THE EFFECT OF SECRETED PRODUCT FROM *PSEUDOMONAS AERUGINOSA* ON IMMUNE CELLS

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen that represents a major burden to the healthcare system as it accounts for a vast number of hospital-acquired infections. *P. aeruginosa* is a particular threat in Cystic Fibrosis (CF) patients. This bacterium expresses an arsenal of secreted virulence factors that play a fundamental role in the invasion of the damaged physical barriers of the host and abrogate immune responses which aid the persistence of *P. aeruginosa* infections. The aim of this study is to elucidate the role of *P. aeruginosa* secretions in the modulation of the immune system and determine the key factors and their specific impact on immune cells such as T helper cells proliferation and monocytes viability.

The present study employed peripheral blood mononuclear cells from healthy donors as a source of immune cells and bacterial supernatants as a source of exoproducts. In this context, the *P. aeruginosa* strain PAO1 was used throughout this project and two sublines of this strain were mainly involved in this work; the PAO1 sublines Lausanne (PAO1-L) and Nottingham (PAO1-N). Different sublines of PAO1 are genetically diverse and this variation was utilised as a tool to identify the key molecules produced by *P. aeruginosa* that are responsible for the observed effect.

The present study demonstrated that *P. aeruginosa* supernatants obtained from PAO1-L has an inhibitory effect on T cell proliferation in response to Staphylococcus Enterotoxin B (SEB), but not α-CD3 and α-CD28 stimulation in peripheral blood mononuclear cells cultures. This effect appears to be

mediated by the cytotoxic effect of *P. aeruginosa* products on monocytes, a precursor of antigen presenting cells. Analysis of different *P. aeruginosa* mutants showed that the cytotoxic activity is controlled by *toxR*. *ToxR* is a regulatory protein which enhances the production of one of the most important virulence factors of *P. aeruginosa*, exotoxin A (*ToxA*; ETA).

The impact of ETA production by *P. aeruginosa* on immune cells was investigated using a newly generated ETA-deficient mutant *ΔtoxA* in PAO1-L (Lausanne background) in comparison with its isogenic wild type (WT). Preliminary observations showed that the generation of apoptotic monocytes correlated with the high expression of ETA in the bacterial supernatants as both PAO1-LΔ*toxR* and PAO1-LΔ*toxA* are less toxic strains than PAO1-L. These findings suggest that the presence of monocytes is crucial to mediate ETA down regulation of T cell proliferation. However, the effect of PAO1-LΔ*toxA* supernatants on T cell proliferation in response to SEB could not be fully assessed because SEB stopped inducing T cell proliferation as before. To overcome this obstacle, phytohaemagglutinin (PHA) was employed as a promoter of T cell division. However, the suppressor effect was only clear on the $CD3^+/CD4^+$ T cell subset after stimulation with PAO1-L (ETA+) supernatant in response to PHA and full T cell proliferation was not recovered in the absence of ETA (PAO1-LΔ*toxA*).

Finally, *P. aeruginosa* and *Candida albicans* are typical opportunistic respiratory tract pathogens and the nature of the relationship between these two pathogens is still not entirely clear. Thus, in this work, an *in vitro* assay to evaluate the impact of *P. aeruginosa* secretions on *C. albicans* was developed. Our results suggest that *P. aeruginosa* secreted products promote *C. albicans*

hyphal growth and the filament formation is not correlated with either the presence of the *toxR* gene or the production of ETA and rhamnolipids. Interestingly, a supernatant prepared from PAO1-L mutant with a 58 Kb deletion induces the growth of the yeast form suggesting that the gene that encodes the key element, which is responsible of inducing the hyphal growth, is located within this region. This work might deliver new understandings on the interactions between *P. aeruginosa* and *C. albicans* in polymicrobial infections particularly in susceptible individuals like those with Cystic Fibrosis and burn victims.

Further work is currently being carried out in our group to identify the active molecules that trigger filamentous growth in *C. albicans* and to determine the contribution of this agent on the interaction between *P. aeruginosa* and *C. albicans* during co-infection.

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LIST OF ABBREVIATIONS

2xYT	Two times yeast extract tryptone
3-oxo-C12	*N*-(3-oxo-dodecanoyl)-L-homoserine lactone
ADP	Adenosine diphosphate
AHL	Acylhomoserine lactone
ANOVA	Analysis of variance
Ap	Ampicillin
APC	Antigen presenting cells
AQ	2-alkyl-4-quinolone
ATP	Adenosine triphosphate
bp	Base pair
C4-HSL	*N*-Butanoyl-L-homoserine lactone
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CF	Cystic fibrosis
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CFTR	Cystic fibrosis transmembrane conductance regulator
CMV	Cytomegalovirus
CR-3	Complement receptor 3
CTL	Cytotoxic T lymphocyte
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribose nucleotide triphosphates

EBV	Epstein–Barr virus
ECD	Phycoerythrin-Texas Red conjugate
EDTA	Ethylenediaminetetracetic acid
eEF-2	Eukaryotic elongation factor-2
ETA	Exotoxin A
ExoS	Exotoxin S
FBS	Foetal bovine serum
FcγR	Fragment crystallisable gamma receptor
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte colony-stimulating factor
GITR	Glucocorticoid-induced tumour necrosis factor receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-2R	IL-2 receptor
IPTG	Isopropylthio-β-D-galactoside
ITAM	Immunoreceptor tyrosine-based activation molecule
kDa	Kilodalton
LAG	Lymphocyte activation gene
LB	Lysogeny broth (also referred to as Luria-Bertani medium)

LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
min	Minute(s)
NAD	Nicotinamide adenine dinucleotide
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor-κB
NK cell	Natural killer cell
OD600nm	Optical density (Absorbance) at 600 nm
ORF	Open reading frame
PAAMP1	58-Kb chromosomal deletion mutant in PAO1-L
PAMP	Pathogen/microbe -associated molecular pattern
PAO1-L	*P. aeruginosa* PAO1 obtained from Lausanne
PAO1-N	*P. aeruginosa* PAO1 obtained from Nottingham
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PC5	Phycoerythrincyanin 5 conjugate
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PLC	Phospholipase C
PMN	Polymorphonuclear
PQS	*Pseudomonas* quinolone signal
PRR	Pattern recognition receptor

QS	Quorum sensing
rpm	Revolutions per minute
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEB	Staphylococcal enterotoxin B
Stats3	Signal transducer and activator of transcription 3
Tc	Tetracycline
TCR	T cell receptor
TG	Tris-Glycine transfer buffer solution
TGF	Transforming growth factor
Th cell	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
Treg cell	Regulatory T cell
uPA	Urokinase plasminogen activator
ΔBif	*Bif* gene deletion mutant in PAO1-L
ΔrhlA	*RhlA* gene deletion mutant in PAO1-L
ΔtoxA	*ToxA* gene deletion mutant in PAO1-L
ΔtoxR	*ToxR* gene deletion mutant in PAO1-L
TAE	Tris-acetic acid-EDTA
HSP	Heat shock protein
MAP	Mitogen-activated protein

1. Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that expresses numerous virulence factors able to alter immune responses and facilitate the establishment of infection in the affected host.

The main objective of this study was to elucidate the effect of the *P. aeruginosa* secreted products on human immune cells, using peripheral blood mononuclear cells (PBMCs), and subsequently define and link potential factors with observed effects. Identifying such factors and their mechanism(s) of action would potentially unveil key contributors to the pathogenesis of *P. aeruginosa* and provide a better understanding of the molecular basis of its ability to modulate the immune system.

1.1 Human Immune System

The immune system is an intricate network composed of soluble and cellular components that work together within the organism to protect the body from invasion and the establishment of infections and diseases. The skin is the largest human organ, it forms the first line of defence and constitutes an effective barrier to infection by preventing pathogens from entering the body. However, when this compacted layer is breached; two distinct arms of the immune system work in concert to initiate an inflammatory response. These two branches provide either an immediate, but less specific, response termed 'innate immune response' (Takeuchi and Akira, 2010) or a more prolonged,

and more specific, response known as the 'adaptive immune response' (Liu *et al.*, 2013).

1.1.1 Innate Immune System

Innate Immunity represents the first line of immune defence (Medzhitov and Janeway, 2000) and initiation of this response predominantly relies on the expression of germ-line encoded pattern recognition receptors (PRRs) (Walsh *et al.*, 2013, Diacovich and Gorvel, 2010). These receptors comprise an essential element of the wide range of non-immune and immune cells that are involved in recognition and sensing of either pathogenic elements known as pathogen/microbe-associated molecular patterns (PMAMPs) (Meylan *et al.*, 2006, Cinel and Opal, 2009); or molecular elements associated to injury, characteristically flagged by products such as uric acid and adenosine triphosphate (ATP) released by stressed cells termed damage-associated molecular patterns (DAMPs) (Ciraci *et al.*, 2012, Foell *et al.*, 2007).

Janeway proposed for the first time the contribution of PRRs in mammalian cells to immune recognition (Janeway, 1989) and these observations were subsequently confirmed by his group (Medzhitov *et al.*, 1997). The most studied PRR family are the Toll-like receptors (TLRs) which play a vital role in the activation of innate responses and signal transduction, acting as environmental sensors (Takeuchi and Akira, 2010, Akira *et al.*, 2006).

For over a decade now, 10 functional TLR isoforms in humans and 12 TLRs in mice have been identified (Gleeson, 2014, Kawai and Akira, 2010, Fitzner *et al.*, 2008). The localisation of specific TLRs is spatially distinct, with some members expressed at the cell surface and others located in the endosomal compartments (Kumar *et al.*, 2011, Barbalat *et al.*, 2011).

The innate immune system comprises diverse groups of cell types. The most relevant in the case of infection with extracellular bacteria (the focus of this thesis) are mononuclear phagocytes (monocytes, macrophages, dendritic cells (DCs)) and polymorphonuclear (PMN) leucocytes (Liu *et al.*, 2013, Weissleder *et al.*, 2014) which are discussed in more detail below.

1.1.1.1 Monocytes

Monocytes comprise ~5-10% of the circulating leucocyte population (Ancuta *et al.*, 2009, Geissmann *et al.*, 2010). Monocytes appear to accumulate at sites of infection to remove either debris or eliminate intruders by phagocytosis (Dale *et al.*, 2008). These phagocytic cells express different surface molecules that facilitate the recognition of pathogens and the phagocytic process. For instance, cluster of differentiation (CD) 14 can engage lipopolysaccharide (LPS) expressed by Gram-negative bacteria and transfer it to the MD2-TLR4 complex leading to cellular activation (Trinchieri and Sher, 2007, Calvano *et al.*, 2003, Tadema *et al.*, 2011). Furthermore, monocytes possess Fc receptors, such as the Immunoglobulin G (IgG) receptor (fragment crystallisable gamma receptor (FcγRI), CD64), which facilitate antibody-mediated phagocytosis, by recognising the Fc portion of IgG immunoglobulins covering foreign particles (Garcia-Garcia and Rosales, 2002, Dale *et al.*, 2008).

An initial study by Passlick et al. revealed that human monocytes exhibit heterogeneity in morphology, size and phagocytic activity (Passlick *et al.*, 1989) and more recently monocytes were categorised into three distinct subgroups depending on the expression of CD14 and CD16 markers (Cole *et al.*, 2010, Ziegler-Heitbrock *et al.*, 2010). As mentioned above, CD14 acts as a

co-receptor for the detection of LPS, whilst CD16 is the low-affinity receptor for the Fc portion of IgG (FcγRIII) (Gordon and Taylor, 2005, Lanier *et al.*, 1991).

The majority of monocytes are characterised by high expression of CD14 and lack of CD16 molecules ($CD14^{++}CD16^{-}$) and are termed classical monocytes, whilst the CD16 expressing monocytes are subdivided into $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ populations, known as intermediate and non-classical, respectively (Ziegler-Heitbrock *et al.*, 2010, Appleby *et al.*, 2013).

The phenotypic variations among monocyte subsets may subsequently lead to differences in their function and behaviour (Appleby *et al.*, 2013). For instance, $CD16^{+}$ monocytes express very high levels of major histocompatibility complex class II (MHC II) and, after engagement with T cell receptors (TCRs), exhibit pro-inflammatory activity due to the production of inflammatory cytokines such as tumour necrosis factor α (TNFα), Interleukin (IL)-6, and reduced production of anti-inflammatory mediators, like interleukin-10 (IL-10), compared to the other monocyte populations (Belge *et al.*, 2002, Gordon and Taylor, 2005, Szaflarska *et al.*, 2004, Serbina *et al.*, 2009).

TNFα is considered one of the most important pro-inflammatory cytokines; it is released in response to infectious agents and so its production is often used as a marker of an inflammatory response (Minoguchi *et al.*, 2004). It has multiple biological consequences on numerous cell types such as PMN cells and macrophages leading to enhanced clearance of pathogens (Bermudez and Young, 1988, Wright *et al.*, 2010, Parameswaran and Patial, 2010, Kumar and Sharma, 2010, Nathan, 1987). IL-6, appears to have a central role in

differentiation of antigen presenting cells (APCs) eventually leading to cell activation (Chomarat *et al.*, 2000). While IL-10 is considered an immune modulatory cytokine based on its ability to repress inflammatory immune responses (Hazlett *et al.*, 2014). IL-10 limits the production of proinflammatory cytokines such as TNF-α and IL-6 and supress the expression of surface receptors on APCs including MHCII and B7 leading to the switch off of T cell-mediated immunity (Jung *et al.*, 2004, Williams *et al.*, 2002). Furthermore, different inflammatory and infectious diseases caused a substantial increase in $CD16^+$ monocytes in blood (Mizuno *et al.*, 2005, Jin and Balthasar, 2005, Emminger *et al.*, 2001, Coley *et al.*, 2015, Fischer-Smith *et al.*, 2001). A study done by Herra et al. showed that numbers of $CD16^+$ monocytes were increased in in blood samples of patients infected with Gram-negative bacteria (Ziegler-Heitbrock, 2007).

In addition to their crucial role in defending the host against invading pathogens, circulating monocytes can give rise to either tissue macrophages or myeloid DCs in the peripheral tissues, which in turn can shape the adaptive immune responses (Figure 1.1) (Imhof and Aurrand-Lions, 2004).

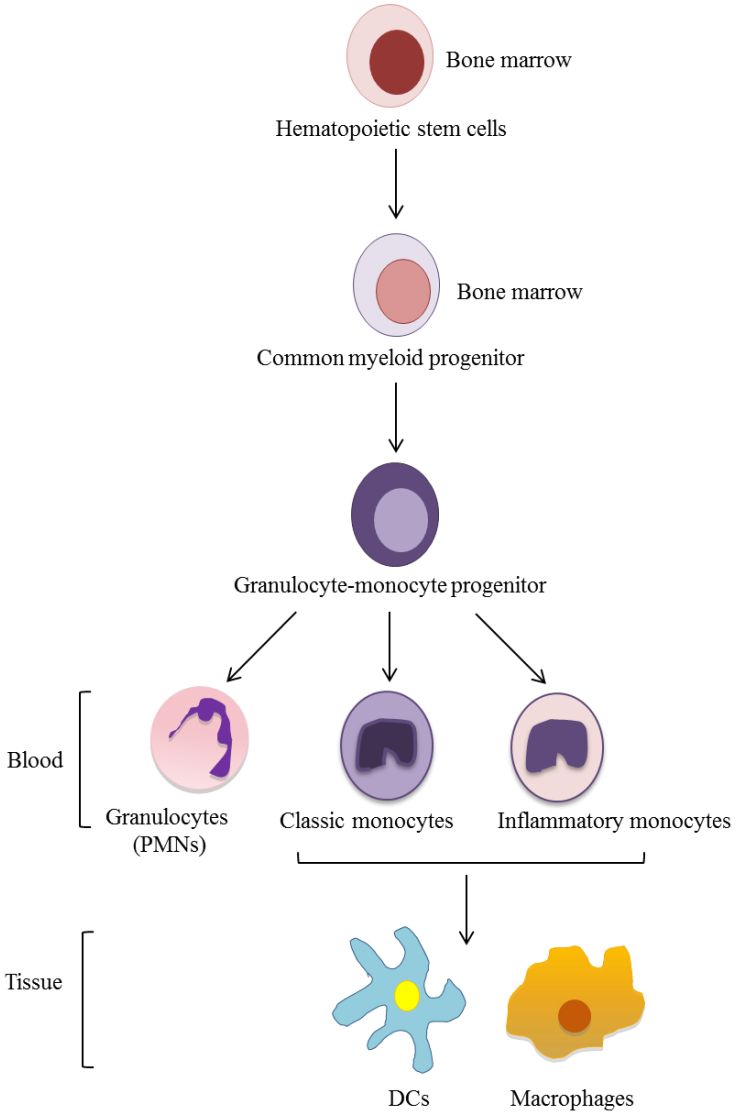

Figure 1.1: Development of myeloid cells.
Myeloid precursors are derived from hematopoietic stem cells in the bone marrow and then differentiate into granulocyte-monocyte originators committed to either macrophage or DC lineage. Adapted from (Imhof and Aurrand-Lions, 2004).

1.1.1.2 Macrophages

The differentiated inflammatory tissue macrophages originate from either monocyte progenitors or the yolk sack with some undergoing replication after their recruitment from the circulation to sites of infection (Jenkins and Hume, 2014, Italiani and Boraschi, 2014). They represent one of the primary phagocytic cells that play a cardinal role in protecting the host from infection.

Macrophages are believed to be responsible for digestion and elimination of foreign molecules and organisms by phagocytosis (Mosser and Edwards, 2008, Shi *et al.*, 2011). In addition to phagocytic activity, macrophages are considered one of the master regulators of the immune responses, modulating the action of other immune cells due to their ability to present antigen to lymphocytes and produce cytokines, chemokines and growth factors (Swirski *et al.*, 2009, Underhill and Ozinsky, 2002).

Macrophages are distributed throughout the organism. After differentiation in distinct microenvironments within tissues, they display diverse phenotypes and play a number of important functions as summarised in figure 1.2. (Murray and Wynn, 2011).

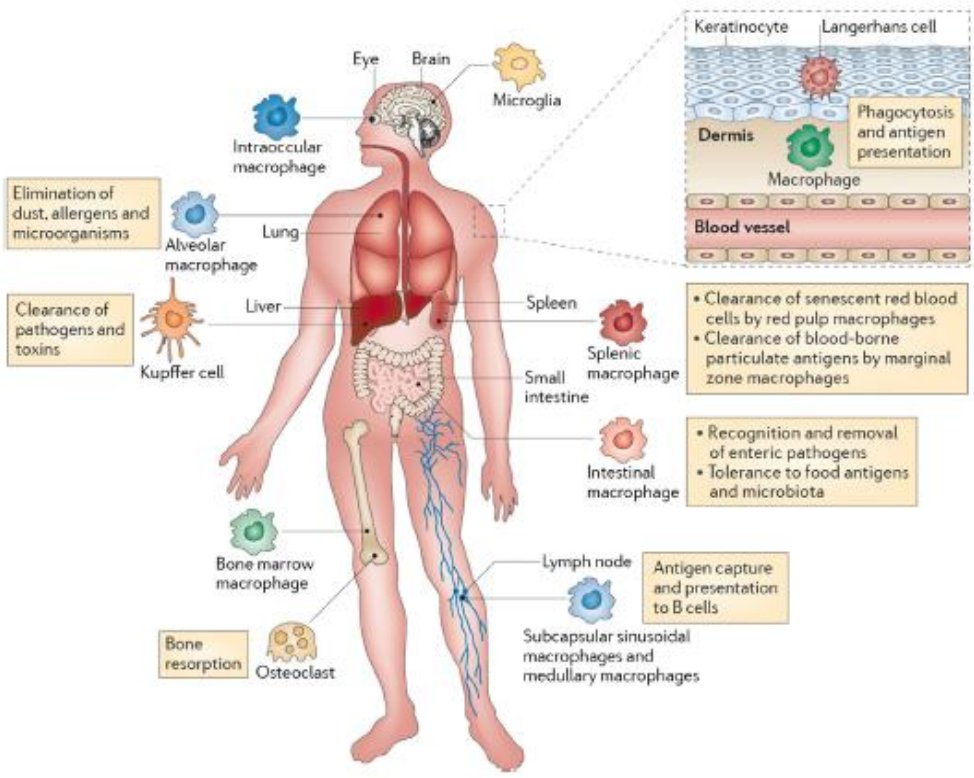

Figure 1.2: Dissemination, differentiation and function of macrophage throughout the body.
Mononuclear myeloid cells, derived from hematopoietic stem cells in the bone marrow, circulate through the body and give rise to macrophages that perform diverse functions such as clearance of debris and immune activation via phagocytosis, production of immune mediators and antigen presentation,(Murray and Wynn, 2011).

The cytokine milieu can switch mature macrophages into two major phenotypic and functional subclasses: classically (M1) and alternatively (M2) activated macrophages (Taylor *et al.*, 2005).

The inflammatory or classically activated M1 macrophage subtype is represented by macrophages activated by interferon-gamma (IFN-γ) plus LPS or TNF α (Martinez and Gordon, 2014). These cytokines promote M1 macrophages to secrete high levels of pro-inflammatory cytokines such as TNF α, and interleukins such as IL-12 and IL-23 promoting the development of Th1 and Th17 cells which further enhance the inflammatory response and clearance of the pathogens (Singh *et al.*, 2014).

In contrast, the alternatively polarised M2 activated macrophages showed significant variations based on their biochemical and physiological characteristics (Edwards *et al.*, 2006). This subset has been further classified into three subpopulations: M2a, M2b and M2c (Martinez *et al.*, 2008). M2a macrophages are generated in response to the cytokines IL-4 or IL-13. M2b, termed regulatory macrophages, are generated by the presence of immune complexes and TLR ligands whereas M2c are induced by IL-10, TGF-β or glucocorticoids (Mantovani *et al.*, 2004, Gordon and Martinez, 2010, Martinez *et al.*, 2009). It has been proposed that M2 macrophages perform an essential role by driving Th2 responses, eliminating parasites and promoting tissue healing and immune suppression (Mantovani *et al.*, 2004).

1.1.1.3 Dendritic Cells (DCs)

Previous studies have highlighted the importance of DCs in antigen presentation to naïve T cells and as important bridges between the innate and

adaptive immune systems (Kumar *et al.*, 2011, Merad *et al.*, 2013, Blum *et al.*, 2013, Collin *et al.*, 2013). DCs were initially identified by Steinman and co-workers in the murine spleen as dendritic-shaped cells (Steinman and Cohn, 1973). They are also termed professional APCs as they are able to capture antigens, process them and present them on MHC to activate naïve T cells (Schott, 2006, Kushwah and Hu, 2011).

