage death after 1 hpi with PA at MOI 20. The contrast between the finding of Sutterwala and colleagues and ours is most likely due to the high MOI used by Sutterwala and colleagues.

In search of potential mechanisms behind our observation of an increased IL-1β in the macrophage/neutrophil co-culture which was curtailed by IFN-γ, we employed Z-VAD (a pan-caspase inhibitor) and EIII (a neutrophil elastase inhibitor) in our infection assays. Since IL-1 β is produced as a pro-cytokine which needs to be processed to the active IL-1β by either caspase-1 or proteases (as detailed above in the introductory section in Chapter 5, refer to Chapter 5.1), we looked at the effect of inhibiting either the activity of caspase-1 by Z-VAD or inhibiting the activity of neutrophil elastase by EIII during the incubation with neutrophils on our infection assays. It was previously confirmed that these inhibitors did not have a direct cytotoxic effect on PA viability (Figure 5.10). However, the ability of macrophage/neutrophil co-culture to control PA growth was reduced especially by Z-VAD although this was not statistically significant (Figure 5.11). Meanwhile, LDH release by infected

macrophage/neutrophil co-cultures were significantly higher in the presence of Z-VAD (Figure 5.14). This might be due to the cells undergoing necroptosis in the presence of Z-VAD. Necroptosis (a programmed form of necrosis where cells undergo a caspase-1 independent form of cellular suicide (Moreno-Gonzalez *et al.*, 2016)) has been described by a number of researchers to occur with Z-VAD treatment in the presence of TNF-α which is produced in our assay conditions (Figure 5.6). TNF-α usually induces cell death by apoptosis (Nagata, 1997). In apoptosis (programmed cell death) the integrity of the cell membrane is retained even after cells disintegrate into apoptotic bodies (Wyllie *et al.*, 1980). The apoptotic cells are taken up by macrophages to avoid their lysis and a consequent inflammatory process (Savill, 1997). In contrast, the cell membranes of cells dying by necrosis is easily lost with a consequent induction of inflammation (Wyllie *et al.*, 1980). Meanwhile, apoptosis induction by TNF-α requires the activation of caspases and accordingly, studies have shown the blockage of TNF-α-induced apoptosis by caspase inhibitors in HeLa and MCF7 tumour cell lines (Miura *et al.*, 1995). However, the use of pan-caspase inhibitors such as Z-VAD-FMK and OMe-FMK are reported to increase TNF-α-induced cell death in mouse fibroblast and in human promonocytic U937 cells (Li *et al.*, 2000). Consistent with our findings is also the study of Wu and colleagues (Wu *et al.*, 2011) which showed that Z-VAD induces necroptosis of L929 cells in the presence of TNF-α. Accordingly, they also showed that the necroptosis induced by Z-VAD was actually inhibited by blocking TNF-α signalling. Since the production of IL-1β in our study was radically reduced in the presence of Z-VAD but not affected by EIII, this confirms the dependency of IL-1β production on caspase activity but not neutrophil elastase under our assay conditions. On the other hand, the use of Z-VAD in our assays also confirmed that IL-1α production was independent of caspase-1 activity (Figure 5.12, IL-1α graph). Indeed, the idea that IL-1α does not require caspase-1 proteolytic cleavage has long been suggested (Howard *et al.*, 1991; Groß *et al.*, 2012). It was initially suggested that IL-1 α does not require any proteolytic cleavage to function based on the study of Mosley and colleagues where they showed that both the inactive pro-IL-1α and the active form (IL-1α) can bind IL-1RA and show bioactivity (Mosley *et al.*, 1987b). However, further studies by the same group (Mosley *et al.*, 1987a) and