DCs can be found in tissues throughout the body and exhibit diversity in terms of phenotype and biological role (Kushwah and Hu, 2011). All DC subsets have been characterised by a high expression of MHC class II and lack of typical lineage surface markers including CD3, CD19/20 and CD56 for T and B lymphocytes and natural killer cell respectively (Collin *et al.*, 2013).

Whilst DCs can be located in various compartments and body tissues, human blood DCs have been significantly more studied and characterised. For example, myeloid DCs (mDCs) were identified by their expression of myeloid antigens such as CD11c+ and this group was further classified into three subgroups based on the presence of distinct surface markers, including $CD1c^+$ DCs, $CD141^+$ DCs and $CD16^+$ DCs, which play different function in immunity (Mittag *et al.*, 2011). Plasmacytoid DCs (pDCs) showed high expression of CD303 (BDCA-2), CD123 (IL-3Rα) and CD304 (BDCA-4) but no CD11c (Lebre *et al.*, 2008). pDCs have a pivotal role in antiviral immunity, through the robust release of type I interferon (IFN) particularly in response to TLR7 and TLR9 ligands including viral nucleic acids (Collin *et al.*, 2013).

1.1.1.4 Polymorphonuclear cells (PMNs)

PMNs or neutrophils constitute about 65% to 75% of all circulating white blood cells and are major cellular components of the innate system (Nathan, 2006). Neutrophils are derived from stem cells and contribute to protection against pathogens (Segal, 2005). During infection or inflammation, host cells such as monocytes, macrophages, epithelial cells and mast cells can produce numerous chemokines and cytokines, such as IL-8, TNF-α, IL-17, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF), that promote recruitment to the site of inflammation and activation and differentiation of neutrophils (Kobayashi *et al.*, 2005, Mantovani *et al.*, 2011b).

Neutrophils have the capacity to kill both intra- and extracellular microorganisms. For instance, they can internalise microorganisms into phagosomes and then destroy them by either: respiratory mechanisms using reactive oxygen species; or production of microbicidal and bacteriostatic molecules located in the cytosolic granules such as cathepsins, lactoferrin, defensins and lysozyme (Borregaard, 2010, Hager *et al.*, 2010). These killing agents can also be delivered to the surrounding environment leading to elimination of external microbes (Kolaczkowska and Kubes, 2013). Neutrophils can also employ an alternative, direct mechanism to kill extracellular pathogens by releasing neutrophil extracellular traps (NETs) which contain antimicrobial and degradative enzymes such as elastase (Phillipson and Kubes, 2011, Papayannopoulos and Zychlinsky, 2009). These traps immobilise microbes, preventing further dissemination throughout the

body, and are subsequently eliminated by phagocytosis (Kolaczkowska and Kubes, 2013).

However, substantial evidence suggests that neutrophils can mediate the pathogenesis of a wide number of diseases, including cancer and autoimmunity (Mantovani *et al.*, 2011a). An excessive activation and infiltration of neutrophils to the site of infection can cause inappropriate damage of host tissues leading to the establishment of chronic inflammatory diseases (Nemeth and Mocsai, 2012, Mocsai, 2013).

Additional work has also drawn attention to other types of granulocytes (eosinophils, basophils and mast cells) and their role in the pathogenesis of allergic disorders and eradication of the extracellular parasites (Gebreselassie *et al.*, 2012, Holgate, 2008, Karasuyama *et al.*, 2011).

1.1.1.5 Natural Killer cells (NKs)

NK cells are a part of the innate immunity that identify and lyse abnormal cells, such as tumour and virally infected cells (Fauriat *et al.*, 2010, Vivier *et al.*, 2008). They represent a minor group of the peripheral lymphoid cells (around 10%) which are originated from CD34 hematopoietic stem cells and then differentiated into two main groups based on expression of the surface adhesion molecule CD56, $CD56^{dim}$ and $CD56^{bright}$ (Orange, 2013). $CD56^{dim}$ cells comprise the majority of the circulating NK cells (approximately 90%) and have a potent cytotoxic capacity as they are equipped with higher levels of perforin, granzymes and cytolytic granules which create holes in the cell membrane and activate apoptotic cell death (Caligiuri, 2008, Poli *et al.*, 2009). However, $CD56^{bright}$ NK cells exhibit less cytotoxicity and are fewer in

number, about 5- 15% of entire NK cells (Freud *et al.*, 2005, Poli *et al.*, 2009). This subset is characterised by antibody-mediated cytotoxicity and a high level of cytokine expression compared to $CD56^{dim}$ such as IFN-γ, TNF-α, GM-CSF, IL-10 and IL-13 based on cues from immune cells (Poli *et al.*, 2009). NK cells have unique receptors that recognise distinct cognate ligands expressed on the surface of transformed cells, leading to the stimulation of NK cells and removal of target cells (Lanier, 2005, Tian *et al.*, 2013).

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1.1.2 Adaptive Immune system

A primary function of the innate immunity is the initiation of specific and long-term immune responses mediated by the adaptive immunise system via the activation of a vast pool of lymphocytic cells including T cells and B cells (Liu *et al.*, 2013, Iwasaki and Medzhitov, 2010).

1.1.2.1 T cells

T lymphocytes represent a fundamental element within the immune system. The initial activation of T cells starts after exposure to a strong stimuli delivered by professional APCs, mainly by DCs (Lanzavecchia, 1996, Banchereau and Steinman, 1998). The engagement of antigen-derived peptides loaded on MHC groups with the TCR-CD3 complex is essential for the initial activation of T cells. The second signal is provided by the ligation of the accessory co-stimulatory molecules (Van Lier *et al.*, 1991, Cantrell, 2002). TCR is composed of α and β chains linked by a disulphide bond. They are associated with CD3 which consists of γ, δ and ε chains occurring as either γ/ε

or δ/ε heterodimers (Tamura and Nariuchi, 1992, Dietrich *et al.*, 1996). After DCs uptake and process foreign antigens, they up-regulate the expression of MHC receptors, co-stimulatory receptors (B7 (CD80/CD86)) and adhesion molecules (such as intercellular adhesion molecules (ICAMs)), which are involved interaction with naïve T cells (Figure 1.3) (Blum *et al.*, 2013, Hammer and Ma, 2013).

T lymphocytes can be divided into two main functional subgroups, $CD4^+$ and $CD8^+$ T cells. The $CD4^+$ T cell family recognises processed peptides presented on MHC class II (MHC II) while peptides loaded on MHC class I (MHC I) are recognised by $CD8^+$ T cells (Zietara *et al.*, 2014).

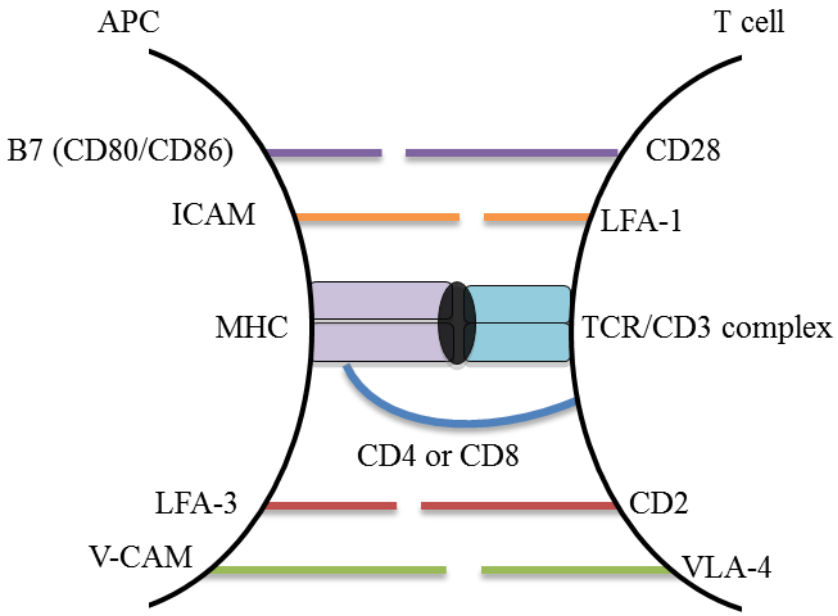

Figure 1.3: Surface molecules involved in APC- T cell interaction.
The first step of T cell stimulation is mediated by the ligation of the TCR:CD3 complex with either MHC I or II class-peptide complexes followed by recognition of accessory molecules which is needed to achieve full T lymphocyte activation .

a) $CD4^+$ T helper cells

$CD4^+$ T cells exert a modulatory function on the immune responses. They are also named T helper (Th) cells as they aid in recruitment and activation of other immune cells (Kim and Lee, 2013). For instance, Th cells enhance the anti-microbial activity of macrophages, antibody production by B cells, recruitment of PMNs to infected areas, and hence collectively modulate the immune responses (Zhu and Paul, 2008).

In fact, there are three events that are required for naïve $CD4^+$ T cell differentiation into several subsets (Magombedze *et al.*, 2013). The first signal includes the concomitant ligation between TCR on T cells with the loaded peptides on MHC II at the surface of APCs followed by a second interaction between CD28 on the T cell and members of the B7 family (CD80/CD86) on APCs (Yamane and Paul, 2012). Furthermore, the cytokine environment determines the terminal lineage commitment and cytokine expression profiles (Zhu and Paul, 2008, Magombedze *et al.*, 2013, Dong, 2006).

In this context, IL-12 cytokine produced by APC is responsible for generation of T helper 1 (Th1) from $CD4^+$ T cells via the up-regulation of the regulatory gene T-bet (Szabo *et al.*, 2000, Lighvani *et al.*, 2001). In contrast, the T helper 2 (Th2) cells are generated in the presence of IL-4 leading to a high expression of GATA-3 (Zheng and Flavell, 1997, Zhang *et al.*, 1997a).

In the case of T helper 17 (Th17) cells, characterised by ROR-γt expression, it has been proposed that both IL-6 and transforming growth factor (TGF)-β in the presence of IL-1β are essential for their development. Whereas induced Regulatory T (iTreg) cells, expressing FoxP3, are generated after secretion of

IL-2 and TGF-β by APCs (Bettelli *et al.*, 2006, Veldhoen *et al.*, 2006, Ivanov *et al.*, 2006, Acosta-Rodriguez *et al.*, 2007, Sakaguchi, 2004, Zhu and Paul, 2008).

It has been demonstrated that each potential subset of $CD4^+$ T cells exerts a distinct and significant role in protecting the body against infection. Contrastingly, they may have damaging effects on host cells and aid in the establishment of several inflammatory and autoimmune diseases as described in the following sections.

1) T helper 1 (Th1)

The central function of Th1 T cell subset is to orchestrate immunity towards intracellular microorganisms such as mycobacterial infections and viral infections, and contribute to progression of some autoimmune diseases (Mosmann and Coffman, 1989, Paul and Seder, 1994, Zhu and Paul, 2008). Such functions are generally mediated by the expression of signature cytokines and chemokines such as IFN-γ which is important for the robust microbicidal activity of macrophages (Zhu and Paul, 2008).

2) T helper 2 (Th2)

Th2 cells are involved in the immune responses against extracellular organisms, particularly helminths, or in hypersensitivity disorders and asthma. These cells produce a wide range of cytokines including IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25 (Zhu and Paul, 2008). IL-4 is essential for Th2 differentiation and immunoglobulin E (IgE) production by B cells which binds to fragment crystallisable epsilon region I (FcεRI) receptor expressed on

basophils, eosinophils and mast cells. This subsequently leads to the release of various active, pro- inflammatory mediators such as histamine and IL-4 and the initiation and promotion of Th2 responses (Zhu and Paul, 2008, Alphonse *et al.*, 2008). IL-5 is required for eosinophil recruitment whilst IL-9 plays a fundamental role in hypersensitivity reactions through either its impact on other cells, like mast cells, or by triggering mucin production by epithelial cells (Coffman *et al.*, 1989, Longphre *et al.*, 1999). With regard to IL-10 and IL-25 cytokines, it has been proposed that IL-10 represents the negative feedback for both Th1 and Th2 response while IL-25 (termed IL-17E), is considered as a positive modulator of Th2 responses by inducing non-lymphoid cells to produce IL4, IL-5 and IL13 (Fiorentino *et al.*, 1989, Fort *et al.*, 2001, Angkasekwinai *et al.*, 2007, Fallon *et al.*, 2006). Finally, IL-13 cytokine is one of the key elements in the removal of helminths and promotes pulmonary hypersensitivity due to its ability to switch B cells towards immunoglobulin E (IgE) production (Wynn, 2003, Urban *et al.*, 1998, Jackson *et al.*, 2009).

3) T helper 17 (Th17)

Th17 cells are a specialized subpopulation of T cells that contribute to protection against extracellular bacteria and fungi (Ye *et al.*, 2001, Chung *et al.*, 2003, Infante-Duarte *et al.*, 2000, LeibundGut-Landmann *et al.*, 2007). The generation of this subset from the CD4 linage depends on the presence of TGF-β, IL- 1β, and IL-6, combined with IL-21 or IL-23 in the inflammatory environment (Acosta-Rodriguez *et al.*, 2007, Manel *et al.*, 2008, Yang *et al.*, 2008, Wilson *et al.*, 2007). Furthermore, it has been reported that IL-23 displays an important role in the survival and expansion of Th17 cells

(Stritesky *et al.*, 2008, Aggarwal *et al.*, 2003b, Langrish *et al.*, 2005). This distinct lineage of CD4 T cells is termed Th17 due to the expression of members of the IL-17 family which includes IL-17A and IL-17F. Furthermore, it also produces a wide range of cytokines including IL-21, IL-22, GM-CSF, and TNF-α (Bedoya *et al.*, 2013, Crome *et al.*, 2010). The majority of studies have focused on the role of the IL-17 family members IL-17A and IL-17F, their implied similarity of function and engagement of IL-17RA (Hymowitz *et al.*, 2001). It has been proposed that IL-17A and F are important in the recruitment and activation of neutrophils which are crucial in the immune defence against extracellular invaders (Kolls and Linden, 2004). IL-21 is believed to be a key for the promotion of IL-17 expression by Th17 cells and sustains Th17 commitment (Wei *et al.*, 2007). While, IL-22 cytokine exhibits both protective and inflammatory functions (Zenewicz and Flavell, 2011). It has been demonstrated that the high expression of IL-22 correlates with a wide range of chronic diseases such as rheumatoid arthritis and skin inflammation (Ikeuchi *et al.*, 2005, Wolk *et al.*, 2009). However, IL-22 exhibits an anti-inflammatory function via the ability to activate anti-apoptotic pathways during hepatitis and enhances survival and tissue repair (Zenewicz and Flavell, 2011). It has been shown that IL-22 receptor (IL-22R) is highly expressed by a wide array of peripheral tissues such as liver, lung, and skin (Aggarwal *et al.*, 2001, Wolk *et al.*, 2004). After the engagement with IL-22, signal transducer and activator of transcription 3 (Stat3) signalling pathways are activated which lead the activation of several pathways such as such as apoptosis (Zenewicz *et al.*, 2007). GM-CSF is a growth factor which can induce the differentiation and

cell growth of granulocytes and macrophages and is considered an immune activator of the innate immune response (Fleetwood *et al.*, 2005).

4) Regulatory T cells (Treg)

Treg cells are a unique subset of CD-4 T lymphocytes that down-regulate immune responses and help to maintain immune tolerance to self-antigens (Sakaguchi, 2004, Baecher-Allan and Hafler, 2006). These cells are also able to either regulate or block the immune responses during allergic disease, microbial infections and transplant rejection (Umetsu and DeKruyff, 2006, Rouse *et al.*, 2006, Waldmann *et al.*, 2006). Tregs can be classified into natural Tregs (nTregs) and inducible Tregs (iTregs). nTregs are generated in the thymus while iTregs are geerated in the periphery. nTregs and iTergs employ different mechanisms to suppress immune responses (Gol-Ara *et al.*, 2012). The immunosuppression by nTregs rely on the expression of surface molecules which mediate cell contact-mediated suppression whereas the suppressive activity of iTregs is contact-independent and based on the expression of IL-10 and TGF-β (Gol-Ara *et al.*, 2012).

Tregs can be identified by the expression of a set of markers: CD25 (IL-2R α-chain) (Jonuleit and Schmitt, 2003, McNeill *et al.*, 2007), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (Read *et al.*, 2000, Takahashi *et al.*, 2000), glucocorticoid-induced tumour necrosis factor receptor family-related gene (GITR) (McHugh *et al.*, 2002), lymphocyte activation gene-3 (LAG-3) (Huang *et al.*, 2004), CD127 (IL-7Rα) (Simonetta *et al.*, 2010) and

the intracellular forkhead /winged-helix transcription factor box P3 (Foxp3) (Hori *et al.*, 2003, Fontenot *et al.*, 2003, Khattri *et al.*, 2003).

Interleukin-2 (IL-2) is highly produced by Th cells after antigen binding to TCR. The up regulation of IL-2 promotes the Treg differentiation and function via expression of IL-2 receptor (IL-2R) by Treg cells (Busse *et al.*, 2010). CTLA-4 mediates the suppression of T cell activation due to its ability to compete with the T cell costimulatory receptor, CD28, for ligation with CD80 and CD86 expressed by of APCs and down regulate TCR signalling (Tai *et al.*, 2012). Expression of GITR is involved in the activity of Tregs due to the activity of IL-10 or the down regulation of Foxp3 expression (Ephrem *et al.*, 2013, Igarashi *et al.*, 2008, Shimizu *et al.*, 2002). Expression of both CD127 and Foxp3 are a hallmark of this T cell subset and are used to discriminate between Tregs and conventional Th cells (Simonetta *et al.*, 2010).

b) $CD8^+$ cytotoxic T lymphocytes (CTLs)

CTLs are a subset of T cells which play a pivotal role in immune protection against viral infections such as cytomegalovirus (CMV) (Gillespie *et al.*, 2000), hepatitis B virus (HBV) (Guidotti *et al.*, 1996), hepatitis C virus (HCV) (Lechner *et al.*, 2000) and Epstein–Barr virus (EBV) (Callan, 2003). The activation of CD8+ T cells involves two steps, the engagement of TCR on $CD8^+$ T cells with peptide-loaded class I MHC expressed on APCs (Zietara *et al.*, 2014) and ligation one of several molecules on the effector T cell, CD28, CD40, 41BB and CD27, with their cognate ligands, B7, CD40, 41BB and CD27, respectively expressed by APCs.(Whitmire and Ahmed, 2000, Denoeud and Moser, 2011, Borst *et al.*, 2005).

CLTs employ one of three pathways to kill the infected cell. Two of the mechanisms involve the direct interaction between TCR and MHC I class on the surface of CTLs and target cells, respectively. The third mechanism requires the secretion of cytokines such as INF-γ and TNF-α upon the stimulation of TCR signalling pathway (Andersen *et al.*, 2006). The direct crosstalk between CLTs and target cells leads to activation of apoptosis which is mediated by either the interaction of Fas ligand expressed on the surface of CTLs with Fas receptor (Fas, CD95) on the target cell or the release of perforin and granzymes in the surrounding environment (Heusel *et al.*, 1994, Bots and Medema, 2006, Voskoboinik *et al.*, 2006, Bolitho *et al.*, 2007, Andersen *et al.*, 2006).

The binding of TNF-α released by CTLs to TNF receptor (TNFR) expressed by infected cells activates caspase activity within the former cells and eventually lead to cell death. INF-γ, is considered as a positive regulator of both MHC I presentation and Fas pathway in infected cells which contributes ultimately to cell lysis (Andersen *et al.*, 2006).

1.1.2.2 B cells

B lymphocytes play a crucial role in humoral immunity and constitute about 5-15% of peripheral lymphocytes and in concert with T cells, protect the host from infections (DiLillo *et al.*, 2011, Fettke *et al.*, 2014). B cells reside within secondary lymphoid organs and after exposure to cognate native antigen and antigen-specific activated T cells (follicular T helper cells), the majority of B cells give rise to two important subsets: plasma and memory cells (Abolhassani *et al.*, 2014, Fettke *et al.*, 2014). Plasma cells are mature B cells

which produce antibody (Cerutti *et al.*, 2012, Wykes, 2003, Ait-Oufella *et al.*, 2014). Memory cells differentiate into plasma cells upon re-exposure to antigen with the aid of Th cells (Tangye and Good, 2007).

In mammals, there are five types of antibody including IgA, IgD, IgE, IgG, and IgM, each with its specific class of heavy chain α, δ, ε, γ, and μ, respectively and a distinctive antigen binding region. These proteins exert a fundamental role in immune protection against infection due to their ability to bind to different antigen determinants and recruit complement and promote cellular activation and phagocytosis through engagement of Fc receptors (LeBien and Tedder, 2008).

1.2 *Pseudomonas aeruginosa*

1.2.1 General Characteristics of *P. aeruginosa*

P. aeruginosa is a Gram-negative, motile, ubiquitous microorganism found in diverse environments. Its large genome allows great genetic and metabolic flexibility enabling the bacterium to thrive within numerous ecological niches (Rossolini and Mantengoli, 2005, Sadikot *et al.*, 2005, Lyczak *et al.*, 2000).

P. aeruginosa can inhabit moist sites of the human body such as perineum, axilla, nasal mucosa and throat and can also be isolated from different terrestrial and aquatic niches (Costa *et al.*, 2010, Rossolini and Mantengoli, 2005). *P. aeruginosa* employs an arsenal of exoproducts that contribute to the invasion of the damaged physical barriers of the host and subsequent abrogation of immune responses which facilitates persistence of *P. aeruginosa* infections (Beattie and Merrill, 1996, Mariencheck *et al.*, 2003, Sadikot *et al.*,

2005, Wilson *et al.*, 1987). Furthermore, many strains of *P. aeruginosa* isolated from multiple infectious diseases exhibited high resistance against a wide array of antibiotics, particularly those isolated from intensive care systems (Livermore, 2001, Goossens, 2003, Deshpande *et al.*, 2004).

During the past two decades, *P. aeruginosa* has emerged as an important pathogen causing nosocomial and severe infections that are associated with a high rate of morbidity and mortality (Hirsch and Tam, 2010). The pathogenicity of this bacterium is related to its large genome (> 6Mbp) that encodes approximately 5,500 open reading frame (ORFs) and coordinate expression of a wide range of virulence factors (Wolfgang *et al.*, 2003a, Wolfgang *et al.*, 2003b, Winsor *et al.*, 2011).

P. aeruginosa cells can exist during chronic infections in unique structures, known as a biofilms that provides an extra degree of protection against harsh environments (Lam *et al.*, 1980, Ma *et al.*, 2009).

1.2.2 *P. aeruginosa Infections*

P. aeruginosa represents a major burden to the healthcare system as it is able to cause severe infections, particularly in immune-compromised, cystic fibrosis (CF), burn and cancer patients undergoing long-term medical treatment (Hauser et al., 2011; Lore et al., 2012; Sadikot et al., 2005; Schaber et al., 2007).

1.2.2.1 Burn Wound Infections

P. aeruginosa is a key contributor to serious complications in thermally injured patients (Tredget *et al.*, 2004, Shankowsky *et al.*, 1994). Although, *P.*

aeruginosa produces a wide variety of cell-associated and secreted products contributing towards virulence, such as alginate, flagellum, pili, elastase, alkaline protease and exotoxin A (ETA), this bacterium requires a deficiency in host defences such as tissue damage to establish infection (Lyczak *et al.*, 2000). The impaired immune defence in wound burn individuals, due to the lack of intact skin, represents a vulnerable target for opportunistic microorganisms (Steinstraesser *et al.*, 2005) such as *P. aeruginosa* which is the most commonly isolated organism encountered in burn wound infection (Weinstein and Mayhall, 2003, Japoni *et al.*, 2009).

After initial colonisation *P. aeruginosa* can disseminate throughout the host tissues due to the production of a wide range of virulence factors. For instance, the exoproduct elastase disturbs the integrity of basement membrane via the digestion of collagenous and non-collagenous host substrates and it is also able to suppress phagocyte chemotaxis which might further impair the elimination of *P. aeruginosa* from burn wounds by phagocytosis (Japoni *et al.*, 2009).

1.2.2.2 Respiratory infections caused by P. aeruginosa

An interesting review by Lau *et al.*, (Lau *et al.*, 2005) proposed two possible strategies that *P. aeruginosa* might adopt to initiate respiratory infections. The first model involves surface attachment of potential adhesins, expressed by planktonic pathogens, such as flagella and pili, to the epithelial lining layer. Upon binding, *P. aeruginosa* secretes a range of virulence determinants, such as Exotoxin S (ExoS), via the type 3 secretion system, to alter cell integrity and viability. At this stage, some bacterial cells may develop a biofilm to increase resistance to the immune defences and also provide a sustainable source of *P.*

aeruginosa cells leading to the establishment of a number of chronic infections (Lau *et al.*, 2005) .

The second strategy involves acquisition of the infecting pathogens, in either a planktonic form or in a biofilm state, from contaminated respiratory equipment into already compromised lungs, particularly those with CF. Either cell debris from injured neutrophils or overexpressed mucus are then exploited to generate the biofilm (Whitchurch *et al.*, 2002).

a) Ventilator-acquired Pneumonia

P. aeruginosa is considered one of the most common Gram-negative bacteria responsible for nosocomial respiratory diseases (Richards *et al.*, 2000). However, in healthy individuals *P. aeruginosa* rarely inhabits the human respiratory tracts, but the rate of colonisation elevates in mechanically ventilated patients, particularly those undergoing long-term hospitalisation (Rossolini and Mantengoli, 2005). Once the infection is established, it can cause chronic respiratory infections with a relatively high mortality rate, ranging from 40% to 60% (Crouch Brewer *et al.*, 1996).

It has been proposed that *P. aeruginosa* is able to breach the lung physical barriers and gain access to the respiratory epithelial layer because of the damage caused by the endotracheal tube and ventilator-induced stress (Pugin *et al.*, 2008).

Furthermore, *P. aeruginosa* can persist and resist in multiple hospital sites like, respiratory equipment, sanitary solutions, sinks, mops and other environments that provide a suitable niche for bacterial growth (Morrison and Wenzel, 1984). For example, *P. aeruginosa* has the ability to grow and form a biofilm

on the endotracheal tubes (Adair *et al.*, 1999). It has also been found that health care personnel contribute to infection by carrying and spreading this pathogen among the intubated patients (Beer *et al.*, 2006).

b) Cystic Fibrosis (CF)

Cystic fibrosis (CF) is an autosomal recessive genetic disorder (Hobbs *et al.*, 2013, O'Sullivan and Freedman, 2009, Hoiby, 2011) caused by mutations within a gene on chromosome 7 that encodes a protein termed the cystic fibrosis transmembrane conductance regulator (CFTR). CF disease can affect a wide number of body parts, but the majority of CF mutations largely affect the respiratory and digestive systems (Lomas, 2014, Hoiby, 2011, Ibrahim *et al.*, 2011, Ramsey, 1996, Giron Moreno *et al.*, 2011).

CF is a consequence of one of many possible mutations (more than 1500) within the CFTR gene (Bye *et al.*, 1994, Gaspar *et al.*, 2013). The most common mutation amongst CF patients is the deletion of three nucleotides encoding phenylalanine at position 508 on the CFTR protein (Boucher, 2004). CFTR is a cyclic adenosine monophosphate (cAMP)-dependent transmembrane chloride channel expressed in a variety of human cells particularly epithelial cells, but also found in macrophages, neutrophils and lymphocytes (Painter *et al.*, 2006, Krauss *et al.*, 1992, Dong *et al.*, 1995, Denning *et al.*, 1992). The CFTR channel regulates the transfer and equilibrium of multiple ions, mainly chloride, within cells (Lyczak *et al.*, 2002, Riordan, 2008). Changes in the CFTR gene lead to hyper-production of mucus and the thickening of the airway surface fluids leading to the impairment of the mucocilliary movement essential for the elimination of foreign inhaled

substances, ultimately resulting in exaggerated immune responses. (Rowe *et al.*, 2005).

Numerous studies have shown that the majority of isolates from lung infections in CF patients are dominated by *P. aeruginosa* which is associated to increased morbidity and mortality in CF patients (Dudley *et al.*, 2008, Geller, 2009, van Westreenen and Tiddens, 2010). Furthermore, it has been demonstrated that *P. aeruginosa* clinical strains from CF patients adapt to the selective conditions within the CF lung such as oxygen deficiency, antibiotic re-exposure, multi-bacterial competition and oxidative stress. It achieves this by employing sophisticated strategies, which aid in the persistence of the bacterial infections, such as biofilm formation, antibiotic resistance and phenotype switching to mucoid-forming bacteria (Sousa and Pereira, 2014).

1.2.3 Virulence Determinants of *P. aeruginosa*

The pathogenicity of *P. aeruginosa* is directly related to the expression of structural and secreted virulence factors (Figure 1.5). The bacterial cell surface structures such as flagella, pili, lipopolysaccharide (LPS) and extracellular polysaccharides play a fundamental role in the first stage of bacterial infection, by facilitating cell adhesion and colonisation of abiotic or biotic surfaces (O'Toole and Kolter, 1998, Hahn, 1997, Mattick, 2002, Rocchetta *et al.*, 1999, Kintz and Goldberg, 2008, Oliver and Weir, 1985). *P. aeruginosa* exoproducts cause cell injury, help bacterial dissemination throughout the host and impair immune defences which results in deregulated inflammatory responses. These secreted factors include several toxins, such as Exotoxin A (ETA), alkaline protease, elastase and phospholipase C. *P. aeruginosa* also possesses a unique

machinery which consists of a needle-like structure able to deliver toxins from bacterial cells into the cytoplasm of infected host cells termed type III secretion system (Kipnis *et al.*, 2004, Kon *et al.*, 1999, Terada *et al.*, 1999, Galle *et al.*, 2012, Webb *et al.*, 2008).

P. aeruginosa possesses a sophisticated signalling system known as Quorum Sensing (discussed further in the following section), which regulates bacterial cellular activity, including virulence factor production and biofilm formation. This regulatory system allows *P. aeruginosa* to behave as an organised community and rapidly adapt to changes in their local environment (Williams and Camara, 2009, Parsek and Greenberg, 2000).

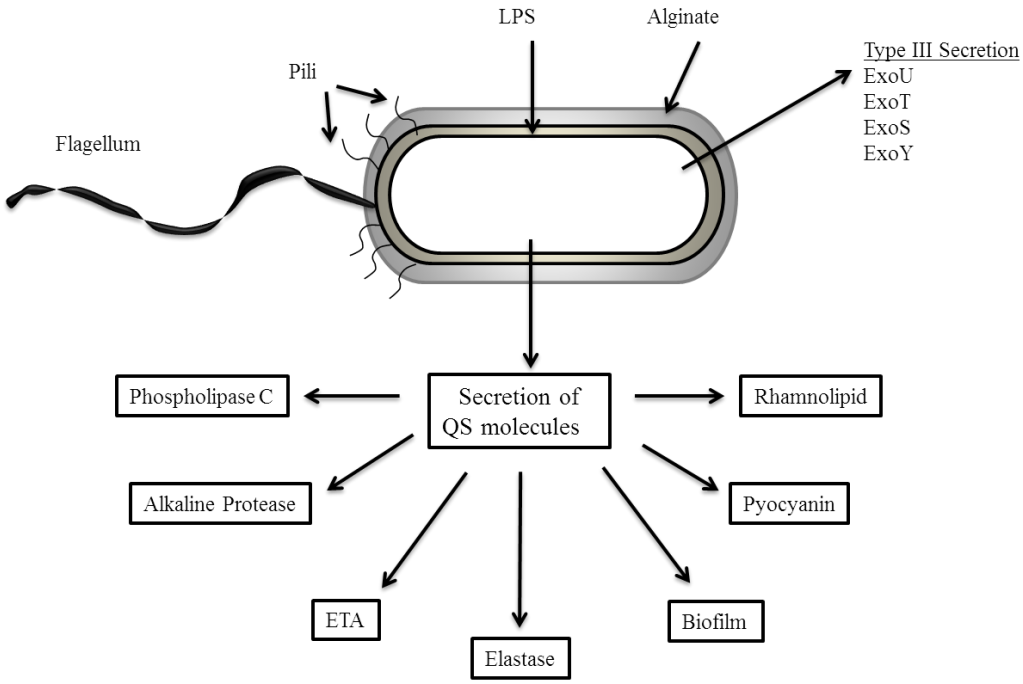

Figure 1.5: Virulence factors of *P. aeruginosa*.
P. aeruginosa has cell-associated virulence factors (flagellum, pili, alginate, and LPS) and extracellular virulence factors (rhamnolipid, elastase, alkaline protease, exotoxin A (ETA), pyocyanin). It can also deliver toxins (ExoU, ExoT, ExoS and ExoY) directly into infected cell cytoplasm via type III secretion system). Adapted from (Winstanley and Fothergill, 2009, Sadikot *et al.*, 2005).

1.2.3.1 Quorum Sensing

Many studies have demonstrated that the primary mechanism used by *P. aeruginosa* to control production of virulence factors and biofilm formation (see 1.2.2.4) is a population-density dependent mechanism known as quorum sensing (QS). QS takes place via the action of small signalling molecules called auto-inducers such as *N*-acylhomoserine lactones (AHLs) and the 2-alkyl-4-quinolones (AQs) (Rumbaugh *et al.*, 2000, Costa *et al.*, 2010, Williams and Camara, 2009).

N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL) are the main examples of AHLs whereas 2-heptyl-3-hydroxy-4 (1H)-quinolone (also termed Pseudomonas Quinolone Signal, PQS) is the main AQ (Williams and Camara, 2009). They auto-regulate three potential integrated QS networks each composed of a signal synthase component responsible for the production of the auto-inducers, which binds to the cognate transcriptional activator.. QS promotes the transcription of over 10% of the bacterial genome (Williams and Camara, 2009, Rumbaugh *et al.*, 2000). The Las system consists of LasI and LasR and is responsible for the production and sensing of 3-oxo-C12-HSL respectively and regulates the secretion of virulence factors such as elastase, the protease LasA, ETA and biofilm formation (Diggle *et al.*, 2003). While, the Rhl system is composed of RhlI that mediates the synthesis of C4-HSL and the regulator RhlR which, upon being activated by this molecule, triggers the production of rhamnolipids, elastase, LasA protease, pyocyanin, and the cytotoxic lectins LecA and LecB.

The PQS synthesis is catalysed by the action of the gene products of the *pqsABCDE* operon the expression of which is controlled by the LysR-type

regulator PqsR upon binding PQS or its immediate biosynthetic precursor molecule 2-heptyl-4(1*H*)-quinolone (HHQ) (Williams and Camara, 2009, Rutherford and Bassler, 2012).

Many studies have demonstrated that auto-inducers directly affect host cell behaviour and viability. Hooi and co-workers observed that 3-oxo-C12-HSL and PQS have an anti-proliferative effect on T cells (Hooi *et al.*, 2004). It has also been reported that 3-oxo-C12-HSL promotes apoptosis in both macrophages and neutrophils (Tateda *et al.*, 2003). In addition, this inducer can suppress IL-12 production (a cytokine which steers Th1 proliferation, see above), lymphocyte proliferation and TNF-α secretion by macrophages stimulated with LPS (Telford *et al.*, 1998a). Furthermore, 3-oxo-C12-HSL modulates neutrophil migration directly (Zimmermann *et al.*, 2006), or via the robust production of IL-8 from fibroblasts and epithelial cell lines, leading to the activation of inflammatory responses (Cooley *et al.*, 2008).

1.2.3.2 Biofilms

In response to external clues *P. aeruginosa* undergoes a phenotypic shift from free living cells (planktonic) to form extensive cellular aggregates and highly structured communities termed biofilms. Biofilm formation comprises a sequence of steps (Figure 1.6), including cell adherence and aggregation onto either abiotic or biotic surfaces. This eventually results in growth of microcolonies embedded within a dense, secreted, extracellular matrix that is mainly composed of polysaccharides, nucleic acid, proteins and cell debris (Wagner and Iglewski, 2008, Ma *et al.*, 2009). The development of a biofilm serves as vital source of bacterial cells and promotes bacterial resistance to

harsh environments and antimicrobial substances. Importantly, it is believed that biofilms maintain and/or promote chronic inflammatory conditions (Costerton *et al.*, 1999).

It has been recognised that the long-term persistence of *P. aeruginosa* in the CF respiratory system might correlate with biofilm development, as biofilms are far more resistant to antibiotics than planktonic cells (Wagner and Iglewski, 2008).

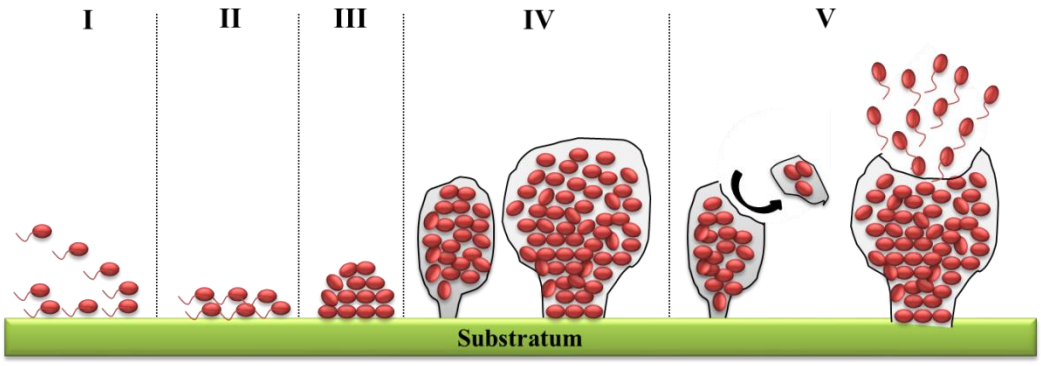

Figure 1.6: Schematic presentation of biofilm formation by *P. aeruginosa*.
Biofilm development undergoes five stages starting with reversible bacterial cell adherence to the target substrate **(I)**, and then irreversible attachment **(II).** After this stage, bacterial cells lose the surface appendages accompanied with the formation of microcolonies **(III)**, followed by the establishment of a mature biofilm consisting of non-motile bacteria aggregated in a unique mixture of extracellular matrix composed of polysaccharides, nucleic acid, proteins and debris **(IV)**. Later, the biofilm structure begins to disperse cells by either the detachment of large portions of existing biofilm or separation of motile cells from a rupture in order to colonise new surfaces or due to surrounding stress and conditions such as nutrient and oxygen depletion within growing biofilms (**V**). Figure adapted from (Wagner and Iglewski, 2008) .

1.2.3.3 Surface-Bound Virulence Determinants

Planktonic *P. aeruginosa* cells possess distinct cellular components that play a cardinal role in establishing the early phase of bacterial infections. The flagellum is a tail-like propeler which is essential for motility and dissemination throughout the host tissues. The response to the bacterial

flagellum is a consequence of the airway epithelia production of IL-8 after activation of Toll like receptor 5 (TLR5) on their apical surface (Adamo *et al.*, 2004, Blohmke *et al.*, 2010). Furthermore, flagella upregulate the expression of TLR5, which is the receptor for the main component of the bacterial flagella termed flagellin, on the surface of myeloid cells in CF and can drive the expansion of myeloid-derived suppressor cells (MDSCs). This in turns inhibits the proliferative response of T cells and impairs the efficient elimination of *P. aeruginosa* (Rieber *et al.*, 2013). In this context, TLR-5 is considered as a target and modifier gene in CF patients as the TLR5-flagellin ligation mediates the establishment of inflammation in response to *P. aeruginosa* (Blohmke *et al.*, 2010).

P. aeruginosa also expresses fine filamentous structures called pili. These appendages have a critical role in twitching motility and rapid bacterial colonisation on the epithelial cells by binding to the GalNAcβ1-4Gal motif present on gangliotetraosylceramide (asialoGM1) eventually leading to the activation of Nuclear factor-κB (NF-κB) and transcription of proinflammatory genes such as IL-8, and mucin production (Hahn, 1997, Mattick, 2002, DiMango *et al.*, 1998, McNamara *et al.*, 2001).

During chronic infections, *P. aeruginosa* cells produce a viscous exopolysaccharide known as alginate. It has been found that the mucoid phenotype of CF isolates are characterised by overexpression of alginate, which forms a unique structure termed the ‘capsule’ (Tielen *et al.*, 2005, Sousa and Pereira, 2014). Alginate production boosts bacterial survival against antibiotics and immune mechanisms, such as phagocytosis, leading to the establishment of chronic infections (Leid *et al.*, 2005, Oliver and Weir, 1985).

Lipopolysaccharides (LPS) is a potential immune activator via interaction with TLR4 (Kopp and Medzhitov, 2003). LPS is an endotoxin found in the outer membrane of Gram-negative bacteria consisting of a hydrophobic lipid A moiety connected to a oligosaccharide with a polysaccharide O-antigen (Rocchetta *et al.*, 1999, Kintz and Goldberg, 2008).

During chronic infections *P. aeruginosa* LPS displays modifications in its acylation patterns to confound recognition by TLR4, possibly as a strategy to minimise innate immune defences in CF and facilitate maintenance of bacterial infection (Ernst *et al.*, 2007, Cigana *et al.*, 2009).

1.2.3.4 Secreted Virulence Determinants

P. aeruginosa has the ability to destroy tissue integrity and disseminate throughout the host due to the production of an arsenal of virulence factors which have the capacity to cause cytotoxicity and attenuate immune responses.

For instance, phospholipase C is a hemolysin that promotes bacterial invasion by triggering fatal injuries in host cells (Sadikot *et al.*, 2005). The ability to supress surfactant function in the lung and neutrophil respiratory burst activity and thus assist chronic persistent infection by *P. aeruginosa* is also attributed to phospholipase C (Terada *et al.*, 1999, Berra *et al.*, 2010).

Alkaline proteases allow *P. aeruginosa* to evade immune recognition through degradation of monomeric flagellin (Bardoel *et al.*, 2011) and multiple immune defence proteins i.e. antibody and complement (Hong and Ghebrehiwet, 1992) as well as cytokines such as IFN-γ and TNF-α (Parmely *et al.*, 1990).

Exotoxin A (ETA) is considered as an important virulence factor in *P. aeruginosa* (Liu, 1974). The enhanced production of this toxin, at the transcriptional level, is controlled by the activity of a set of genes such as *toxR* (also known *RegA*), *RegB, lasR* and *PvdS* (the iron starvation sigma factor) (Davinic *et al.*, 2009, West *et al.*, 1994, Hamood *et al.*, 1996a). However, the structural gen *toxA* is directly involved in ETA production by *P. aeruginosa* (Gaines et al., 2007) under the control of the regulatory element *toxR* (Hedstrom et al., 1986). Thus, the regulatory gene *toxR* is the most extensively studied gene among the positive regulators. A work has been done by Hedstrom and co-workers showed that *toxR* enhances the expression of ETA 10 fold (Hedstrom *et al.*, 1986). The toxic nature of this enzyme is due to its ADP-ribosylation activity which catalyses the transfer of adenosine diphosphate ribose (ADP-ribose) from nicotinamide adenine dinucleotide (NAD^+) to eukaryotic elongation factor-2 (eEF-2), thereby blocking protein synthesis and leading ultimately to cell death (discussed in more detail in Chapter 3) (Webb *et al.*, 2008).

Elastase exerts a number of effects on host cells by breaking down immunoglobulins (Ig), surfactant protein A and D and affecting epithelial integrity (Wretlind and Pavlovskis, 1983, Mariencheck *et al.*, 2003).

P. aeruginosa also secretes a potent pigmented metabolite referred to as pyocyanin (Denning *et al.*, 1998). This product has been shown to be responsible for direct host damage at several levels including disruption of tight junctions in bronchial epithelial cells and mucociliary movement (Wilson *et al.*, 1987, Sadikot *et al.*, 2005). It has been also reported that pyocyanin can

block antioxidant defences and catalase activity, thus amplifying local inflammatory responses (O'Malley *et al.*, 2003b, O'Malley *et al.*, 2003a).

Rhamnolipids are functional bio-surfactants that have a pivotal role during chronic *P. aeruginosa* infection. Rhamnolipids are mixtures of glycolipids with mono- or di-rhamnose moieties linked to 3-hydroxyalkanoyloxy alkanoic fatty acids (HAA), which have the ability to solubilise various insoluble substances, reduce surface tension (Beal and Betts, 2000, Caiazza *et al.*, 2005, Deziel *et al.*, 2003) and ultimately increase cell motility, such as swarming (Kohler *et al.*, 2000). Furthermore, the toxins that are delivered by the type III secretion machinery from bacterial cells into the cytoplasm of infected host cells are able to alter immune responses and enhance bacterial pathogenicity (Kim *et al.*, 2005, Rietsch and Mekalanos, 2006). To date, *P. aeruginosa* has been shown to secrete four different effector proteins in this way, ExoS, ExoT, ExoU and ExoY, with each exerting different effects on target cells (Hauser, 2009).