others later showed that the proteolytic cleavage of pro-IL-1α by inflammatory proteases (such as granzyme B, elastase and chymase) to the active form (IL-1α) makes it far more biologically active than the pro- IL-1α (Afonina *et al.*, 2011). Another study has proposed that IL-1α processing might be either dependent or independent on caspase-1 and the NLRP3-inflammasome depending on the type of stimuli activating the inflammasome (Yazdi *et al.*, 2013). However, the study of Al Moussawi and colleagues (Al Moussawi *et al.*, 2014) showed that IL-1α production upon infections with PA was independent of caspase-1 and this is consistent with our data. Thus the dependency of IL-1α on caspase-1 activity might not only be due to the type of stimuli activating the inflammasome but also the type of pathogen and the type of inflammasome as well (as PA uses the NLRC4 inflammasome as detailed earlier). Also, the use of the pan caspase inhibitor Z-VAD showed that there was no significant effect in IL-1α (Figure 5.12, IL-1α graph). This contrast with the work of England and colleagues (England *et al.*, 2014) where they showed that necroptosis induced by Z-VAD in mice bone marrow derived macrophages induced IL-1α processing and release independent of IL-1β processing (England *et al.*, 2014). They also showed that IL-1β release was rather dependent on apoptosis in a caspase-8 dependent manner (England *et al.*, 2014). The contrast in the study of England and colleagues with regards to necroptosis increasing IL-1α release and our results are likely due to the difference in experimental models.

Our data from the confocal analysis of FAM-FLICA labelled samples confirmed that positivity for caspase-1 activity was dependent on infections with PA. The nuclei staining with Hoechst gave an indication of the presence and numbers of cells per field observed. Meanwhile, there were no obvious differences between IFN-γ and IL-17A-treated cultures. Interestingly, however, the confocal analysis of FAM-FLICA labelled infected macrophage/neutrophil co-cultures in the presence of either IFN-γ or IL-17A showed the presence of caspase-1 in both samples, but there seemed to be more cells being negative for FAM-FLICA in the IL-17A treated conditions (Figure 5.17). This observation was highly unexpected as there were more IL-1β produced in the IL-17A treated conditions compared to IFN-γ treated conditions (Figure

5.4). Further studies undergoing in our laboratory seeks to quantify caspase-1 under the various treatment for more insight to the current observation. Considering the fact that the FAM-FLICA used was specific for caspase-1, we had expected a direct proportionality between the IL-1β observed from the supernatants and caspase-1 from the FAM-FLICA labelling. One possible explanation behind our observation is that perhaps there were not enough pro-IL-1β to be processed by caspase-1 to the active IL-1β in the IFN-γ treated conditions which led to less IL-1β being measured from the supernatants. One way to confirm this will be to test for pro-IL-1β and active IL-1β by western blot in the cultures. It may also be that the processing of IL-1β under the IFN-γ treated conditions were ineffective compared to the IL-17A treated conditions or that the neutrophils under the IFN-γ treated conditions were undergoing apoptosis (Muhl *et al.*, 2003) and hence unable to shoot up IL-1β production. Another possibility is that under IFN-γ treated conditions, the function of caspase-1 is not primarily to convert pro-IL-1β to active IL-1β but rather to induce cell death as an anti-inflammatory mechanism (Muhl *et al.*, 2003). Besides the function of caspase-1 in converting pro-IL-1β to active IL-1β, it also induces a rapid form of programmed cell death called pyroptosis (Denes *et al.*, 2012) as mentioned earlier. Pyroptotic cell death helps in removing the bacteria niche by the demise of the infected cells (Denes *et al.*, 2012). Apoptosis contrast from pyroptosis in the fact that, while pyroptosis is dependent on caspase-1, apoptosis is dependent on apoptotic caspases (i.e. caspase-2, 3, 6, 7, 8, 9, 10) (Siegel, 2006; Sollberger *et al.*, 2014). Furthermore, during pyroptotic cell death, cells swell and rupture their cytoplasmic membranes while maintaining the integrity of their mitochondrial membranes (Sollberger *et al.*, 2014). This consequently increases the release of inflammatory mediators such as pro-IL-1α, high mobility group box 1 (HMGB1) protein, ATP, etc. which leads to neutrophils recruitment and induction of inflammation (Sollberger *et al.*, 2014). Contrarily, apoptotic cells attract macrophages to phagocytose them without inducing an inflammatory response (Sollberger *et al.*, 2014).

In a study by Sutterwala and colleagues (Sutterwala *et al.*, 2007b), using mice models showed that a PA strain lacking the T3SS effector protein ExoU caused macrophage death in an IPAF and caspase-1-dependent manner while inducing IL-1β and IL-18 secretions. The group also showed that infections of macrophages with ExoU efficient PA strain caused macrophage death independently of caspase-1, and that ExoU had the ability to inhibit caspase-1 activity with a resultant inhibition of IL-1β and IL-18 secretions (Sutterwala *et al.*, 2007b). Since the strain of PA used in this study (i.e.PAO1) is known to lack the PA T3SS: ExoU but expresses only ExoS, ExoT, and ExoY (Stover *et al.*, 2000; Vance *et al.*, 2005), macrophages and /or neutrophils may be undergoing a caspase-1-dependent cell death in the presence of IFN-γ in our assay conditions.

However, some studies have shown the induction of caspase-1 dependent pyroptosis which was independent of IL-1β processing in infections with *S. typhimurium* (Monack *et al.*, 2001) and in infections with NLRC4-activating pathogens (Miao *et al.*, 2010). Likewise, mouse peritoneal macrophages treated with LPS are shown to undergo a caspase-1 dependent cell death which was entirely independent from IL-1 secretion (Le Feuvre *et al.*, 2002). Thus these studies support a direct involvement of caspase-1 in cell death. Meanwhile, there is no evidence yet for caspase-1 involvement in direct cell death in human cells but the current findings in our study might be a starting point for such investigations. However, since there was no difference in the levels of LDH in our assay conditions (Figure 5.8 and Figure 5.9), the type of cell death seen in our assays (confocal microscopy analysis) is more likely to be apoptosis rather than pyroptosis or necrosis as both pyroptosis and necrosis are characterised by the release of LDH (Reisetter *et al.*, 2011; Rayamajhi *et al.*, 2013), but more work will be needed to confirm this. Future studies involving the use of specific caspase-1 inhibitors (such as Y-VAD) along with TNF-α blockers (to avoid necroptosis) will confirm if the cell death occurs and is actually dependent on caspase-1 or not. Also, microscopic analysis of cell membrane integrity can reveal the type of cell death in our assays as both necrotic and pyroptotic cells lose their membrane integrity while apoptotic cells do not (as described earlier).

5.5 Summary and conclusions

This Chapter demonstrates the potential anti-inflammatory property of IFN-γ in the context of PA infection by comparing the differences between IFN-γ and IL-17A dominated inflammation on IL-1β production and perhaps an induction of apoptosis by IFN-γ in macrophages and neutrophils infected with PA.

For samples to be obtained for CFU and supernatants, monocyte-derived macrophages were pre-treated with either IFN- γ or IL-17A for 24 h prior to infections. Treated or untreated human macrophages were infected with PA at MOI 1 and incubated for 2 h. Half of the samples were obtained 2 hpi for CFU and/or supernatants and freshly isolated neutrophils were added at this time. Samples were further incubated for 60 min and samples obtained 3 hpi again for CFU and/or supernatants. Supernatants were frozen at -80^{o}C until ready for analysis for either cytokines, LDH or neutrophil elastase.

For samples to be obtained for confocal microscopy, again monocyte-derived macrophages treated with either IFN- γ or IL-17A for 24 h prior to infections were used. Human macrophages were initially infected with PA at MOI 1 and incubated for 2 h and samples labelled with FAM-FLICA and incubated for 45 min before processing for confocal microscopy (i.e. in the case of macrophage only infections). But for macrophage/neutrophil co-cultures, half of the samples were removed 2 hpi and freshly isolated neutrophils were added to the other half. Macrophage/neutrophil co-culture samples were further incubated for 30 min before labelling with FAM-FLICA and incubated for 45 min before samples were obtained and processed for confocal microscopy. The following conclusions could be drawn from the results obtained:

1. Infections with PA increased cytokine production by macrophages and macrophage/neutrophil co-cultures.

2. Macrophages and neutrophils collaborate for the production of IL-1β and IL-1α, there was increased IL-1β production after addition of neutrophils to the macrophage cultures and this was dependent on caspase activity.

3. There was a significant reduction of IL-1β and IL-1α in the presence of IFN-γ compared to IL-17A.

4. IFN-γ had a tendency to increase TNF-α production in macrophages in some donors but there was no effect with neutrophil addition to the macrophage cultures.

5. IFN-γ drastically increased MCP-1 production in macrophages with no effect observed with neutrophils addition to the macrophages.

6. There was no significant effect of IFN-γ and IL-17A in IL-6 and MIP-1α production in both macrophages and macrophage/neutrophil co-cultures.

7. IL-17A either did not affect or slightly increased cytokine production by either the macrophages or macrophage/neutrophil co-cultures with values of IL-17A treatment tallying with untreated conditions or slightly more than untreated conditions.