ExoS and ExoT are GTPase activating proteins considered responsible for actin depolymerisation, cell detachment and eventually cell death (Finnan *et al.*, 2004, Kaufman *et al.*, 2000). ExoU is a potent phospholipase that targets a broad array of molecules, such as phospholipids, leading to the impairment of cell membrane integrity and cell lysis (Sato and Frank, 2004, Phillips *et al.*, 2003, Sato *et al.*, 2003, Finck-Barbancon *et al.*, 1997). ExoY is an adenylyl cyclase protein that exerts cytotoxic effects in various cell types, such as yeast and mammalian cells (Yahr *et al.*, 1998, Arnoldo *et al.*, 2008, Cowell *et al.*, 2005, Sayner *et al.*, 2011).

1.2.4 Immune recognition of *P. aeruginosa.*

The effective elimination of *P. aeruginosa* at the sites of infection requires the collaboration of components of both the innate and adaptive immune system.

1.2.4.1 Myeloid Cells

Myeloid innate cells are recognised as vital in host immunity against *P. aeruginosa* due to their ability to recognise foreign molecules through expression of membrane and cytosolic PRRs, (McIsaac *et al.*, 2012b). TLRs expressed by immune cells are able to alert the immune response to pathogens through the stimulation of specific signalling pathways (see Section 1.1.1).

Murine studies have indicated the importance of TLR2, 4 and 5 as signalling receptors that coordinate the protection against *P. aeruginosa* (Elson *et al.*, 2007, Feuillet *et al.*, 2006, Power *et al.*, 2004, Ramphal *et al.*, 2005, Ramphal *et al.*, 2008, Skerrett *et al.*, 2007). In this context, the potential mechanisms by which pseudomonas LPS and flagella can be recognised by TLR4 and TLR5, respectively, have been identified (Mariathasan *et al.*, 2004, Franchi *et al.*, 2007, Sutterwala *et al.*, 2007, Miao *et al.*, 2008). In addition, the extracellular slime-glycolipoprotein (slime-GLP), alginate, and ExoS are considered potential ligands for TLR2 (Lagoumintzis *et al.*, 2008, Flo *et al.*, 2002, Epelman *et al.*, 2000).

It has been also hypothesised that CFTR can bind *P. aeruginosa* and act as a sensor and receptor that could aid in controlling bacterial infection (Schroeder *et al.*, 2002, Campodonico *et al.*, 2008, Cohen and Prince, 2012). It has been established that neutrophils are responsible for the clearance of *P. aeruginosa* (Sadikot *et al.*, 2005), primarily in the lung, and this defensive role has been

confirmed in murine models (Koh *et al.*, 2009). Whilst TLRs are now known to be important in the control of this pathogen, Gyetko and his colleague showed that CD87, an urokinase plasminogen activator (uPA) receptor, expressed by neutrophils is also involved in the murine defence against *P. aeruginosa* (Gyetko *et al.*, 2004, Gyetko *et al.*, 2000, Elghetany *et al.*, 2003). During infection, neutrophils migrate towards the invaded region in response to a wide spectrum of cytokines and chemokines produced by various cells, such as macrophages, epithelial cells, and lymphocytes (Sadikot *et al.*, 2005). However, it has been observed that their protective and killing functions are compromised when *P. aeruginosa* cells adopt a biofilm state during chronic infections, such as those common to CF patients (Jesaitis *et al.*, 2003, Jensen *et al.*, 2007). Furthermore, *P. aeruginosa* can abolish the action of neutrophils by the production of virulence proteins which either cause cell injury, such as pyocyanin and rhamnolipids, or impair their phagocytic and killing activities (Bayer *et al.*, 1991, Usher *et al.*, 2002, Jensen *et al.*, 2007, Mishra *et al.*, 2012, Sun *et al.*, 2012). The induction of neutrophil cell death by *P. aeruginosa* may explain the strategy by which this organism can survive host defences and persist for a long time in hostile tissue environments (Usher *et al.*, 2002, Tateda *et al.*, 2003).

In vivo studies in murine models also revealed that innate immune cells, such as neutrophils and macrophages, appear to express CFTR and that CFTR in these cells can contribute to phagocytic activity (Deriy *et al.*, 2009, Di *et al.*, 2006). Many reports attributed the impaired elimination of *P. aeruginosa* by phagocytic cells in CF patients to the defect in CFTR function, which may be responsible for subsequently poor endosomal acidification and less killing

activity (Di *et al.*, 2006, Deriy *et al.*, 2009, Yoshimura *et al.*, 1991, Bruscia *et al.*, 2009, Del Porto *et al.*, 2011, Sorio *et al.*, 2011).

Interestingly, CFTR control of neutrophil function causes intense inflammatory disorders in CFTR knock-out mice (Su *et al.*, 2011). Furthermore, Ratner and co-workers observed that neutrophils showed a dramatic reduction in phagocytic activity in CF patients and this may be attributed to the impairment of function in the mutated CFTR (Ratner and Mueller, 2012).

Further studies into the impact of CFTR on neutrophil activity have revealed that knock-down of CFTR expression in neutrophils disturbs the chloride ion pumping into the cytosolic phagosomes, which manifests as a significant decrease in killing activity and IL-8 production, and ultimately cell death (Painter *et al.*, 2010, Painter *et al.*, 2006, Painter *et al.*, 2008, Porro *et al.*, 2001, Watt *et al.*, 2005). The role of eosinophils in *P. aeruginosa* infections has also been investigated. Eosinophils can recognise bacterial components via the expression of TLRs on the cell membrane, including TLR2, 4 and 5 (Wong *et al.*, 2007). Work by Linch and co-workers in mice, showed that eosinophils exhibit antimicrobial activities against *P. aeruginosa* through the production of cationic secondary granule proteins, which may suggest a role in pathogen control (Linch *et al.*, 2009).

Macrophages also play an important role in *Pseudomonas* clearance, but possibly in their capacity to alert and activate adaptive responses (Sadikot *et al.*, 2005). In the lung, alveolar macrophages, beside phagocytic activity, represent the main source of secreted cytokines and chemokines, such as IL-1β and IL-8, following *P. aeruginosa* invasion (Williams *et al.*, 2010). The

production of TNF-α by the resident lung macrophages has also been linked to the ability to eliminate invading *P. aeruginosa*, as TNF-α-deficient mice are less effective in controlling bacterial growth (Lee *et al.*, 2003).

As well as TLR, several other surface molecules have been reported as *P. aeruginosa* receptors in macrophages including complement receptor 3 (CR-3) (Mueller-Ortiz *et al.*, 2004, Heale *et al.*, 2001), CD14 (Wilkinson *et al.*, 2009, Heale *et al.*, 2001), Fc receptor and mannose receptor (Speert *et al.*, 1988). These receptors have been shown to be vital in the early recognition of this pathogen. The mannose receptor, in synergy with TLR2 on human monocytes, significantly promoted the pro-inflammatory responses against bacterial infection (Xaplanteri *et al.*, 2009).

Inflammasomes are powerful protein platforms that are triggered upon exposure to potential pathogenic components and lead to the activation of cysteine protease enzymes, i.e. caspase-1, and subsequently to the proteolytic maturation of pro-inflammatory cytokines, such as IL-1β and IL-18 (Schroder and Tschopp, 2010, Poyet *et al.*, 2001). A number of studies have highlighted the presence of inflammasomes in myeloid cells, such as macrophages, and implicated inflammasomes in the stimulation of innate immune defences. The inflammasome components, ice protease activating factor (IPAF), can be activated by the type III secretion system and flagellin, ultimately leading to cell death, referred as pyroptosis, and inflammatory responses (Franchi *et al.*, 2006, Franchi *et al.*, 2007, Fink and Cookson, 2005).

However in chronic respiratory tract infections, *P. aeruginosa* develops new strategies to impair bacterial clearance by immune cells, especially in patients with CF. There is clear evidence that *P. aeruginosa* cells abolish the

expression of different virulence factors, like exoenzymes excreted through the type III secretion system, which results in long-term bacterial survival and invasion (Mahenthiralingam *et al.*, 1994, Jain *et al.*, 2004).

DCs act as a bridges between the innate and adaptive immune responses (Collin *et al.*, 2013). They capture and process antigens from *P. aeruginosa*, leading to either local cytokine release by DCs or migration toward the lymph nodes to present the processed peptides to T lymphocytes, thereby initiating the adaptive immunity (Kushwah and Hu, 2011, Schott, 2006). However, in chronic infections, such as those seen in CF patients, DC differentiation is significantly slower than in healthy counterparts, and this delay is believed to be directly related to the delay in initiating the adaptive responses (Xu *et al.*, 2009).

1.2.4.2 Lymphocytes

There is clear evidence in the literature that points to the importance of $CD4^+$ and $CD8^+$ T cells in the fight against *P. aeruginosa* in murine models (Dunkley *et al.*, 1994, Markham *et al.*, 1984, Pier and Markham, 1982, Markham *et al.*, 1985). Furthermore, *in vivo* studies also suggest that the type of T cell-dominated immune response impacts on survival. Th2 responses showed a lower resistance to *P. aeruginosa* pulmonary infections compared to Th1 responses (Tam *et al.*, 1999, Moser *et al.*, 1999). Interestingly, similar results were exhibited by CF patients and led to the proposal that the presence of Th1 cells in the lung may be essential for effective resistance to *P. aeruginosa* due to IFN-γ production and subsequent $CD8^+$ T cell activation (Moser *et al.*, 2002, Moser *et al.*, 2000, Moser *et al.*, 1999, Moser *et al.*, 1997). It has been

reported that CF sufferers exhibit predominantly Th2 pulmonary responses, which might explain the deficiency in lung function and bacterial elimination (Hartl *et al.*, 2006).

In the context of *P. aeruginosa* infection many studies have demonstrated a strong humoral immune response during chronic *P. aeruginosa* infections in CF patients (Moser *et al.*, 2000) and in murine models (Song *et al.*, 2003), which are dominated by T helper 2 cells (Th2) and the expression of IL-4 and IL-13 leading to the generation of M2 macrophages. M2 macrophages are less microbicidal and pro-inflammatory and their presence correlate with poor lung function in CF patients (Hoiby *et al.*, 2001, Hartl *et al.*, 2006, Mantovani *et al.*, 2004, Martinez *et al.*, 2009)

There is a rapidly growing body of evidence in support for a vital role for IL-17 and IL-23 produced by Th17 for protection against a number of extracellular Gram-negative pathogens such as *P. aeruginosa* (Kuestner *et al.*, 2007) and several studies have shown enhanced IL-17 production in CF patients (Brodlie et al., 2011) . Elevated level of IL-17 and 23 cytokines in CF patients might be responsible for the exacerbated inflammatory response and neutrophilic inflammation in the CF lung (Dubin and Kolls, 2007, Brodlie *et al.*, 2011, McAllister *et al.*, 2005).

This would be in agreement with results from our group showing that IFN-γ and IL-17A production by stimulated PBMCs positively and negatively correlate with lung function in CF patients (Singh *et al.*, 2015).

Besides $CD4^+$ and $CD8^+$ T cells, a small subset of T cell termed NKT can contribute to protection against *P. aeruginosa*. NKT cells recognise and ligate with distinct glycolipids expressed by phagocytic macrophages in the context

of CD1 MHC class 1-like proteins (Terabe and Berzofsky, 2008). Nieuwenhuis and his co-workers (Nieuwenhuis *et al.*, 2002) demonstrated that the activity of CD1d-restricted T cells is required for efficient bacterial killing by mice alveolar macrophages (Nieuwenhuis *et al.*, 2002).

1.3 Aims and Objectives

T cells play a cardinal role in controlling infection caused by bacterial invasion. However, the protective role of these cells relies on their clonal expansion to increase in cell numbers. The aim of the present study was to assess the impact of *P. aeruginosa* exo-products on human immune cells using bacterial supernatants as a source of secreted virulence factors. Peripheral blood mononuclear cells from healthy donors were employed as models to study human immune cells. In addition this thesis explored the potential effect of *P. aeruginosa* exo-products on *C. albicans*.

The specific objectives of this thesis were as follows:

1. Analysis of the effect of *P. aeruginosa* supernatants on T-cell proliferation.
2. Assessment of the impact of *P. aeruginosa* supernatants on the viability of T cells and monocytes.
3. Identification of components responsible for observed effects and construction of a *P. aeruginosa* deficient strain
4. Explore the effect of *P. aeruginosa* supernatants on the behaviour of the opportunistic pathogen *C. albicans*

2. Effect of *P. aeruginosa* supernatants on T cell proliferation

2.1 Introduction

T cells play a fundamental role in immune protection by orchestrating the immunological cascade. They exert this function after activation which is governed by a number of factors including, the nature of antigen, presence of co-stimulatory molecules in antigen presenting cells, and environmental factors such as cytokine and chemokine profiles (D'Elios *et al.*, 2011). Full T cell activation and extensive clonal expansion requires multiple interactions with APCs such as the ligation of the T-cell receptor (TCR/CD3) complex with MHC-peptides, in parallel with co-stimulatory binding of the CD28 molecules on T cells with B7 ligands expressed by APCs (as described in Section 1.1.2.1) (Lenschow and Bluestone, 1993, Thompson *et al.*, 1989, Azuma *et al.*, 1993, Freeman *et al.*, 1993, Linsley *et al.*, 1990).

The majority of T lymphocytes express αβTCRs which consist of disulfide bonds-connected α and β chains associated with CD3γδε and ζ2 chains (Kuhns *et al.*, 2006). The CD3 subunits play a crucial role in cellular signaling transduction upon the ligation of TCR by the cognate peptide-MHC complex leading to the activation of NF-κB cascade which is responsible for T cell expassion and differentiation (Paul and Schaefer, 2013, Sun *et al.*, 2013). An early event during TCR activation involves the phosphorylation of cytosolic signalling molecules termed immunoreceptor tyrosine-based activation molecule (ITAM) associated with CD3 chains by Src family tyrosine kinases

which leads to the recruitment of z-chain associated protein kinase (Zap-70) (Burbach *et al.*, 2007, Cheng *et al.*, 2011, Cronin and Penninger, 2007). Zap-70 activates and phosphorelates a number of substrates such as trans-membrane adaptor protein LAT which promotes the expression of the second messengers like diacylglycerol (DAG) followed by activation NF-κB signalling pathway (Cheng *et al.*, 2011).

CD28 is a 44-kDa homodimeric glycoprotein expressed at the surface of T cells. In the presence of TCR signalling engagement of CD28 with corresponding ligands triggers synthesis of the T cell growth factor IL-2 which promotes T cell expansion and, in turn, T cell-dependent responses (Thompson *et al.*, 1989, Ledbetter *et al.*, 1990, Nelson, 2004, Sharpe and Freeman, 2002, Appleman *et al.*, 2000).

Peripheral blood is the most common source of T lymphocytes for *in vitro* studies to evaluate human T cell-mediated immune responses (Clay *et al.*, 2001). T cell function in terms of cell expansion and cytokine secretion has been traditionally measured using either whole blood (Deenadayalan *et al.*, 2013), or PBMCs (Lin *et al.*, 2009, Starska *et al.*, 2011, Kaabinejadian *et al.*, 2013, Deenadayalan *et al.*, 2013) or purified specific T cell subsets (Aggarwal *et al.*, 2003a, Kronin *et al.*, 2000, Kaminitz *et al.*, 2010).

A wide number of agents can specifically or non-specifically induce T cell activation. Purified APCs are widely used to activate T cells (Eken *et al.*, 2011). Furthermore, antibody against CD3 and CD28 are used in many T cell proliferation protocols because of their ability to interact directly with their cognate antigens on a large percentage of responder T cells (Riddell and

Greenberg, 1990, Klapper *et al.*, 2009, Rosenberg and Dudley, 2004, Li and Kurlander, 2010, Aggarwal *et al.*, 2003a).

Super-antigens, which are proteins derived from microorganisms, are also widely used to activate T cells in culture. For instance, staphylococcal enterotoxin B (SEB) is a powerful mitogen for both murine and human T cells. SEB is expressed by *Staphylococcus aureus* (*S. aureus*) and plays a crucial role in the activation of T cells via the association of the Vβ chain of the T-cell receptor (TCR) with HLA class II molecules on antigen-presenting cells (El Hage *et al.*, 2010, Bisset *et al.*, 1993, Marrack and Kappler, 1990). The mitogen Phytohaemagglutinin (PHA) is a plant lectin that stimulates, T cell expansion in the presence of soluble factors, such as IL-6, elaborated by accessory cells (Ceuppens *et al.*, 1988, Greaves *et al.*, 1974).

Extensive studies in animal models have demonstrated that T cell-based immune responses increase resistance against *P. aeruginosa*. The protective role of T cells against bacterial infections has been assumed to be limited to promotion of antibody production. However, it has been demonstrated that T cell immunity to invading bacteria such as *P. aeruginosa* may have a crucial role in preventing bacterial growth in the host and promote killing of *P. aeruginosa* cells (Stevenson *et al.*, 1995, Markham and Powderly, 1988a, Dunkley *et al.*, 1994, Kondratieva *et al.*, 2000, Powderly *et al.*, 1986, Markham *et al.*, 1984, Markham *et al.*, 1985, Markham *et al.*, 1988, Markham *et al.*, 1991, Markham and Powderly, 1988b). However, *P. aeruginosa* possesses an arsenal of structural and secreted virulence factors with the ability to modulate immunity of the infected host and aid to the persistence of bacterial infection (described in Section 1.2.2.3).

2.2 Hypothesis

P. aeruginosa expresses numerous virulence factors that play a crucial role in the modulation of immune responses and might facilitate the establishment of infection in the host. T lymphocytes are adaptive immune cells, which when activated expand and contribute to the control of bacterial dissemination. We hypothesise that *P. aeruginosa* supernatants could contain molecules capable of modulating T cell activation. In this chapter we describe the effect of supernatants from different *P. aeruginosa* strains on T cell proliferation in PBMC cultures stimulated with SEB with the aim to identify key factors that could be exploited to attenuate bacterial pathogenicity.

2.3 Aims

1. Elucidate the effect of *P. aeruginosa* secretions on T cell proliferation.
2. Determine the key factors produced by *P. aeruginosa* that regulate T cell proliferation and their specific impact on immune cells such as T helper cells and monocytes.

2.4 Materials and Methods

2.4.1 Isolation of peripheral blood mononuclear cells (PBMCs) from human blood.

All procedures were approved by the Faculty of Medicine Ethics Committee. Fresh blood samples were collected in ethylenediaminetetracetic acid (EDTA) vacutainers (BD Diagnostics-Pre analytical Systems, UK) from healthy donors after obtaining consent. Blood was diluted with an equal volume of Hank's balanced salt solution (Sigma H9394) and layered gently, using a 25ml pipette, over Histopaque -1077 (Sigma HH8889) in 50 ml falcon tubes (BD Biosciences, UK). Samples were centrifuged at 800 g, slow acceleration, and no brake, at room temperature for 30 minutes. After centrifugation, PBMCs, were collected using a sterile plastic Pasteur pipette and washed twice with Hank's balanced salt solution before suspension in RPMI complete medium [RPMI-1640 (Sigma R0883) containing 10% human AB serum (Sigma H4522), 2 mM L-glutamine (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma) and 10 mM HEPES (Invitrogen)].

2.4.2 Preparation of (PAO1) supernatants.

Bacterial strains PAO1 sub-line Lausanne (PAO1-L); PAO1-L*ΔtoxR* and PAO1 sub-line Nottingham (PAO1-N), were kept in glycerol (50%) at -80°C . To prepare bacterial inoculum, bacteria were streaked on Luria-Bertani (LB) agar plates and incubated at 37°C for 24 hours. A single colony was inoculated into a 50ml falcon tube containing 5 ml of X-Vivo 15 media (Lonza BE04-744Q) and incubated overnight at 37°C, 200 rpm. The next day, cultures were

diluted into 25ml of fresh X-Vivo 15 media in either 250 ml glass flasks or Costar flasks (Sigma) as required, such that the optical density at 600nm (OD_{600nm}) of the new culture was 0.01. Cultures were incubated for 4 hours under the above conditions, centrifuged at 10,000 rpm for 10 minutes in a Beckman Coulter, Allegra X-30R centrifuge. Supernatants were collected, filtered through a 0.22 µm filter, aliquoted and stored at -80°C until use.

2.4.3 Analysis of T cell proliferation in response to Staphylococcus enterotoxin B (SEB).

Purified PBMCS were washed twice with Hank's balanced salt solution and re-suspended in 1 ml RPMI complete medium before labelling with 5µM Carboxyfluorescein succinimidyl ester (CFSE, Fisher Scientific, VXC1157) for 5 minutes in the dark at room temperature. PBMCs were then washed 3 times in RPMI complete medium and re-suspended in the same medium at 2.1 X 10^6 PBMCs/ml. Cells were added to each well of a sterile U-shape bottom 96-well plate (1.5 X 10^5 PBMCs in 70µl, Thermo Fisher Scientific, 163320) and treated with different amounts of supernatants in the presence of 10 ng/ml SEB (Sigma, S4881) in a final volume of 100 µl.

After 5 days incubation at 37°C, 5% CO_2, PBMCs were transferred to 12x75mm polystyrene, FACS tubes (Elkay, 2052-003) and washed with 1ml RPMI containing 2% fetal bovine serum (FBS). PBMCs were then labelled for T cell surface markers using conjugated primary antibody and appropriate isotype-matched controls: CD3-PC5 (Beckman-Coulter, IM2635U) alone or in combination with either CD4-ECD (Beckman-Coulter, 6604727) or CD8-ECD (Beckman-Coulter, 737659) for 30 minutes at 4°C in the dark. PBMCs were

subsequently washed with 1 ml PBA (0.5% bovine serum albumin (Sigma A3294) and 0.1% sodium azide (Sigma 100958771) in Phosphate-buffered saline (PBS), re-suspended in 0.5ml PBA and fixed by the addition of 0.5 ml 1% paraformaldehyde (PFA) in PBS, and stored at 4°C until to analysis. Samples were analysed using a Beckman Coulter FC500 flow cytometer and the data were analysed using Weasel software.

2.4.4 Analysis of T cell proliferation in response to α-CD3 and α-CD28 antibody.

Wells of flat-bottom 96 well plates were coated with 100 µl of 2µg/ml human α-CD3 antibody (clone OKT3, kindly provided by Lucy Fairclough) for 90 min at 37°C, 5% CO_2 and washed twice with 100 µl and 200 µl cold PBS, respectively. PBMCs were labelled with CFSE as described in section 2.4.3 and re-suspended at 2.1 x 10^6 PBMCs/ml in RPMI complete medium (see section 2.3). 70µl of this PBMC suspension (1.5 X 10^5 PBMCs), either alone or in the presence of 2µg/ml of human CD28 (Clone 37407, R & D Systems, cat.no: MAB342, kindly provided by Dr Lucy Fairclough), was added to each well. Wells containing PBMCs exposed to either α-CD3 alone or α-CD3/α-CD28 were either left untreated or treated with PAO1-L supernatants prepared in glass flask (20%, v/v) to a final volume of 100 µl. Controls included un-stimulated PBMCs plated in uncoated wells in the absence or presence of the bacterial supernatants and PBMCs activated with SEB.