8. The neutrophils alone did not produce any of the cytokines tested under our experimental conditions (IL-1α, IL-1β, TNF-α, IL-6, MCP-1 and MIP-1α) except IL-8, and the IL-8 production was neither affected significantly by IFN-γ or IL-17A.

9. There was no LDH release by PA only control conditions both with and without IFN-γ or IL-17A.

10. LDH was released by infected macrophages, neutrophils and macrophage/neutrophil co-cultures with no difference under all treatments.

11. LDH released in uninfected versus infected conditions of macrophages, neutrophils and macrophage/neutrophil co-cultures were not significantly different under all conditions although this was only with one donor sample.

12. The pan-caspase inhibitor (Z-VAD) and the neutrophil elastase inhibitor (EIII) had no effect on PA viability.

13. There was a tendency to increased CFU of PA with macrophage/neutrophil co-culture conditions in the presence of Z-VAD.

14. There was a significant increase in LDH released by macrophage/neutrophil co-cultures in the presence of Z-VAD (indicative of necroptosis).

15. The production of neutrophil elastase was drastically declined by the neutrophil elastase inhibitor EIII confirming the specificity of the inhibitor

16. The detection of caspase-1 activity using FAM-FLICA was dependent on infection with PA.

17. There were no obvious differences in FAM-FLICA signal between infected macrophages in the presence of IFN-γ or IL-17A.

18. There seemed to be more cells showing negativity for FAM-FLICA labelling in the IL-17A-treated macrophage/neutrophil co-cultures compared to IFN-γ treated macrophage/neutrophil co-cultures.

Chapter 6

General discussion and future directions

Chapter 6: General discussion and future directions

Numerous researchers have investigated the interaction between PA and immune cells, but most of these studies focused on animal models. The strength of this study lies in the use of human cells and also the use of a co-culture model comprising of macrophages and neutrophils. Indeed, this study is the first to give an insight on the collaboration between human macrophages and neutrophils in controlling the opportunistic pathogen PA. The findings from this study provide a better understanding of PA pathogenesis and possible therapeutic targets.

The study employs a macrophage/neutrophil co-culture infection model which aims to represent an *in vitro* model of an acute PA infection. The model takes into consideration the contribution of complement activation by the use of normal human serum in opsonising the bacteria. The initial addition of macrophages symbolises the initial involvement of tissue resident macrophages as sensors and triggers of inflammation. The inflammatory products released by these infected macrophages could modulate the function of the neutrophils that were subsequently added. Although the neutrophils are known for their effective antimicrobial activity, they can also cause tissue damage if not properly controlled by releasing neutrophil proteases (Pineda *et al.*, 2010; Serhan, 2010; Soehnlein *et al.*, 2010; Jenner *et al.*, 2014). The use of a higher neutrophil to macrophage ratio intended to mimic the higher numbers of neutrophils *in vivo* at infection sites although our ratio of neutrophil: macrophage 5:1 was not close to the massive numbers of neutrophils at infection sites (Soehnlein *et al.*, 2010). The MOI of 1 was chosen to enable following up infection with time as higher bacteria numbers were cytotoxic to the macrophages, and the timing was chosen because at 2 hpi, our preliminary data had shown that the macrophages had already produced a number of cytokines and chemokines that could modulate the neutrophilic response. The use of the various cytokine treatments versus untreated allowed us to investigate the immune response to PA infection under different inflammatory conditions. Meanwhile, the model had its limitations such as not considering neutrophil migration and the enormous numbers of neutrophil recruited to inflammation sites (Soehnlein *et al.*, 2010) as well as the use of only

one strain of PA. However, this model still serves as a solid foundation which can be improved by the use of different isolates of PA, use of PA biofilms, incorporation of neutrophil migration, using varying cell densities and sampling at varied time points.

Based on our initial observation of a positive correlation between IFN-γ produced from PBMCs from CF patients with lung function and a negative correlation between IL-17A and lung function (Singh *et al.*, 2015), we hypothesised that IL-17A-treated macrophage/neutrophil co-cultures would enhance the clearance of PA but with a potential to increasing tissue damage by increasing the release of enzymes such as neutrophil elastase (Sutherland *et al.*, 2014), which would be in agreement with the detrimental role of Th-17 cells in chronic inflammation. IFN-γ on the other hand, was proposed to enhance the ability of neutrophils to clear PA infections in the macrophage/neutrophil co-cultures through the exposure of neutrophils to a more pro-inflammatory cytokine milieu produced as a result of the initial immune response mounted by the macrophages (Singh *et al.*, 2015), this we believed would be alongside minimising tissue damage by reducing neutrophil elastase release. But rather, our data showed that neutrophil elastase was released by infected neutrophils and infected macrophage/neutrophil co-culture conditions but the release of neutrophil elastase was neither affected by IFN-γ nor IL-17A treatment. The specificity of the assay was proven by the fact that the release of neutrophil elastase was dependent on the presence of neutrophils as conditions of infected macrophages only and PA only were negative for neutrophil elastase activity.

It was clear that there was a marginal increase in PA clearance by the IL-17A-treated macrophage/neutrophil co-cultures (Figure 4.11). This was most likely due to a direct effect of IL-17A on neutrophils as IL-17A significantly increased PA clearance by neutrophils (Figure 4.15). To the best of our knowledge, this is the first study to demonstrate such findings. More work will however be needed in the future to determine possible mechanisms behind this observation but in the meantime, preliminary data indicate that it could possibly be associated with increased phagocytosis of PA by neutrophils in the presence of IL-17A-treatment as there

appeared to be more bacteria inside neutrophils with IL-17A-treatment (Figure 4.10, B) compared to without IL-17A-treatment in our assays (Figure 4.10, A).

Our study has also revealed that IFN-γ reduced the ability of macrophage/neutrophil co-cultures to control PA growth (Figure 4.1) without directly affecting the ability of neutrophils to clear PA infections (Figure 4.5). This might be due to IFN-γ-treated macrophages displaying its anti-inflammatory properties (Muhl *et al.*, 2003; Sun *et al.*, 2008) and subsequently dampening the activity of neutrophils in the macrophage/neutrophil co-cultures as discussed earlier in Chapter 5. The exact mechanism(s) behind this observation is however unclear but still under investigation in our laboratory. In the meantime, one possible mechanism to explain the above observation may be due to the reduced levels of IL-1β detected in our assays in the presence of IFN-γ as the reduction of pro-inflammatory cytokines has been earlier reported as one of the anti-inflammatory mechanisms of IFN-γ (Muhl *et al.*, 2003). The levels of IL-1β and IL-1α were increased in infected macrophage/neutrophil co-cultures compared to infected macrophages only cultures (Figure 5.4), notwithstanding the fact that supernatants and bacteria from these macrophage/neutrophil co-cultures were diluted two fold by the addition of neutrophils to the macrophage cultures. Although NF-κB is the key transcription factor which controls the induction of pro-IL-1β, caspase-1 is the major protease involved in the processing of pro-IL-1β to the active IL-1β through the activation of the inflammasomes (Guo *et al.*, 2015) as detailed earlier in Chapter 5. PA induces IL-1β production through the NLRC4 inflammasome (Sutterwala *et al.*, 2007b). The study of Schultz and colleagues has shown that deficiency of IL-1β and treatment with IL-1RA were protective against PA (Schultz *et al.*, 2002). Schultz and colleagues also showed that the deficiency of IL-1β correlated with decreased neutrophil recruitment. This is consistent with another study by Cohen and colleagues who reported an improved PA clearance and decreased lung pathology in murine models of acute pneumonia with the deletion of NLRC4, caspase-1 inhibition and the deletion of IL-1R and IL-18R (Cohen *et al.*, 2013). A chronic PA infection could be favoured by a neutrophilic inflammatory response by promoting biofilm formation which could hinder bacterial clearance

(Walker *et al.*, 2005). Therefore, IFN-γ could be protective in CF in spite of not promoting bacterial clearance through its direct effect on IL-1β production and possibly induction of neutrophil apoptosis (as hinted in the confocal microscopy and reduced killing of PA under FN-γ treated conditions). Thus the beneficial effect of IFN-γ in CF could be by promoting a more balanced neutrophilic inflammatory response during infection with extracellular bacteria such as PA, which could protect organ function.

Inhibition of IL-1β production by IFN-γ during infection has been shown in other infection models (Mayer-Barber *et al.*, 2011a). Inflammasome activation and IL-1β secretion in response to PA are preserved in CF immune cells, therefore these findings are relevant to an understanding of lung infection and inflammation in CF (Tang *et al.*, 2012). The contribution of IL-1β to PA pathogenesis may however be dependent on the infection model as IL-1β has been shown to promote bacterial clearance during corneal infections with PA (Karmakar *et al.*, 2012).