After 5 days incubation at 37°C, 5% CO_2, PBMCs were transferred to FACs tubes and analysed for T cell surface markers by flow cytometry: as described previously (see section 2.4.3).

2.4.5 Analysis of the effect of PAO1-L supernatants on T-cell and Monocyte viability.

Purified PBMCs were prepared as described previously (section 2.4.3) and re-suspended at a density of 2.1 x 10^6 PBMCs/ml. 70µl of this PBMC suspension (1.5 X 10^5 PBMCs) was added to each well of U-shape bottom 96 well plates. PBMCs were treated with 10 ng/ml SEB (Sigma), PAO1-L supernatant (20%, v/v) or both in a final volume of 100 µl and then incubated for either 24 or 48 hours at 37°C, 5% CO_2. Cellular apoptosis was assessed using an Annexin-V-FITC based kit (Serotec Inc., ANNEX300F) according to manufacturer's instructions. In brief PBMCs were transferred to FACs tubes, washed with PBS and re-suspended in binding buffer to a cell density of 5 x 10^5 PBMCs/ml. 195µl of PBMCs in binding buffer were labelled with 5µl Annexin-V-FITC for 15 minutes in the dark at room temperature. The labelled cells were washed with 190µl of binding buffer, centrifuged (350 x g, 10 minutes) and fixed in a solution of 500µl binding buffer/500µl 2% PFA) for 10 minutes on ice. After fixation, PBMCs were washed twice with 1ml PBS and subsequently labelled with α-CD3-PC5 and its isotype control (see section 2.4.3).

Based on initial observations, further studies were performed using flat bottom ultra-low attachment 96-well plates (Appleton Woods). Purified PBMCs were treated with either SEB (10ng/ml), 20% PAO1-L supernatant alone or both for 4 and 6 hours. Untreated PBMCs were used as negative control and Staurosporine (5µM, Sigma, S4400)-treated PBMCs as positive control. At required times PBMCs were collected and labelled with CD3-PC5 and CD14-ECD antibody (as described above) for identification of T cells and monocytes

respectively. Samples were analysed using a Beckman Coulter FC500 flow cytometer and the data analysed using Weasel software.

2.4.6 Analysis of the effect of PAO1-L supernatant on adherent cells in PBMC cultures.

Sterile, acid-etched coverslips (12 mm, Scientific Laboratories Supplies) were placed at the bottom of 24-well plates. PBMCs were purified, labelled with CFSE and re-suspended in RPMI complete medium as described in section 2.3. The CFSE-labelled PBMCs were plated at a density of 4.5 x 10^5 PBMCs/well in a final volume of 210 µl. PBMCs were treated with either 10ng/ml SEB, PAO1-L supernatant (20%, v/v) or both in a final volume of 300 µl and incubated for 5 days, 37°C, 5% CO_2. At the required times, coverslips were fixed with 4%PFA (16% paraformaldehyde, EM grade, Electron Microscopy Sciences, USA) in PBS and stored at 4°C until staining using Hemacolor staining kit (Merck Millipore) as per the manufacturer's instructions. Concurrent T cell proliferation at day 5 was assessed independently using conjugated primary antibody: CD3-PC5 in combination with either CD4-ECD or CD8-ECD (for complete description of method see section 2.4.3).

2.4.7 Monocyte cytotoxicity assay

To assess the cytotoxic effect of PAO1-L supernatants on monocytes at two different time points (12 and 24 hrs), microscopic examination using Trypan blue and the lactate dehydrogenase (LDH) colorimetric assay, were employed. Monocytes were isolated from PBMCs using human CD14 MicroBeads and

MS MACS columns (Miltenyi Biotec, UK) as per the manufacturer's instructions. $CD14^+$ cells (monocytes) were re-suspended at a density of 7.14 X 10^5cells/ml in RPMI medium supplemented with 1% human AB serum (Sigma F2442) and 50ng/ml rhM-CSF (R&D Systems, UK).

Monocytes were added to wells of a flat bottom 96-well plate (5 $X10^4$ cells per well) and treated with PAO1-L supernatant (20%, (v/v)) in a total volume of 100µl, in the absence or presence of 10ng/ml SEB. Background and negative controls included PAO1-L supernatant alone, untreated monocytes and SEB only-treated monocytes. As a positive control (100% cell death), monocytes were treated with 1% Triton X-100. At various time points, the supernatants from all conditions were collected and stored at -80°C until use. At various time points, the supernatants from all conditions were collected and stored at -80°C until use. The plate was then washed with PBS and stained with 0.1% Trypan blue for 1 min, washed and examined.

Supernatants were used to measure LDH activity using the Cytotoxicity Detection Kit (LDH) (Roche Applied Science, UK). Briefly, 50 µl of sample was mixed with 50 µl of reaction mixture (consisting of diaphorase /NAD+ mixture, iodotetrazolium chloride, and sodium lactate) in a 96-well microtitre plate (Corning Life Sciences, the Netherlands) and incubated at room temperature in the dark for 25 min. After incubation, the absorbance of sample and control wells was measured at 492 nm and at 650 nm (reference wavelength used to correct for differences in absorbance between wells due to imperfections in the material of the microtitre plate) using a Labsystems Multiskan EX plate reader.

2.4.8 Comparison of monocyte morphology in response to PAO1 supernatants.

Purified monocytes prepared as described in section 2.4.7 were re-suspended in RPMI complete medium supplemented with 50 ng/ml rhM-CSF at a density of 1.7 X 10^6 cells/ml and plated in duplicate onto 12 mm acid-etched coverslips in 24 well plate at a density of 3.5 x 10^5 per well in a final volume of 300 µl. Monocytes were treated with PAO1 supernatants and incubated at 37°C, 5% CO_2 for 16 hrs. Negative controls were left untreated.

After incubation, the coverslips were washed twice with cold PBS, fixed with 4% formalin at 4°C for 10 min and examined under bright field microscope prior staining using the Hemacolor staining kit (Merck Millipore) as per the manufacturer's instructions. Coverslips were mounted and assessed using Nikon Diaphot 300 inverted microscope and spot software. The supernatant from each culture was collected and spun on to the surface of a glass slide using a Shandon Cytospin 3 Cytocentrifuge at 400 rpm, medium acceleration for 10 min at room temperature. The slides were allowed to air dry at room temperature and stained using the Hemacolor staining kit, mounted and examined as above.

2.4.9 Western blot analysis of Exotoxin A in PAO1 supernatants.

Presence of ETA in PAO1 supernatants was detected using western blot. Bacterial supernatants were mixed with 4X sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (10% (w/v) SDS, 20% (v/v) glycerol, 0.2M Tris-HCl pH 6.8 and 0.05% (w/v)

bromophenol blue) to a final volume of 40 µl, boiled for 5 min and subjected to 15% SDS-PAGE using Tris-Glycine-SDS Buffer (25mM Tris-HCl, 200Mm glycine, 0.1 % (w/v) SDS, Bio-Rad). Gels were transferred overnight to nitrocellulose membrane (GE Healthcare) using Tris-Glycine transfer buffer solution (TG) (Bio-Rad) containing 20% (v/v) methanol. Non-specific binding was blocked using blocking buffer (5% (w/v) non-fat milk in PBS 0.1% (v/v) Tween20), and ETA was detected using a rabbit α-ETA primary antibody (Sigma, 1:20,000) and horseradish peroxidase (HRP)-conjugated goat α-rabbit secondary antibody (Sigma, 1:5,000). Antibody binding was visualised using an enhanced chemiluminescence detection reagent (Amersham Biosciences), high performance chemiluminence film (Hyperfilm, Amersham) and Biomax Cassette (Kodak).

2.4.10 Quantification of ETA in PAO1 supernatants

To semi-quantify the amount of ETA in PAO1 supernatants, western blot analysis was employed using pure ETA (P 0184, Sigma) as standard. Briefly, 45 µl of different concentrations of pure ETA (500, 100, 50 and 25 ng) were prepared in X-Vivo 15 medium containing protease inhibitors and mixed with loading buffer (4X) to a final volume of 60 µl. Different amounts of PAO1-L supernatant (45, 30, and 15 µl) in a final volume of 60 µl were electrophoresed and analysed by western blot as above. In the case of Δ*toxR* supernatants 60 µl of supernatant in a final volume of 80 µl was also used.

2.5 Results

2.5.1 PAO1 supernatants inhibit proliferation of $CD3^+$ T cells in response to SEB.

To determine the effect of *P. aeruginosa* soluble virulence factors on T cell proliferation, PBMCs were incubated with SEB in the presence and absence of PAO1-L supernatant prepared in X-Vivo 15 as described in materials and methods. Initial results using supernatants prepared from cultures grown in glass flasks revealed a negative impact of PAO1-L supernatants on T cell proliferation. CFSE-labelled PBMCs were incubated with SEB alone or together with different amounts of PAO1-L supernatant (5%, 10%, 20% (v/v)) for 5 days and cell proliferation was assessed by flow cytometry. The gating strategy used for all experiments was to first gate on live cells and then gate on total CD3+ cells or on CD3+/CD4+ or CD3+/CD8+ T lymphocytes within the total T cell population. The results showed a significant decrease of T cell division in response to SEB in the presence of 20% PAO1-L supernatant (Figure 2.1). However, there was no clear dose-dependent effect and the percentage of proliferated CD3+ T cells similarly decreased in the presence of 5% and 10% bacterial supernatant (Figure 2.1B). When CD3+ T cells were divided into CD4+ and CD8+ a significant inhibition of SEB-stimulated proliferation of CD3+/CD4+ and CD3+/CD8+ T cell in the presence of PAO1-L supernatants was observed. A clear dose effect was observed in the case of CD3+/CD4+ T cells (24% inhibition for 5% (v/v) PAO1-L; 30.7% inhibition for 10% (v/v) PAO1-L; 49.6% inhibition for 20% (v/v) PAO1-L (Figure 2.1C) but this was not the case for the CD3+/CD8+ T cells.

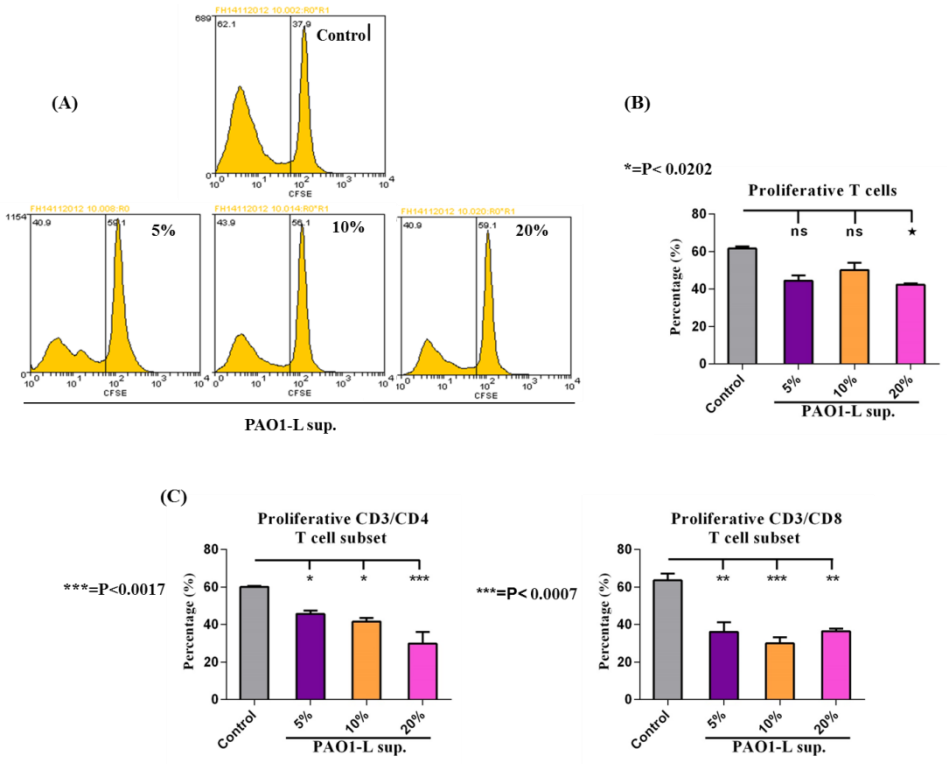

Figure 2.1: Reduction of T lymphocyte proliferation in the presence of different amount of PAO1-L supernatant.
A. Representative results of CFSE dilution upon stimulation with 10ng/ml SEB alone (control) or in combination with different concentrations of PAO1-L supernatant (5%, 10%, 20% (v/v)) for 5 days. **B and C.** Graphs represent results obtained from three independent experiments. One-way ANOVA and post-hoc Dunnett multiple comparison tests were used to calculate significance.

Based on these preliminary observations, further studies were performed using preparations of supernatants from PAO1-L and PAO1-N grown in single use polypropylene Costar flasks to confirm the results. Costar flasks were selected to grow the bacteria to avoid the possibility of impurities derived from the glass flasks affecting the results. Labelled PBMCs were stimulated with SEB in the presence of the new PAO1 supernatants (20%, v/v) and cultures treated with PAO1-L supernatants prepared in glass flasks were used as control. Work performed by the Quorum sensing group discovered that PAO1 strain used in the Nottingham laboratory (PAO1-N) has a large chromosomal deletion (58 kb) encompassing the *toxR* gene which may reduce the virulence of this strain.

ToxR is a regulatory gene involved in exotoxin A (ETA) (Hedstrom *et al.*, 1986) and rhamnolipid production (unpublished). Thus, the genetic differences between PAO1-N and PAO1-L provide an ideal tool to identify the potential factors regulated by *toxR* and responsible for potential differences observed between PAO1-L and PAO1-N supernatants.

According to the flow cytometric analysis (n=2) (Figure 2.2 B) a marked decrease in SEB-induced CD3 T cell proliferation was observed in the presence of both sets of PAO1-L supernatants (20%, v/v) compared to SEB stimulation alone although in this case the differences were only significant in the case of PAO1-L supernatants prepared in Costar Flasks. The PAO1-N supernatant had a minor effect (Figure 2.2B). As before the reduction in T cell proliferation is more marked when looking at CD3+/CD4+ and CD3+/CD8+ independently (Figure 2.2C).

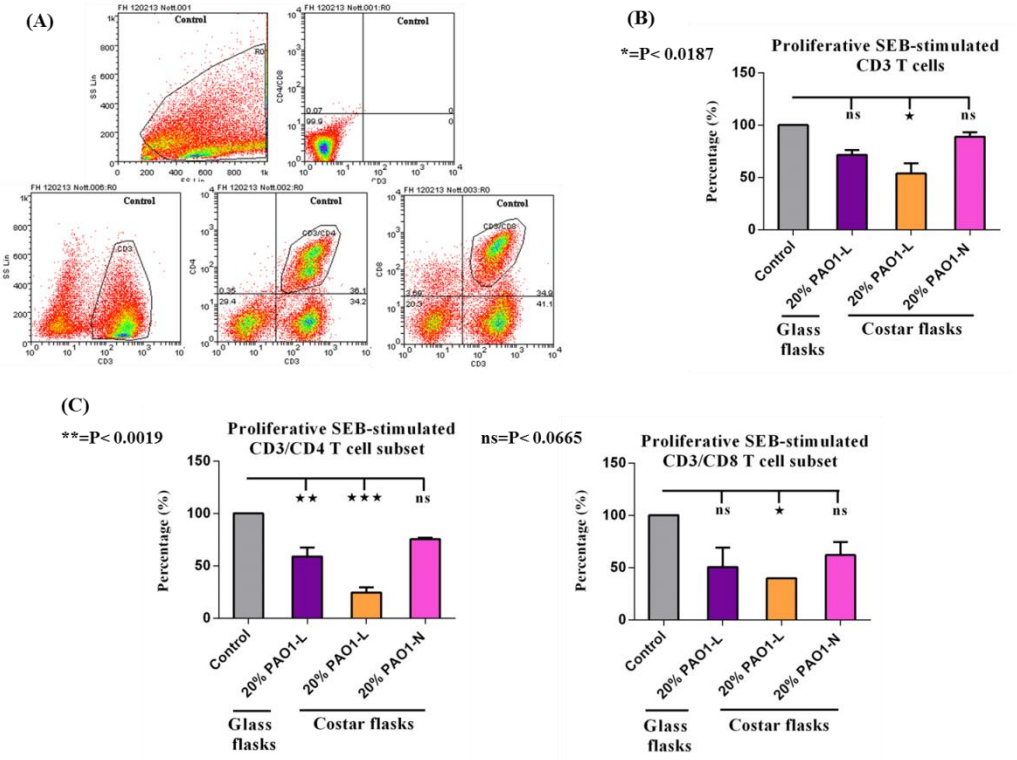

Figure 2.2: Reduction of CD3/CD4 and CD3/CD8 T cell division in response to SEB in the presence of PAO1-L supernatants.
A. Dot plot analysis of CD3, CD3/CD4 and CD3/CD8 T cells from PBMC cultured in RPMI complete medium for 5 days in the presence of 10ng/ml SEB. Data presented are from one representative experiment of two. **B**. Percentage of T cell proliferation in CFSE-labelled PBMCs cultures stimulated with SEB (10ng/ml) alone or in combination with either PAO1-L supernatant (prepared in either glass flasks or Costar flasks) or PAO1-N supernatant (prepared in Costar flask). Control PBMCs were treated SEB alone. **B** & **C.** Comparison of CD3 (B) and CD3/CD4 and CD3/CD8 (C) T cell proliferation in response SEB in the presence of PAO1 supernatants normalised to the level of SEB-induced proliferation. One-way ANOVA and post-hoc Dunnett multiple comparison tests were used to calculate the significance. Data were obtained from two independent experiments

2.5.2 PAO1-L supernatant does not affect the proliferation of CD3 T cells in response to stimulation with α-CD3/CD28 antibody.

In order to determine if the effect of PAO1-L supernatant on T cell proliferation depended on the antigen presenting cells present in the PBMC

cultures, studies were performed in which PBMCs were stimulated with α-CD3 and α-CD28 antibody as described in materials and methods.

PBMCs were stimulated with α-CD3 antibody in the presence and absence of α-CD28 antibody and in the presence and absence of 20% of PAO1-L supernatant (20 %, glass flasks). Statistical analysis of the data revealed that the percentage of CD3, CD3/CD4 or CD3/CD8 cell division in response to α-CD3 antibody in the presence and absence of α-CD28 antibody was not affected by PAO1-L supernatants (Figure 2.3). The percentage of α-CD3 or αCD3/CD28-induced T cell proliferation were normalised to 100% and used as a baseline to compare among different experiments.

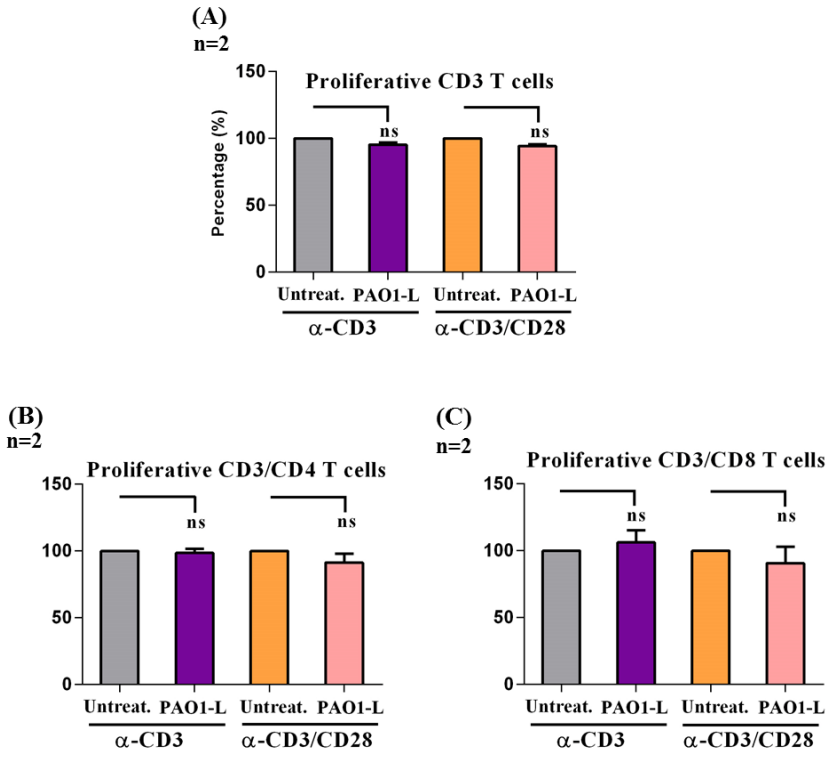

Figure 2.3: The effect of PAO1-L supernatant on the proliferative response of CD3 T cells and their subsets in response to α-CD3 antibody alone or in the presence of α-CD28 antibody.
A, B and C. CFSE-Labelled PBMCs were stimulated with α-CD3 alone or in combination with α-CD28 in the presence or absence of 20% PAO1-L supernatant (20%, Glass flask) for 5 days. Data were obtained from two independent experiments. Statistical significance was calculated by one-way ANOVA and post-hoc Tukey's multiple comparisons test.

To confirm that the bacterial supernatant has no inhibitory effect on α-CD3/CD28-stimulated T cell division further experiments were performed using new preparation of PAO1-L and PAO1-N supernatants prepared in Costar flasks (Figure 2.4). The data demonstrated that there are no significant differences in the proliferation of α-CD3/CD28-stimulated T cell and their subpopulations in the presence of 20% of either PAO1-L or PAO1-N supernatants prepared in Costar flasks. The percentage of α-CD3/CD28-induced T cell proliferation were normalised to 100% and used as a baseline to compare among different experiments.