Our findings from the use of the pan-caspase inhibitor Z-VAD and the neutrophil elastase inhibitor EIII confirms that IL-1β production in our assays was dependent on caspase-1 activity and not neutrophil elastase. This however does not exempt the possibility of other neutrophil proteases involved in IL-1β processing, but more work will be needed in future studies to clarify this. Also, the cellular source of caspase-1 is currently not clear from the current study but future work involving microscopic and biochemical analysis of caspase activation, pro-IL-1β processing and testing for the requirement for cell contact may clarify this. In the meantime, our preliminary data from the confocal microscopy examination of active caspase-1 labelling showed the infected macrophages to be positive for caspase-1 as seen in the macrophage only cultures (Figure 5.15 and Figure 5.16). The difference between the macrophages and neutrophils in the macrophage/neutrophil co-cultures were not apparent and thus, future studies involving labelling for the individual cells will give more insight on this.

Previous studies where IFN-γ and IL-17A have been targeted as therapeutic agents have been reported. Indeed, the use of IFN-γ as therapy has been under study for a number of diseases including mycobacterial infections, mycoses, rheumatoid arthritis, atopic dermatitis and chronic granulomatous disease (CGD) (Åhlin *et al.*, 1999). Even though some data on diseases like atopic dermatitis are not convincing, results on the use of IFN-γ in CGD has been very convincing and the use of IFN-γ therapy in CGD is accepted and commonly used (Åhlin *et al.*, 1999).

The IL-17 family of cytokines although protective against invading microorganisms, have also been implicated in a number of pathological conditions involving chronic inflammation and autoimmune diseases including multiple sclerosis, psoriasis and rheumatoid arthritis (Onishi *et al.*, 2010). Researchers are intensively studying the possibility of blocking IL-17 signalling as a therapeutic target at the clinical level (Lubberts, 2008; Gaffen, 2009a) and it is interesting to know that there have been some successful benefits in patients especially with psoriasis where the IL-17 inducer IL-23 is targeted (Onishi *et al.*, 2010; Kopp *et al.*, 2015).

Taking a look at the current management guidelines in CF, it includes ways of improving airway clearance, medications, nutritional supplements and exercises. The use of medications is based on the specific CFTR mutations (such as ivacaftor for mutations of either G1349D, G1244E, R117H, S549N, S1251N, S1255P, G178R, S549R or G551S, or and the use of a combined medication of lumacaftor / Ivacaftor for the most common CFTR mutation: ΔF508) . Thus because of the specificity of the drug targets on the various mutations, it is important to perform genetic testing to identify the specific mutation before either of these drugs could be used to ensure efficacy. Although there are age specific guidelines for treatments, CF patients may actually be prescribed a combination of antibiotics to clear bacterial infections if any, bronchodilators, anti-inflammatory drugs, or medicines that will aid in mucus clearance (Chmiel *et al.*, 2005). Focusing on the use of anti-inflammatory drugs in CF, it is necessary to select the ones with minimal side effects as the current popular ones (i.e. corticosteroids and Non-Steroidal Anti-Inflammatory Drugs) have a number of side effects (Chmiel *et al.*, 2005).

Meanwhile, there are a number of targets for anti-inflammatory therapy including the use of receptor antagonists and cytokines such as IL-10 and IFN-γ (Chmiel *et al.*, 2005). Although some studies have begun, there will be the need for more exhaustive research to implement any of these as therapy. In a study by Chmiel and colleagues (Chmiel *et al.*, 2002) there was prolonged inflammation in IL-10 knockout mice upon infections with PA (Chmiel *et al.*, 2002). Chmiel and colleagues showed that, even though both IL-10 knockout mice and the wild type cleared the bacteria by day 6, there were more neutrophils in the BAL of knockout mice compared to wild type (Chmiel *et al.*, 2002). Also, their work showed that there were high levels of pro-inflammatory cytokines in knockout mice on day 2 which persisted on day 4 while the levels in wild type were only detected on day 2 and undetected by day 4 (Chmiel *et al.*, 2002). Likewise, it was shown that exogenous administration of IL-10 enhanced the survival of IL-10 knockout mice (Chmiel *et al.*, 1999). These data support the suitability of IL-10 as a potential anti-inflammatory agent in CF.