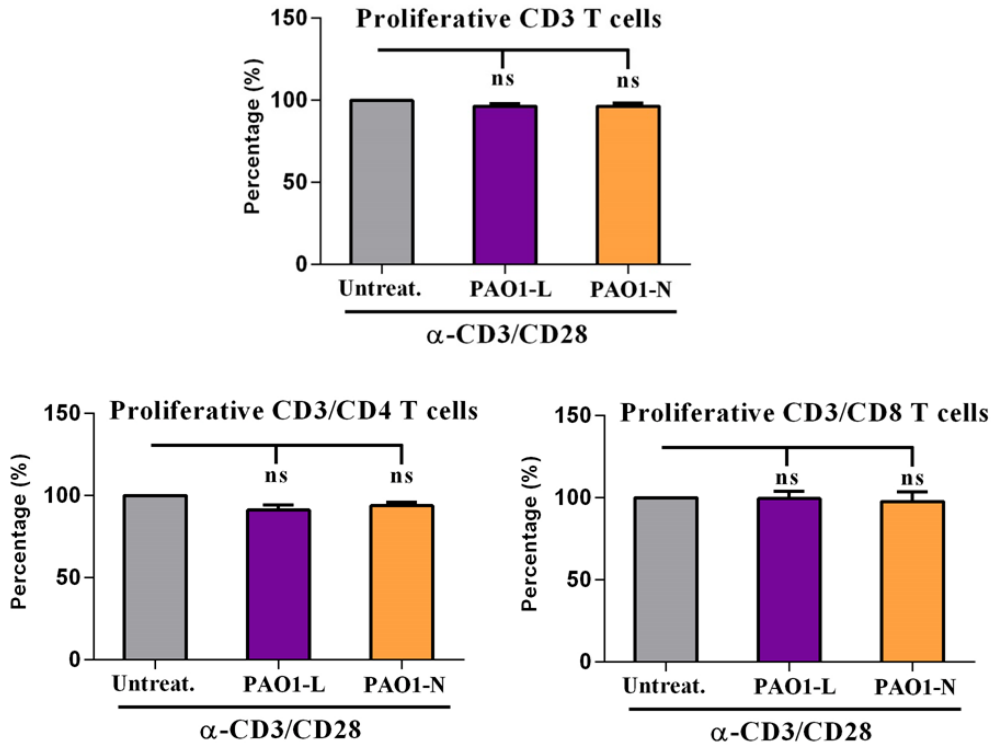

Figure 2.4: PAO1 supernatants do not affect T cell proliferation in response to α-CD3/CD28-stimulation.
CFSE-labelled PBMCs were incubated with 20% of PAO1 supernatants from either PAO1-L or PAO1-N in the presence of α-CD3 (2µg/ml) and α-CD28 (2µg/ml) antibody. One-way ANOVA and post-hoc Tukey's multiple comparisons test was used to calculate significance. Data were obtained from two independent experiments.

Thus, it would seem that the PAO1 supernatants did not affect lymphocyte proliferation after engagement of CD3 and CD28 with α-CD3 and α-CD28 respectively. Because SEB requires the presence of APCs for T cell proliferation we hypothesised that APC were required for the suppression of T cells proliferation by PAO1 supernatants.

2.5.3 Analysis of apoptosis of T cells and monocytes in response to PAO1 supernatant.

Because of the potential toxicity of PA supernatants on immune cells the effect of PAO-L supernatant on the viability of T lymphocytes was tested using Annexin-V. Annexin-V labelling detects the translocation of phosphatidylserine (PS) to the cell surface which is characteristic of apoptotic cells. PBMC cultures prepared in a low attachment 96 well plate and treated with PAO1-L supernatants for 4 and 6 hours were labelled with Annexin-V and α-CD3 and α-CD14 antibody were used to identify T cells and monocytes respectively.

Figure 2.5 depicts the percentage of Annexin V+ T cells in PBMCs cultures treated with SEB (10/ng) in the presence and absence of PAO-L supernatant (20%, v/v). Results indicate that PAO1-L supernatant, with or without SEB, do not have a direct cytotoxic effect on T lymphocytes compared to staurosporine-treated PBMCs, in the presence or absence of SEB.

A minor percentage of CD14+ monocytes were detected by flow cytometry probably due to high degree of attachment to the wells in the PBMC cultures (data not shown). In spite of this, a population of CD14+, Annexin-V+ cells

can be detected in stautosporin-treated cultures. This observation suggests that viable monocytes remained attached to the bottom of the well and just the dead cells were floating in the media (Figure 2.6).

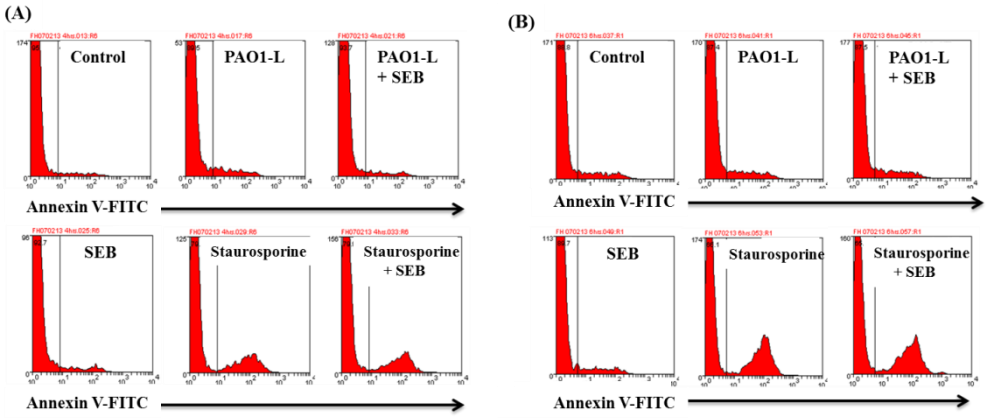

Figure 2.5: Analysis of T cells apoptosis in response to PAO1-L supernatant for 4 and 6 hours.
Purified PBMCs were treated with SEB (10ng/ml), 20% PAO1-L supernatants alone (glass flasks) or 20% PAO1-L supernatants in combination with SEB (10ng/ml). Staurosporine-treatment was used alone or in combination with SEB (10ng/ml) as a positive control while untreated PBMCs were considered a negative control. Cultures were incubated for 4 hours **(A)** and 6 hours **(B)**. T cells were gated based on CD3 labelling. Data were obtained from a single experiment.

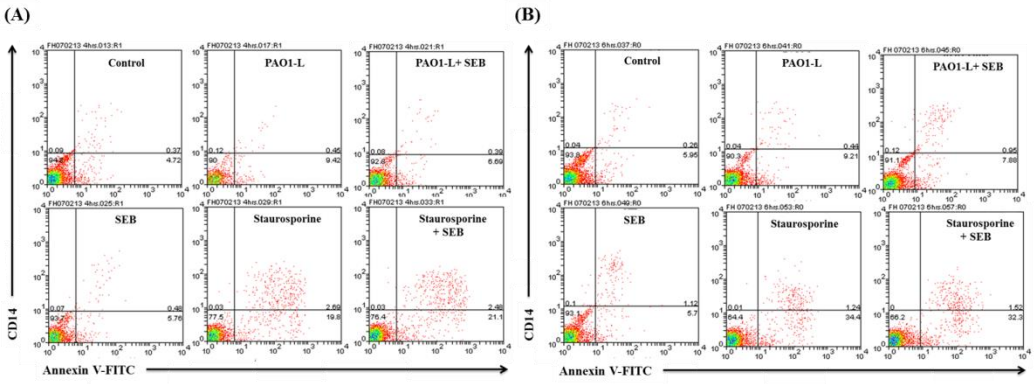

Figure 2.6: Analysis of the generation of apoptotic monocytes in response to PAO1-L supernatant, SEB and stautosporin treatment for 4 and 6 hours.
Purified PBMCs were treated with either SEB (10ng/ml), 20% PAO1-L supernatants alone (glass flasks) or 20% PAO1-L supernatants in-conjugation with SEB (10ng/ml). Staurosporine-treatment was used alone or in-conjugation with SEB (10ng/ml) as a positive control while untreated PBMCs were considered a negative control. Cultures were incubated for 4 hours **(A)** and 6 hours **(B)**. Monocytes were identified based on CD14 labelling. Data were obtained from a single experiment.

2.5.4 Altered monocyte morphology in the presence of PAO1-L supernatant.

To visualise the potential effect of PAO1-L supernatant on monocytes, microscopic examination of Hemacolor-stained adherent cells from PBMC cultures was used. Purified PBMCs were plated on acid-etched glass coverslips and treated with either 20% PAO-L (glass flasks), SEB (10ng/ml) or both, and incubated for 2, 3, 4 and 5 days. At the required time, the coverslips were fixed and stained using Hemacolor stain as described in materials and methods. Preliminary observations revealed that adherent cells (monocytes) displayed an abnormal morphology in response to PAO1-L supernatant in comparison to their untreated counterparts. Adherent cells in PBMC cultures stimulated with SEB showed a noticeable increase in the cell size and number (Figure 2.7).

T cell proliferation was also measured in these cultures after 5 days using flow cytometric analysis as above. Figures 2.8A and 2.9A depict the T cell proliferative response. The data confirmed the previous observations as there is a marked decrease in T cell division after stimulation with SEB in the presence of PAO1-L supernatant compared to SEB-treated PBMCs (Figures 2.8 and 2.9) and that there is no proliferative response in the presence of the bacterial supernatant alone.

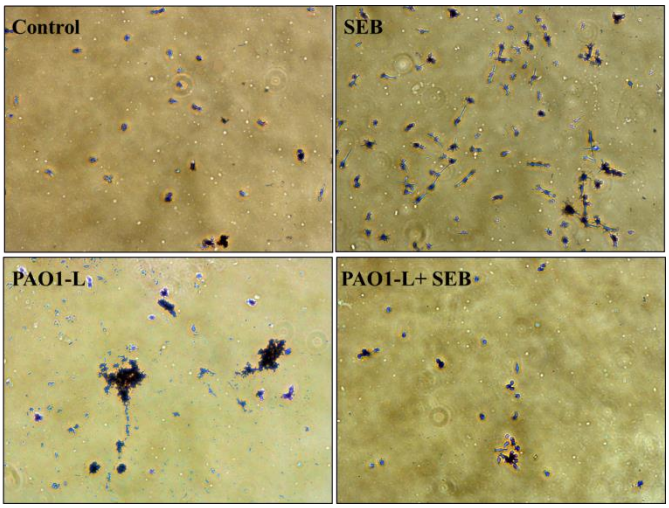

Figure 2.7: Altered morphology of adherent cells from PBMC cultures in response to PAO1-L supernatant after 2 days.
Purified PBMCs were plated on 12 mm coverslips and treated with either SEB (10ng/ml) or 20% PAO1-L (glass flasks) alone or in combination with SEB for 2 days. The coverslips were fixed with 4% PFA and stained using Hemacolor staining kit. Images are from one preliminary experiment.

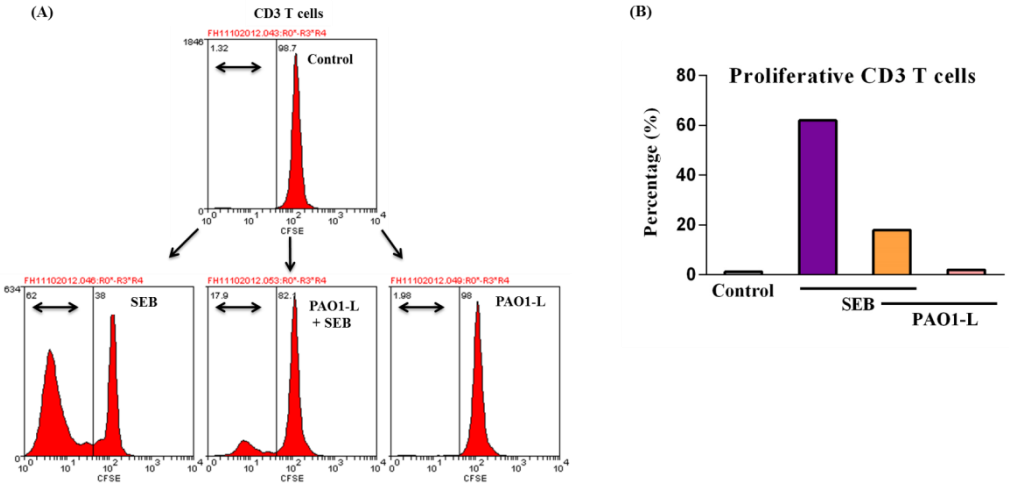

Figure 2.8: The effect of PAO1-L supernatant on CD3 T cell proliferation is not affected by culture conditions.
A. Purified PBMCs were labelled with CFSE (5µM) and stimulated with SEB (10ng/ml) alone or in combination with 20% PAO1-L supernatants (glass flasks). PBMCs were treated with 20% PAO1-L alone or left untreated as controls. **B.** Graphic representation of data shown in A. Data from a single experiment.

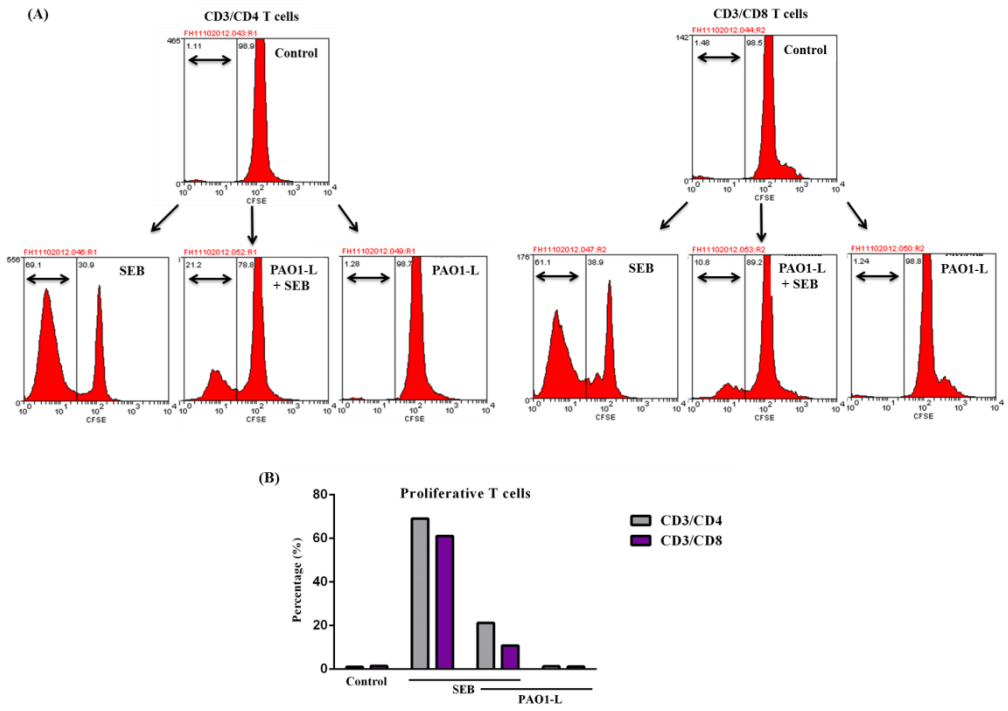

Figure 2.9: The effect of PAO1-L supernatant on CD3/CD4 and CD3/CD8 T cell proliferation is not affected by culture conditions.
A. Purified PBMCs were labelled with CFSE (5µM) and stimulated with SEB (10ng/ml) alone or in-conjugation with 20% PAO1-L prepared in glass flasks). PBMCs were treated with 20% PAO1-L alone or left untreated as controls. **B.** Graphic representation of data shown in A. Data from a single experiment shown in Figure 2.8.

2.5.5 Monocyte death in the presence of PAO1-L supernatants

In view of the altered morphology displayed by adherent PBMC cells in the presence of PAO1-L supernatants, viability of purified monocytes in the presence and absence of PAO1-L supernatants was assessed by measuring lactate dehydrogenase (LDH) activity in the supernatant using the Cytotoxicity Detection Kit (LDH). LDH is a cytosolic enzyme found in all cells that is released when the integrity of the plasma membrane is impaired. The amount of LDH released into the media is proportional to cell damage and thus LDH is considered as a biomarker for cell toxicity (Decker and Lohmann-Matthes, 1988).

Monocytes were purified and re-suspended in RPMI complete medium supplemented with rhM-CSF, plated onto U-shaped bottom 96 well plate and treated with either 20% PAO1-L supernatant, SEB (10ng/ml) or both and incubated for 4 and 6 hours and for 12 and 24 hours. Negative controls were untreated monocytes and monocytes treated with 20% X-Vivo 15 medium or SEB (10ng/ml). No differences were observed in the concentration of LDH between untreated and PAO1-L (+/-SEB)-treated monocytes after 4, 6, 12 and 24 hours (Figure 2.10).

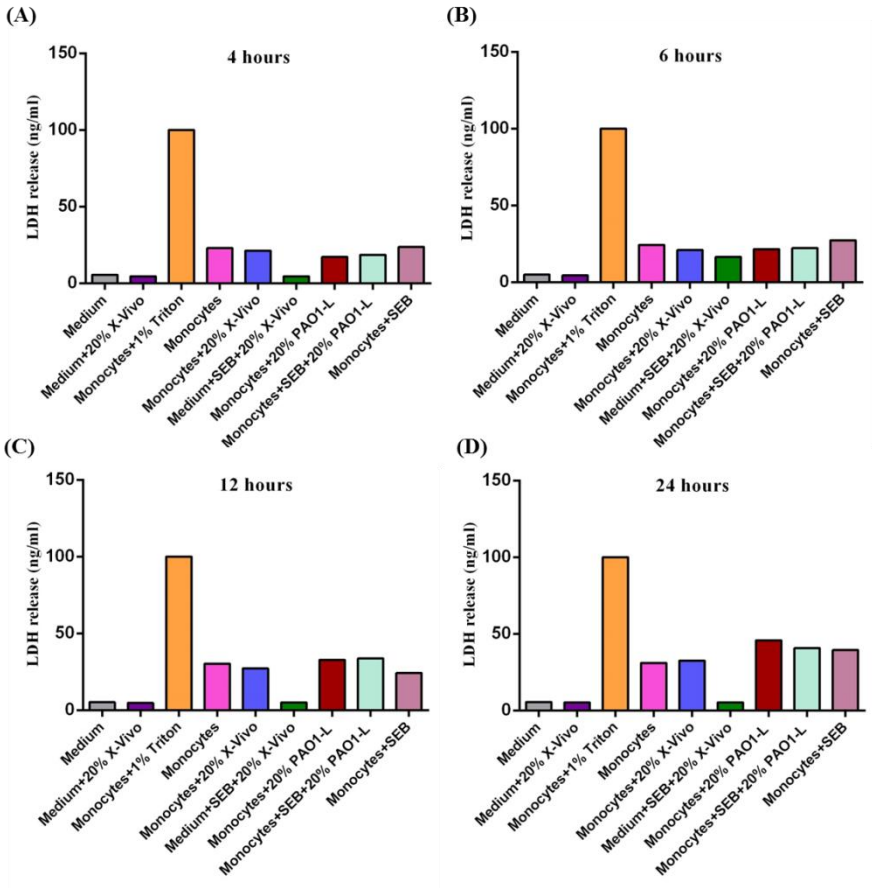

Figure 2.10: LDH release from monocytes after exposure to 20% PA01-L supernatant.
Monocytes were purified and treated with PAO1-L supernatant (20% (v/v)) alone or in combination with SEB (10 ng/ml) for 4 (**A**), 6 (**B),** 12 (**C**) and 24 (**D**) hours. Culture supernatants were collected and assayed for LDH activity which is proportional to cell death. No differences in LDH release were observed between untreated and treated monocytes. The percentages of cell death were normalised to Triton-X100-treated cells (100% cell death). Data were obtained from a single experiment.

2.5.6 Altered monocyte morphology in response to supernatants from different PAO1 strains.

As mentioned above the PAO1-N subline has a large chromosomal deletion (58 kb) including the *toxR* gene which may reduce its virulence. *ToxR* is a regulatory gene that controls ETA (Hedstrom *et al.*, 1986) and rhamnolipid (unpublished) production. Thus, the genetic differences between PAO1-N and PAO1-L provide an ideal tool to identify the potential factors responsible for the observed cytotoxic effects on monocytes. Furthermore, in an attempt to determine whether the presence of *toxR* in the bacterial genome is required for PAO1 cytotoxicity, monocyte morphology was assessed in response to different PAO1 supernatants (20% (v/v), Costar Flask) using microscopic examination. For this purified monocytes were re-suspended in RPMI complete medium in the presence of M-CSF and plated on coverslips in 24 well plate at a density of 3.5 x 10^5 per well in a final volume 300 µl. Monocytes were treated with supernatants from either PAO1-L (*toxR*+), PAO1-N (*toxR*-) or PAO1-LΔ*toxR* mutant strain for 16 hours. After incubation, culture supernatants were collected, and centrifuged onto the surface of a glass slide. Images of unstained and hemacolor-stained coverslips (Figure 2.11A and B respectively) revealed that monocytes showed a dramatic reduction in the cell size and number after treatment with 20% PAO1-L supernatant (*toxR*+), compared to PAO1-N (*toxR*-) and PAO1-L Δ*toxR* treated-monocytes. This observation suggests that the ability of PAO1-L to impact on monocytes viability correlates with the activity of *toxR*.

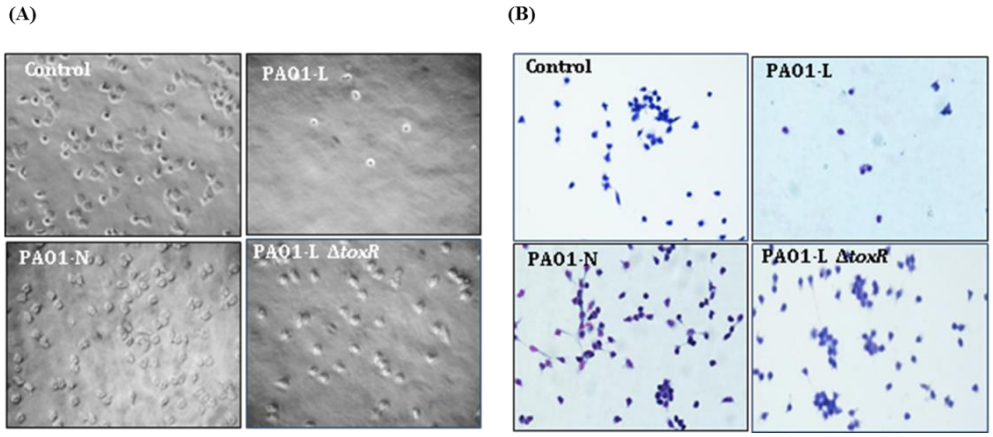

Figure 2.11: *ToxR* is responsible for the altered monocyte morphology after exposure to PAO1-L supernatants.
Monocytes were seeded on glass coverslips and treated with different PAO1 supernatants (20% (v/v)) for 16 hours. The samples were fixed with 4% Formalin and examined. The same samples were then stained with Hemocolor staining, mounted and analysed. Images showed that the PAO1-L strain causes changes on monocyte morphology and number compared to PAO1-N (*toxR*-) and PAO1-L Δ*toxR*. Data are representative from three independent experiments. Magnification=400x.

Fewer cells were attached to the coverslips after stimulation with the PAO1-L supernatant. To characterise monocytes that remained in suspension the culture supernatants from the same plate were employed for microscopic examination in an attempt to further understand the impact of PAO1 supernatants on monocyte viability. The images showed that most of the monocytes displayed altered morphology in response to PAO1-L supernatant compared to untreated cells (Figure 2.12). These effects were reduced after exposure to the same amount of PAO1-L Δ*toxR* supernatant. These preliminary observations indicate the importance of *toxR* for causing cytotoxicity in monocytes.

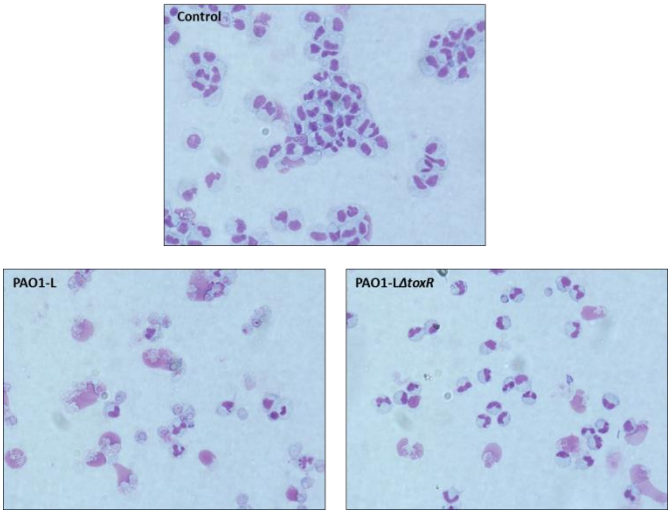

Figure 2.12: Impact of PAO1 supernatants on the morphology of monocytes in suspension.
Cytospins of 16 hours-treated monocyte cultures were stained with Hemacolor stain, mounted and viewed using an inverted microscope. Many of the cells died after stimulation with 20% PAO1-L supernatant compared to untreated counterpart. The number of the damaged cells was reduced in response to 20% PAO1-L Δ*toxR*. Magnification = 400x. Images presented are from one experiment.

2.5.7 Exotoxin A (ETA) is highly expressed by PAO1-L

ETA is a extracellular toxin secreted by a wide number of clinical isolates of *P. aeruginosa* (Liu, 1974). This protein is encoded by the *toxA* gene (Gaines *et al.*, 2007) and, as mentioned above, its expression is controlled by the regulatory element *toxR* (Hedstrom *et al.*, 1986). Based on the data obtained from the previous study, we hypothesise that the cytotoxic effect of PAO1-L supernatants is linked to the presence of ETA. To investigate this possibility the presence of ETA in PAO1 supernatants was detected by western blot as described in materials and methods. Besides PAO1-LΔ*toxR*, a number of mutant strains in the Lausanne (PAO1-L) background were employed in this study including: (i) PAAMP1strain, which has the 58 kb deletion found in PAO1-N and lacks *toxR*. (ii) PAO1-LΔ*Bif* strain which lacks another gene

missing in PAO1-N (role unknown). (iii) PAAMP1Δ*Bif* strain, which has both mutations. As shown in Figure 2.13, PAO1-L and PAO1-LΔ*Bif* expressed high amount of ETA compared to other strains which lack the regulatory gene *toxR*. Keeping in mind that ETA production is dependent on *toxR* activity, this result confirms the findings that *toxR* enhances the expression of ETA 10 fold (Hedstrom *et al.*, 1986). Suggesting that, the production of this toxin by *P. aeruginosa* may relate to its ability to cause monocyte cytotoxicity and supress lymphocyte proliferation.

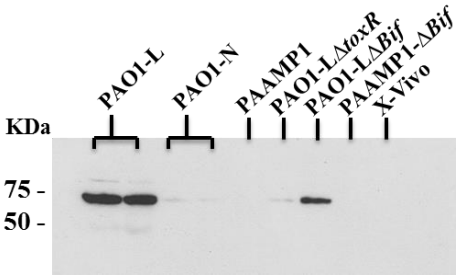

Figure 2.13: Western blot analysis of Exotoxin A production by different PAO1 strains.
ETA is expressed in high amounts by PAO1-L and PAO1-LΔ*Bif* strains in comparison with the *toxR* deficient strains used in this study. X-Vivo 15 medium was used as negative control. Data are representative of two independent experiments.

2.5.8 Quantification of ETA in PAO1 supernatants.

In order to estimate the concentration of ETA released in the bacterial supernatants, different amounts of either PAO1-L or PAO1-LΔ*toxR* supernatants were prepared and diluted in X-Vivo 15 medium. To avoid protein degradation, purified ETA was prepared in X-Vivo 15 supplemented with protease inhibitors. The samples were mixed with loading buffer, boiled for 5 min and electrophoresed. Previous results showed that there is a reduction in ETA in PAO1-LΔ*toxR* supernatants. In this instance a higher amount of *toxR* supernatant (60 µl) was included. After 10 min exposure,

approximately 3.3 µg/ml of ETA was detected in PAO1-L supernatants while less than 166 ng/ml of ETA was detected in the supernatants from the *toxR* mutant (Figure 2.14). This result further supports that the high production of ETA in PAO1-L supernatants could be responsible for its cytotoxicity on monocytes and ultimately inhibitory response on T cells proliferation. The OD of the bacterial cultures was similar among all strains so the differences are not due to reduced bacterial growth.

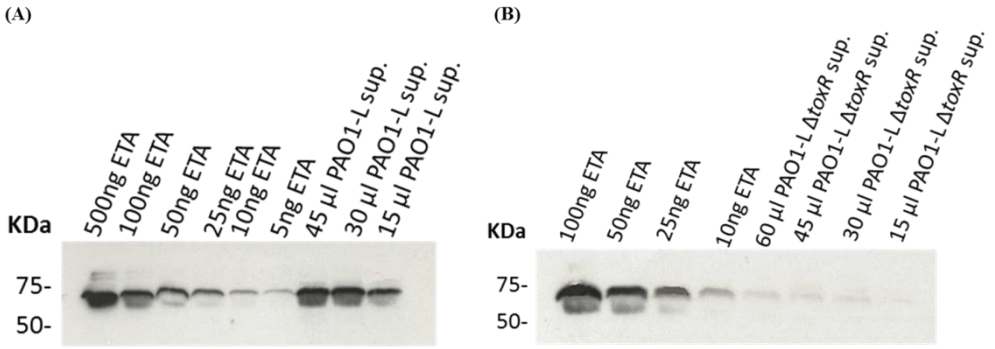

Figure 2.14: Quntification of ETA in PAO1 supernatants using western blot.

ETA in bacterial supernatants was quantified in both PAO1-L **(A)** and PAO1-L Δ*toxR* **(B)** using different concentrations of pure ETA as standard. Data are from a single experiment.

2.6 Discussion

The main finding of the present study is that PAO1-L supernatant has the ability to cause cytotoxicity in monocytes and this activity correlates with the presence of ETA. PAO1-L supernatant does not exert a direct cytotoxic effect on T cells. The cytotoxic effect of PAO1-L supernatants on monocytes, important antigen presenting cells, might supress their ability to digest and process antigen and consequently have a negative impact on T cell activation and expansion.

T cells play a crucial role in the development of immunity against *P. aeruginosa* in both mice (Chapman *et al.*, 2009, Roy *et al.*, 2010) and humans (Hedstrom *et al.*, 1986, Iglewski and Kabat, 1975). However, the development of T cell responses depends on a prompt increase in numbers of T cells specific for a particular antigen. In this study, we aimed to investigate the immunomodulatory effects of PAO1 supernatants on human T lymphocytes and we initiated the study looking at cellular proliferation *in vitro*.

To achieve the study goal, we used PBMCs purified from fresh peripheral blood donated by healthy donors rather than purified T cells, to provide a more physiological model, which may mimic the immune system by taking into consideration the contribution of various immune cell types that can interact with each other through soluble factors and cell contacts (Sullivan *et al.*, 2000, Schildberger *et al.*, 2013, van 't Wout *et al.*, 2008). However, PBMC purification is associated with considerable cellular loss thus requires large blood volumes (Deenadayalan *et al.*, 2013). It has been reported that the delayed PBMC purification and handling may have a negative impact on assay outcomes because of down regulation of cellular immune functions (Hanekom

et al., 2008, Palmirotta *et al.*, 2012, Bull *et al.*, 2007). Furthermore, for optimum cell growth, PBMCs are cultured in media supplemented with natural products such as serum which may introduce foreign material to cell culture and interfere with the study outcomes (Sula *et al.*, 1980).

Interestingly, it has been shown that using the whole blood is a potential alternative avenue to study cytokine production and lymphocyte proliferation mainly when the blood volume is a limiting factor as it requires less blood volume compared to the conventional PBMC assays (Deenadayalan *et al.*, 2013, Silva *et al.*, 2013)

To monitor T lymphocyte division, we employed the fluorescent dye CFSE which is considered one of the most powerful tools to evaluate cell proliferation (Quah and Parish, 2012). Using CFSE, multiple divisions of each daughter cell can be visualized using flow cytometry histograms to visualise low CFSE intensity (proliferated T cells) compared to high CFSE intensity (non-proliferated T cells). Measuring the fluorescence intensity in the new cells allows us to quantify the number of progenitor cells that enter division (Lyons and Parish, 1994, Weston and Parish, 1990, Kurts *et al.*, 1997, Bird *et al.*, 1998, Gett and Hodgkin, 1998, Marzo *et al.*, 2000, Jedema *et al.*, 2004). Optimisation of CFSE labelling, flow cytometry settings and compensation were carried out based on results from early experiments (data not shown) as the emission spectrum of CFSE bleeds through into the ECD channel (CD4 and CD8 antibody).

The recognition of the cell surface markers is considered as a main tool to identify cells subpopulations. For T cell, the expression of CD3 protein complex is a defining marker of the T cell lineage. CD3 is a T cell co-receptor

that binds with the T cell receptor (TCR) and initiates the intracellular signalling pathway after the engagement TCR with MHC on APCs (Smith-Garvin *et al.*, 2009, Chetty and Gatter, 1994, Lanzavecchia *et al.*, 1999). Furthermore, the expression of either CD4 or CD8 marker on the surface of CD3 T cells is used to classify T lymphocytes into either helper or cytotoxic T cell subsets respectively (D'Acquisto and Crompton, 2011).

Therefore, after 5 days of culture, the cells were harvested and these PBMCs were stained with α-CD3 alone or in combination with either α-CD4 or α-CD8 antibody and analysed by flow cytometry to assess T cell proliferation

The quantitative analysis of cell division revealed that PAO1 supernatants significantly inhibit the proliferative response of T cells in response to SEB based on the reduction of the CFSE fluorescence intensity (Quah and Parish, 2012). SEB is a potent antigen produced by *S. aureus* and plays a crucial rule in the activation of T cells via the engagement of T cell receptor (TCR) particularly the Vß chain on αß T cells and the outside of the peptide binding groove of MHC class II (El Hage *et al.*, 2010, Herman *et al.*, 1991). It has been revealed that this superantigen is able to induce robust activation of 5-30 % of αß T cells (Krakauer, 2005)

Issekutz and Stoltz, stimulated PMBCs with both *P. aeruginosa* and *S. aureus* and observed that *P. aeruginosa* inhibited T cell proliferation in the presence of *S. aureus* (Issekutz and Stoltz, 1985). Our results using *P. aeruginosa* supernatants are in agreement with these observations. We showed a substantial reduction in the percentage of SEB-induced CD3-T cell division and its subsets in response to 20% PAO1-L supernatant (Figure 2.1). In this context, it should be considered that a number of studies have demonstrated

that cell-mediated immune responses are supressed after exposure to *P. aeruginosa* in mice (Floersheim *et al.*, 1971, Petit *et al.*, 1982) by suppressing lymphocyte proliferation (Chhabra *et al.*, 2003, Telford *et al.*, 1998b) and cytokine production (Telford *et al.*, 1998b, Chhabra *et al.*, 2003, Ritchie *et al.*, 2003, Smith *et al.*, 2002, Ritchie *et al.*, 2005) due to the negative activity of the quorum sensing molecule 3-oxo-C12-HSL on immune cells such as macrophages (Tateda *et al.*, 2003) and B cells (Ritchie *et al.*, 2005).

To assess whether the bacterium supresses proliferative response of T cells in PBMC cultures by interfering directly with T cell function, purified PBMCs were stimulated with α-CD3 and α-CD28 antibody in an attempt to activate T cell in an APC-independent manner. α-CD3 and α-CD28 antibody are considered powerful mitogens that promote robust T cell activation via engagement of CD3 and CD28 respectively (Katayose et al., 1996; Van Wauwe et al., 1980). It has been proposed that the co-stimulatory receptor, CD28, is crucial for full T activation by providing an additional signal for T cell differentiation and expansion(Damle *et al.*, 1983, Appleman *et al.*, 2000, Sharpe and Freeman, 2002, Fraser *et al.*, 1991, Lindstein *et al.*, 1989).

The results of this study indicate that there are no differences in the cellular division of either CD3 T cells or their subsets in the presence of PAO1-L supernatant (20% (V/V)) when cells were stimulated with α-CD3 alone or in the presence of α-CD28 (Figure 2.3). Therefore, this observation provides conclusive evidence that *P. aeruginosa* exoproducts do not appear to exert a direct effect on T cell proliferation but might act by affecting APC activity which is required in the case of SEB-induced T cell proliferation (Issekutz and Stoltz, 1985).

We also hypothesised that the down regulation of SEB-stimulated T cell proliferation and decrease in cell numbers in response to *P. aeruginosa* supernatants was attributed to the cytotoxic effect of the *P. aeruginosa* secreted products on T cells leading to cell death and the presence of apoptotic bodies (Bruno *et al.*, 2000, Epelman *et al.*, 2002, Bruno *et al.*, 1998, Mody *et al.*, 1995). To determine whether the decreased number of divided T cell upon treatment with PAO1-L supernatants was due to programmed cell death, Annexin-V was used to detect the presence of exposed phosphatidylserine (PS) (Brumatti *et al.*, 2008, Vermes *et al.*, 1995, Zhang *et al.*, 1997b). In the early stages of apoptosis, the cell morphology and membrane integrity are altered and lead to the translocation of PS molecules to the outer surface of the cell membrane which can bind efficiently Annexin-V (Vermes *et al.*, 1995). Flow cytometry analysis revealed that there are no noticeable differences in the percentages of the apoptotic T cells between un-treated and PAO1-L supernatant-treated cultures suggesting that *P. aeruginosa* does not exert a direct cytotoxic effect on T cells and might have an impact on other immune cells which impairs T cell proliferative response (Figure 2.5). These data are in agreement with a work by Issekutz et al suggesting that the inhibition of T cell division by *P. aeruginosa* is correlated to the negative impact of this organism on monocytes (Issekutz and Stoltz, 1985).

Monocytes are APCs and precursor of dendritic cells (DCs), which are considered professional APC (Paquette *et al.*, 1998). APCs play a cardinal role in processing and presenting the antigen on MHC which ultimately promotes T cell expansion and protects against cell death (Smith-Garvin *et al.*, 2009). The interaction between the antigen-loaded on MHC and T cell receptor (TCR)

leads to the activation and expansion of naïve T cells (Smith-Garvin *et al.*, 2009, Steinman and Hemmi, 2006). Furthermore, the ligation of the co-stimulatory CD28 expressed on T cell surface with CD80/CD86 ligands located on DCs promotes T cell expansion and protects against cell death (Smith-Garvin *et al.*, 2009). Previous studies demonstrated that *P. aeruginosa* has a toxic effect on macrophages, inducing apoptosis and impairing secretion of cytokines IL-12 and TNF-α, via the action of 3-oxo-C12-HSL (Telford *et al.*, 1998a, Tateda *et al.*, 2003, Miyairi *et al.*, 2006, Glasser *et al.*, 2008). This further suggests that the suppression of the proliferative response of T cells may be due to alteration of APC function, and cytokine profile, which make them unable to successfully promote T cell activation. Based on these observations, we investigated the pro-apoptotic effect of PAO1-L supernatants on monocytes using an Annexin-V-based assay. However, we were unable to accurately determine the percentage of apoptotic monocytes, as most, if not all, live cells (unstained cells) remained adhered to the bottom of the tissue culture plate.

Necrosis is a major type of cell death that occurs due to a wide range of physical and chemical factors such as trauma and bacterial toxins (Chan *et al.*, 2013, Essmann *et al.*, 2003, Kennedy *et al.*, 2009, Kim *et al.*, 2013, Zong and Thompson, 2006). It is characterised by the irreversible damage of the cell membrane due to a large increase in cell volume and organelles which leads to cell rupture and leakage of the intracellular contents (Kroemer *et al.*, 2009). The release of the cytosolic enzyme, LDH, from cells to the environment is used classically as a marker of necrotic cell death (Chan *et al.*, 2013). However, the findings of the cytotoxicity assay did not correlate with the

microscopic observation of monocytes as there were no significance differences in the concentration of LDH released from PAO1-L (+/-SEB)-treated monocytes compared to untreated counterparts at different time points in spite of the major phenotypic differences observed.

In light of this, the impact of *P. aeruginosa* supernatant on monocytes was assessed by microscopic examination. As mentioned above preliminary observations revealed that adherent cells within the PBMC cultures (monocytes) displayed differences in morphology and number after stimulation with PAO1-L supernatant in the absence and presence of SEB for 2 days compared to untreated cells (Figure 2.7). Further studies were done to assess whether the presence of *toxR* in the bacterial genome is required for PAO1 cytotoxicity. Preliminary observations showed that PAO1-L exerts a negative impact on monocyte viability and the bacterial cytotoxicity is mediated by the activity of *toxR* regulatory gene as monocytes appeared less damaged when using PAO1-N or PAO1-L Δ*toxR* supernatants (Figure 2.11).

In this study the effect of PAO1-N supernatants on T cell proliferation and monocyte viability was compared to that of PAOI-L. As mentioned above PAO1-N has a large chromosomal deletion (approximately 58 Kb). The regulatory gene *toxR* is one of the genetic elements located within the deletion and this transcriptional regulator positively regulates the expression of ETA 10 fold (Hedstrom *et al.*, 1986) thus making PAO1-N a *toxR* negative strain. Furthermore, the genetic differences between PAO1-N and PAO1-L provided an ideal tool to identify the key elements that might responsible for the observed cytotoxic effects on monocytes. Furthermore, *toxR* is involved in the regulation of the Rhl quorum sensing system as a *toxR* deficient strain

produces less C4-HSL autoinducer than the wild-type leading to lower production of the biosurfactant rhamnolipid (unpublished work). Therefore, a *ΔtoxR* strain constructed in the Lausanne background was used to test the effect of *toxR* on monocyte viability. Interestingly the viability of monocytes was not affected by the presence of 20% PAO1-L*ΔtoxR* supernatant. This further supports the hypothesis that the reduction in SEB-dependent T cell proliferation caused by PAO1-L supernatants is due to a cytotoxic effect on monocytes.

Based on work by Liu et al, exotoxin A (ETA) is considered an important virulence factor secreted by *P. aeruginosa* (Liu, 1974) due to its ability to block protein synthesis and ultimately cell death (Siegall *et al.*, 1989). Furthermore, ETA down regulates the expression of co-stimulatory molecules including CD80, CD86, ICAM-1 and HLA-DR on IFN-γ treated monocytes (Michalkiewicz *et al.*, 1999) and block T cell expansion (Michalkiewicz *et al.*, 1998). Therefore, the expression of ETA in PAO1 supernatants was tested. Importantly, presence of ETA on supernatants correlated their cytotoxic effect on monocytes.

To confirm that ETA is responsible for the effect caused by PAO1-L supernatant on T cell proliferation and monocyte cytotoxicity, a *toxA* mutant strain was constructed in the PAO1-L background and characterised in comparison with the wild type strain (chapter 3).

Together these observations suggest that PAO1-L supernatant inhibits T cell proliferation but PAO1 sub-line Nottingham does not and the activity of the regulatory gene *toxR* is very important for this effect. However, PAO1-L does

not exert a direct cytotoxic effect on T cells and its effect on T cell proliferation is dependent on the presence of APCs.

3. Construction of a Mutant of *Pseudomonas aeruginosa* Deficient in Exotoxin A

3.1 Introduction

P. aeruginosa is a versatile bacterium that causes serious opportunistic infections, especially in susceptible individuals such as cystic fibrosis patients (Lyczak *et al.*, 2000, Finnan *et al.*, 2004). The severe infections caused by *P. aeruginosa* are attributed to the expression of a large number of cell-associated and extracellular products which aid in resistance and persistence of this bacterium in host tissues (Strateva and Mitov, 2011)

Strateva and Mitov described three different stages of bacterial infection based on distinct determinants expressed by *P. aeruginosa (Strateva and Mitov, 2011)*. Colonisation represents the initial stage of bacterial infection and is mainly mediated by a large number of structural molecules such as type IV pili. This is followed by invasion which relies on an extensive production of extracellular determinants controlled by QS. The final stage is dissemination throughout host tissues which involves both cell associated and secreted virulence determinants (Hahn, 1997, Strateva and Mitov, 2011).

Many virulence factors secreted by *P. aeruginosa* have been reported in the literature as involved in the development of infection. ETA is considered the most well researched secreted virulence protein. It was initially described by Liu and colleagues in 1973 (Liu, 1973, Liu *et al.*, 1973). ETA is highly expressed by *P. aeruginosa*, particularly by strains that have been isolated from a variety of infection sites (Van Delden and Iglewski, 1998, Vasil *et al.*,

1986). Furthermore, this toxin is a fundamental virulence determinant in CF, as samples collected from patients have shown a high accumulation of α-ETA antibody due, most likely, to the recurrent exposure to the ETA antigen. In addition, it has been reported that the high titres of α-ETA antibody titres are positively correlated to mortality in CF patients (Jaffar-Bandjee *et al.*, 1995, Klinger *et al.*, 1978, Moss *et al.*, 1986) .