IFN-γ on the other hand reached clinical trials where inhaled IFN-γ was randomly administered to CF patients (500 μg and 1000 μg) and these were compared with patients randomly receiving placebo (Moss *et al.*, 2005).The 500 μg was well tolerated by patients but patients who received 1000 μg had more hospital admissions due to pulmonary exacerbations (Moss *et al.*, 2005). However, data from this trial did not show any improvement in pulmonary function and there was no alterations in inflammatory mediators when the treated and placebo were compared (Moss *et al.*, 2005). These data halted the use of IFN-γ for treatment in CF. Meanwhile, IFN-γ still has the potential of being a therapeutic agent in CF and this may be more realistic in the use of IFN-γ in combination therapy (more research is however needed to substantiate this).

In summary, this study has made evident the cooperation between human macrophages and neutrophils for IL-1β and IL-1α production in response to infections with PA. The production of IL-1β was found to be dependent on caspase activity. The potential anti-inflammatory property of IFN-γ was also evident by (i) the ability of IFN-γ to curtail the production of IL-1β

and IL-1α in the infected macrophage/neutrophil co-cultures compared to untreated, although the reduction in IL-1β and IL-1α was more significant when comparing IFN-γ treated to IL-17A treated cultures, (ii) the increase in MCP-1 in the infected macrophages which correlates with resolution of inflammation and (iii) possibly the induction of apoptosis; although this still needs further investigation.

PA clearance was however retarded in the presence of IFN-γ in the macrophage/neutrophil co-cultures while IL-17A appeared to increase PA clearance through a direct effect on neutrophil-mediated killing of PA. However, IL-17A did not significantly affect cytokine production by either the macrophages, neutrophils or the macrophage/neutrophil co-cultures, as cytokine values from IL-17A treated conditions tended to either tally with untreated or slightly higher than untreated conditions. In general, the production of most of the cytokines tested was found to be dependent on infections with PA as only negligible amounts were produced under uninfected conditions.

Thus the therapeutic potential of this current study lies in comparing the effect of these two cytokines, IFN-γ or IL-17A, on the pathogenesis of PA in terms of bacteria clearance, cytokine production and inflammatory potential. However further studies involving the use of clinical isolates as well as PA biofilms in our infection model will be instrumental in highlighting more on the potential of IFN-γ or IL-17A as therapeutic targets. But for now, it is obvious that the employment of either cytokine as a therapeutic agent will be a compromise on either bacteria clearance or inflammatory status. Thus, they must be selected carefully based on disease state and type of infection. For instance, in CF patients where the majority of morbidity and mortality is as a result of tissue damage, perhaps the most important issue under such circumstances will be to control inflammation and hence IFN-γ could be employed. Another possibility is the use of combination therapy as suggested above where bacteria multiplication could be controlled alongside controlling inflammation. For instance, antibiotics could be used to control bacterial infection and IFN-γ as an anti-inflammatory agent (although this will need to be further investigated for suitability). Meanwhile, some antibiotics already have been

shown to have anti-inflammatory properties (Labro, 2002; Buret, 2010) so this will have to be carefully factored in when using a combination of antibiotics and other anti-inflammatory agents. Again, PA virulence could be controlled by blocking quorum sensing molecules in PA (Reisetter *et al.*, 2011; O'Loughlin *et al.*, 2013) and then using IFN-γ alongside as an anti-inflammatory agent (again this will need to be further investigated for suitability).

Therefore, this study has not only provided a better understanding of the pathogenesis of PA but also provides a starting point in the design of novel therapeutic approaches against PA infections which could be aimed at boosting the arm(s) of the human innate immune system responsible for PA control, thus enhancing a better quality of life for especially the highly vulnerable group to PA infections.

To conclude, this study highlights successfully the collaboration of macrophages and neutrophils in controlling PA infections. It also demonstrates the key role for IFN-γ and IL-17A in balancing IL-1β/IL-1α and MCP-1 production and modulating neutrophil activity during PA infections. The findings as well support a role for IFN-γ in regulating neutrophil-mediated inflammation alongside reduced neutrophil-mediated killing, IL-1β synthesis and possibly induction of neutrophil death. More so, the macrophage/neutrophil co-culture described in this study will be invaluable to obtain a better understanding of human phagocyte cross-talk during infection with extracellular bacteria and how it is affected by inflammatory mediators, antibiotics and virulence factors of microbes.

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