ETA is encoded by the *toxA* gene (Gaines *et al.*, 2007) and its expression is enhanced by the regulatory element *toxR* (previously referred to as *regA*). ETA is regulated by the *las* quorum-sensing system (Hedstrom *et al.*, 1986, Gambello *et al.*, 1993, Storey *et al.*, 1998, de Kievit and Iglewski, 2000, Lory, 1986, West *et al.*, 1994). Many studies have determined that the iron levels in the media have an impact on ETA production (Liu, 1973, Bjorn *et al.*, 1978, Bjorn *et al.*, 1979) with high iron concentrations in the growth medium reducing the expression of ETA (Chen *et al.*, 1987, Grant and Vasil, 1986, Hindahl *et al.*, 1987, Lory, 1986, Vasil *et al.*, 1986) and resulting in an accumulation of the *toxR* transcript (Frank and Iglewski, 1988, Prince *et al.*, 1991).

ETA is a single polypeptide chain (66.6 kDa) composed of 613 amino acids that are arranged in three structural and functional domains (Benhar *et al.*, 1994) (Figure 2). The binding domain Ia (residues 1–252) is located at the N-terminus (Mohammed *et al.*, 2012) and is responsible for the recognition of the target receptor (α2 macroglobulin receptor/low density lipoprotein receptor, CD91) and internalisation within cells (Kounnas *et al.*, 1992). Domain II (residues 253–364) has a crucial role in translocation of the toxin into the cytosol while the function of domain Ib (residues 365–404) is to facilitate toxin

secretion (Siegall *et al.*, 1989). The last 4 residues (aa 400–404) of domain Ib together with domain III (aa 405–613), form the catalytic part of ETA and is required for the inhibition of protein synthesis (Siegall *et al.*, 1989). There is an important amino acid squence (aa 274–280) located in domain II and exposed on the outer surface of ETA. This motif is cleaved by the protease furin and this cleavage is esssential for ETA cytotoxicity (Gordon *et al.*, 1995, Nakayama, 1997). The toxic nature of this enzyme is due to its ADP-ribosylation activity which catalyses the transfer of adenosine diphosphate ribose (ADP-ribose) motif from nicotinamide adenine dinucleotide (NAD^+) to eukaryotic elongation factor-2 (eEF-2) blocking protein synthesis and leading ultimately to cell death (Webb *et al.*, 2008).

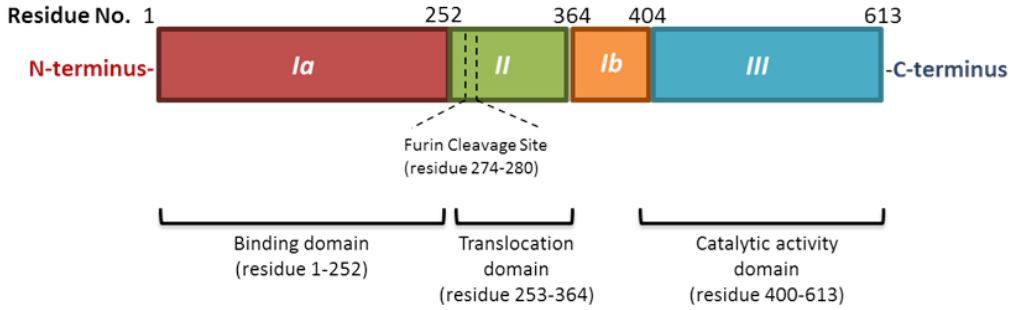

Figure 3.1: The structure and function of exotoxin A (ETA) produced by *P. aeruginosa.*
ETA is composed of three domains. Domain Ia (aa 1-252) which is vital for the recognition of its target receptor. Domain II (253-364) helps in internalisation of the toxin into cells. The last four residues of Domain Ib and Domain III mediate the enzymatic activity (ADP-ribosyltransferase) of ETA. Adapted from Wolf and Elsasser-Biele (Wolf and Elsasser-Beile, 2009).

It has been shown that the mortality rate among patients infected with ETA-producing *P. aeruginosa* strains is three times higher than in patients infected with non-ETA producing counterparts (Cross *et al.*, 1980). Furthermore, the impact of ETA as a toxic product has been studied in several models. It has

been demonstrated that ETA impaired respiratory tract tissue viability in hamster tracheas (Baker, 1982). ETA is also responsible for ocular damage in mice (Hazlett *et al.*, 1981) and corneal ulceration and cell death in rabbits (Iglewski *et al.*, 1977).

ETA has cytotoxic effects on murine and human PMNs which impair the phagocytosis and elimination of *P. aeruginosa* (Miyazaki *et al.*, 1995). The enzymatic motif of this toxin has been fused to cell specific antibody and growth factors to eliminate cancer cells, virally infected cells and mast cells (Jenkins *et al.*, 2004). Furthermore, ETA arrests the expression of co-stimulatory molecules including CD80, CD86, CD54 and HLA-DR on IFN-γ-induced monocytes (Michalkiewicz *et al.*, 1999) and inhibits human granulocyte and macrophage progenitor cell proliferation (Middlebrook and Dorland, 1977, Pollack and Anderson, 1978, Snell *et al.*, 1978).

Interestingly, a study by Moestrup and colleagues revealed that CD91, the ETA receptor is largely restricted to monocytes and their precursors and erythroblasts (Moestrup *et al.*, 1992). This observation is in agreement with our results; i.e. monocytes died in response to PAO1-L supernatants which contain high amount of ETA leading to arrested T cell proliferation. It has also been found that purified ETA modulates monocyte activation and stimulates mononuclear phagocytes to produce toxic oxygen radicals leading to lung tissue damage during chronic *P. aeruginosa* infections (Patzer *et al.*, 1989a).

The ETA regulatory gene, *toxR*, positively promotes rhamnolipids production by *P. aeruginosa* and is under the control of the *rhl* QS system (unpublished data by Quorum Sensing group). These biosurfactants are glycolipids consisting of hydrophobic and hydrophilic motifs and have the ability to

liquefy insoluble substances, reduce surface tension (Beal and Betts, 2000, Caiazza *et al.*, 2005) and ultimately lead to increased swarming motility (Kohler *et al.*, 2000). Most of the virulence factors regulated by QS systems, such as ETA and rhamnolipids, are crucial for the establishment of an effective *P. aeruginosa* infection in the eukaryotic host (Bjarnsholt and Givskov, 2007). Although different studies have shown that ETA is one of the most toxic extracellular products involved in *P. aeruginosa* pathogenicity, in this study, we tried to identify, using an unbiased approach, effector molecule(s) in PAO1 supernatants which dominates the *P. aeruginosa* cytotoxicity on monocytes and, in turn, reduces T cell proliferation.

As seen in the previous chapter, *toxR* deficient strain was less cytotoxic when compared to its parental strain due to the lack of some genetic elements that altered the expression of ETA and rhamnolipids, and ultimately the immune response. This was achieved by exploiting the genetic differences between PAO1 strains to identify the key factors which are responsible of the observed effect. To determine whether ETA activity is responsible for the findings in the previous chapter a *toxA* deletion in the PAO1-L background was constructed. Previously we assessed the effect of ETA in bacterial supernatants from different mutants which contain a plethora of secreted molecules, this chapter involves the generation of a tool to assess the actual impact of ETA more closely to provide a better understanding of the fundamental role of this toxin on monocyte viability.

3.2 Hypothesis

Based on our previous results, PAO1-L secreted products inhibit lymphocyte proliferation in response to SEB, which requires functional APCs. Furthermore, the morphology of monocytes was altered upon stimulation with PAO1-L supernatant and this ability is associated with the presence of *toxR r*egulatory element in the bacterial genome. We hypothesise that the cytotoxic activity of PAO1 sub-line Lausanne is mediated by a high expression of ETA that could potentially influence the monocyte viability and ultimately supresses T cell proliferation.

3.3 Aim

In an attempt to elucidate the role of ETA in *P. aeruginosa*-mediated cytotoxicity on immune cells, a *toxA* deficient strain was constructed by in-frame deletion of the *toxA* gene in the PAO1-L background. This chapter details the generation and phenotypic characterisation of PAO1-LΔ*toxA*.

3.4 Materials and Methods

3.4.1 Constructing PAO1-L*ΔtoxA* Mutant Strain

Site directed mutagenesis using homologous recombination was used to create an in-frame deletion mutant. Briefly, the upstream and downstream ~500 base pair (bp) regions flanking the *toxA* gene were cloned into the suicide vector pME3087. *E. coli* S17-1 λ *pir* was used to harbour and conjugate the pME3087 construct into PAO1-L. The chromosome and suicide vector were co-integrated via homologous recombination. Colonies of trans-conjugants were selected on LB medium supplemented with 150 µg/ml tetracycline and 15 µg/ml nalidixic acid. The strain was cured of the pME3087 backbone by tetracycline sensitive enrichment. Candidates were screened for deletion using PCR (Figure 3.2).

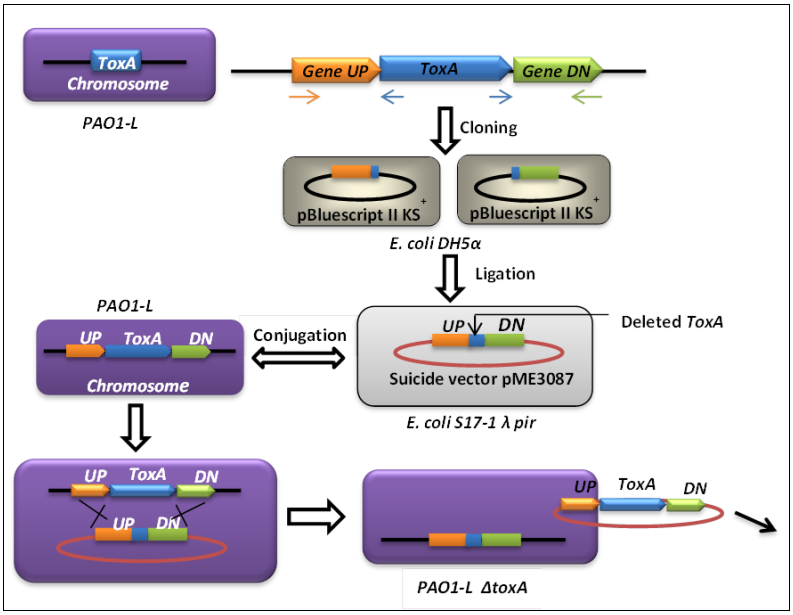

Figure 3.2: Schematic presentation of the stages to generate the mutant strains. Cloning two DNA fragments derived from the flanking regions located in up and down stream region of ToxA gene (signed as UP and DN) using pBluescript II KS+ plasmid. After that, both PCR products were fused in the suicide plasmid pME3087. Then, the mutagenesis was done by the conjugation of PAO1-L strain with *E. coli S17-1 λ pir* containing the suicide plasmid with the deletion construct.

3.4.2 Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are shown below in Table 3.1

Strain/Plasmid	Characteristics	Reference/Source
Strain: *Pseudomonas aeruginosa*		
PAO1-L	Wild-type, Lausanne strain	From Dieter Haas, Lausanne, Switzerland.
PAO1-LΔ*tox*A	*Tox*A chromosomal deletion mutant derived from PAO1-L	This study
Strain: *Escherichia coli*		
DH5α	Cloning strain	Grant et al., 1990
S17-1λpir	strain for suicide plasmids	Simon et al., 1983
Plasmids		
pBluescript-II KS+	Cloning vector; ColE1 replicon; ApR	Stratagene
pME3087	Suicide vector; ColE1 replicon;TcR	Voisard et al., 1994

Table 3.1: Bacterial strains and plasmids used in the construction of PAO1-LΔ*toxA*.

3.4.3 Nucleotide Primers

In order to delete *toxA* gene in the Lausanne background, oligonucleotide primers were designed using the Pseudomonas Genome Database (Winsor *et al.*, 2011) (Figure 3.3) and synthesised by Eurofins (UK). Primers incorporated restriction sites at their 5' end. All primers are listed in Table 3.2.

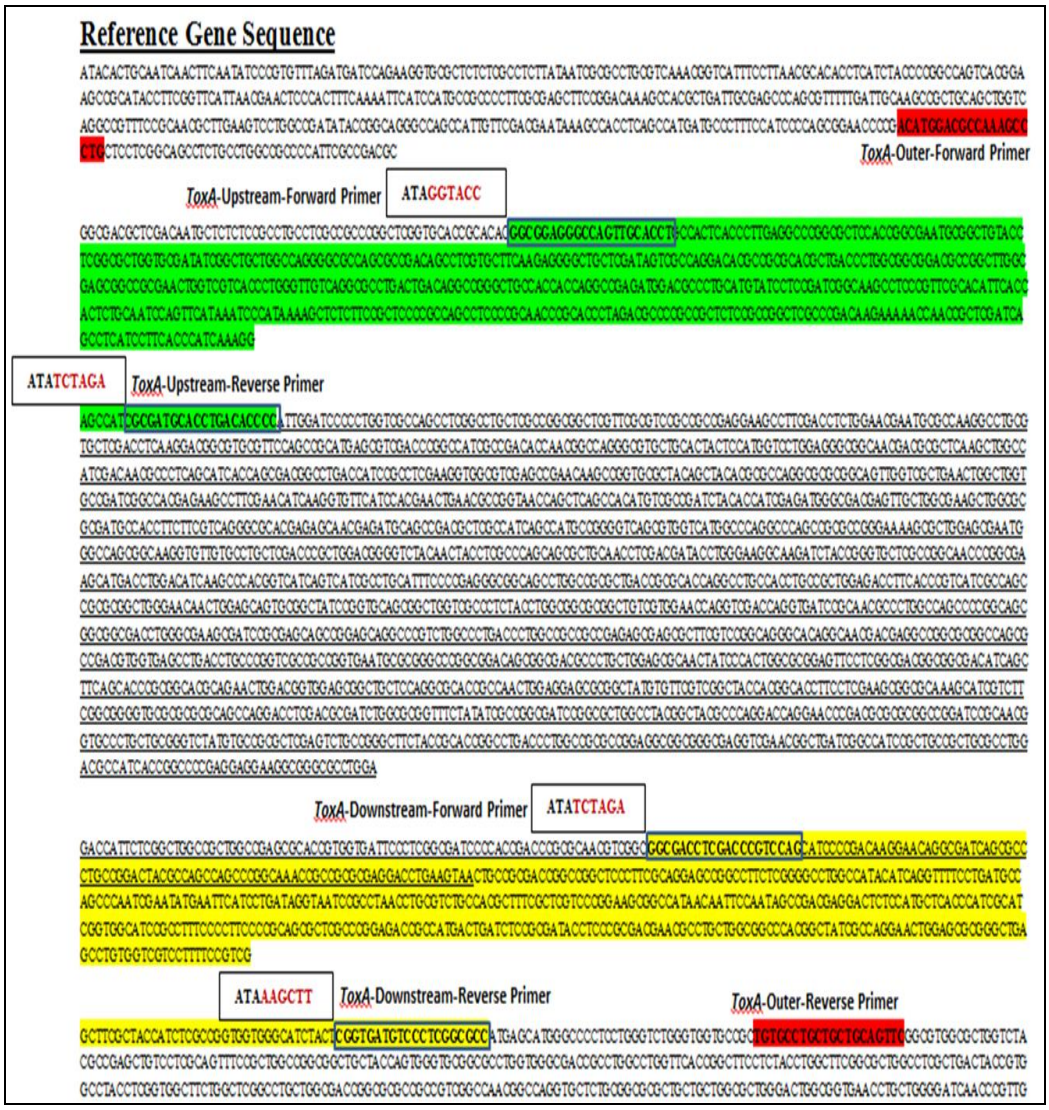

Figure 3.3: *ToxA* gene and its flanking regions.
The underlined nucleotides represent *toxA* gene. The *toxA* upstream and downstream regions were shaded with either green or yellow respectively. Red-shaded nucleotides represent both forward and reverse outer PCR primers.

Primer	Sequence (5' to 3') with restriction site	Restriction Enzyme	Function
ToxA-UP-F	ATAGGTACCGGCGGAGGGCCAGTTGCACCT	KpnI	To amplify upstream flanking region of toxA gene
ToxA-UP-R	ATATCTAGAGGGGTGTCAGGTGCATCGCG	XbaI	
ToxA-DN-F	ATATCTAGAGGCGACCTCGACCCGTCCAG	XbaI	To amplify downstream flanking region of toxA gene
ToxA-DN-R	ATAAAGCTTGGCGCCGAGGGACATCACCG	HindIII	
ToxA-Check-F	ACATGGACGCCAAAGCCCTG		To identify PAO1-LΔtoxA
ToxA-Check-R	GAACTGCAGCAGCAGGCACA		

Table 3.2: Oligonucleotide primers used in constructing PAO1-LΔ*toxA*.

3.4.4 Chemical Reagents

All general chemicals were obtained from Sigma (UK) unless otherwise stated.

3.4.5 Growth Media

Media were prepared using deionised water and autoclaved at 121°C for 20 min at 15 pound-force per square inch (p.s.i.).

3.4.6 Antibiotic / X- Gal/ IPTG

Stock solutions of antibiotics were prepared according to Sambrook et al. (1989), and stored at -20 °C. Ampicillin (Amp) was prepared to a stock concentration of 100 mg/ml in distilled water and tetracycline (Tc) stock was prepared to 50 mg/ml in methanol (Fisher). 20 mg/ml of 5-bromo-chloro-3 indoyl β-D-galactoside (X-Gal) was prepared in DMSO. Isopropylthio-β-D-galactoside (IPTG) was prepared to a concentration of 0.1 mM in distilled

water. All reagents were sterilised before use by filtering through 0.22µm sterile filter.

3.4.7 Lysogeny Broth

Lysogeny Broth (LB) (10 g/l tryptone, Oxoid, 5 g/l yeast extract, Oxoid, 10 g/l NaCl and NaOH to pH 7.0) was made into solid media with the addition of 1.5% (w/v) Technical Agar No. 3 (Oxoid). *P. aeruginosa* was grown in LB while the cloning plasmid pBluescript KS II (+) was maintained in *E. coli DH5α* in LB supplemented with ampicillin (Ap) 100 µg/ml

3.4.8 Two Times Yeast Extract Tryptone (2xYT) Broth

The suicide plasmid, pME3087 was maintained in *E. coli S17-1λ pir* in 2xYT broth supplemented with 25 µg/ ml Tc.

3.4.9 Growth conditions

Liquid cultures were grown in LB at 37 °C with agitation at 200 rpm in a Gallenkamp incubator (Gallenkamp Ltd.,UK). The bacterial growth was monitored by measuring the absorbance at a wavelength of 600 nm using a Novospec II visible spectrophotometer (Pharmacia LKB Ltd.,Cambridge, UK).

3.4.10 Storage and Reconstitution of Bacterial Strains

Bacterial strains were grown to stationary phase and then mixed with glycerol to a final concentration of 15% v/v before being frozen at -80 °C for future use.

All strains were revived on solid media. Strains were revived for each individual step and not stored for greater that 3 days at 4 °C.

3.4.11 DNA Manipulation

3.4.11.1 Plasmid DNA Isolation

Isolation of DNA plasmid was done using Qiagen Miniprep kit (Qiagen, UK) according to the manufacturer's instructions. Briefly, cells from 1 ml of an overnight bacterial culture were lysed using alkaline solution, neutralised and centrifuged at 13,000 rpm for 10 min to remove cellular debris. The lysate was then filtered through a silica-gel filter, washed and plasmid DNA eluted using RNAse and DNAse free H_2O.

3.4.11.2 Isolation of Chromosomal DNA

The extraction of genomic DNA was performed as per manufacturer's instructions using the Wizard Genomic DNA Purification Kit (Promega, UK). Briefly, bacteria were cultured in LB broth overnight, centrifuged for 2 min at 13,000 rpm and lysed by the addition of 600 µl of Nuclei Lysis solution at 80°C for 5 min. The samples were then cooled at room temperature (RT) and incubated with 3 µl of RNase solution at 37 °C for 15-60 min. After this step, proteins were precipitated by adding 200 µl of Protein Precipitation Solution, followed by 5 min on ice and centrifugation in a microfuge (MSE Micro Centaur, Sanyo, UK) at 13,000 rpm for 3 min. The supernatant was added to 600 µl of isopropanol and centrifuged for 10 min at 13,000 rpm. Later, the supernatant was removed and the pellet was mixed with 600 µl of 70 % (w/v)

ethanol, mixed and centrifuged at 13,000 rpm for 5 min. The ethanol was aspirated away and the DNA pellet air-dried for 10-15 min. The pellet was then rehydrated in 100 µl Rehydration Solution for 3 h at 65 °C.

3.4.11.3 Plasmid and Strain Construction

a) Optimisation the annealing temperature of upstream and downstream primers of Δ*tox*A mutant strain

The flanking regions of the target gene were amplified using PCR. The annealing temperature of the primers to the single stranded DNA template was adjusted. For general PCR reaction, 0.125 µl crimson Taq DNA polymerase (M0324S, BioLabs) in 5 x buffer (B0324S, BioLabs) was used to a final volume 25 µl. The reaction mixture contains 2 µM of each primer, 2 mM dNTPs and approximately 0.5 µg DNA templates. PCR reactions were done in the presence or absence of 2 % (v/v) DMSO to lower the melting point and aid in the dissolution of secondary DNA structures.

PCR amplifications of the upstream and downstream fragments were performed to a final volume 50 µl. In this reaction, 1 µl DyNAzyme (F-505S, Promega) and 5 µl 10x buffer (Promega) were used. 2 µM of each primer was also used in the presence of 2 mM Deoxyribose nucleotide triphosphates (dNTPs) and approximately 0.5 µg DNA templates. Amplification reactions of *P. aeruginosa* DNA were carried out in absence of presence of 2% DMSO in a Techno Thermal Cycler (Progene) using the conditions shown in Table 3.3. A no template control was always included to check for DNA contamination of the reagents. Reaction mixes were then stored at 4 °C until used.

	Stage 1	Stage 2			Stage3	Hold
	Initial denaturation	Denaturation	Annealing	Elongation	Final extension	-
Cycles	1	←40→			1	-
Temperature	95°	95°	59-70°	70°	68°	4°
Time*	2:0	00:30	00:30	1:00	10:00	∞
*: (minutes : seconds)						

Table 3.3: PCR conditions used in constructing PAO1-LΔ*toxA*.

3.4.11.4 Cloning of PCR Products

This process included clean-up of PCR products, digestion, an extra clean-up and ligation. This method is based on the recombination of the digested PCR fragment with a linearized vector with the help of ligase enzyme. This recombinant DNA was then electroporated into *E. coli DH5α*. Trans-formants containing recombinant clones were identified by blue/white screening on LB agar plates containing the appropriate antibiotics and sequenced.

3.4.11.5 DNA Purification

DNA to be used in subsequent cloning steps was purified using the QIAquick PCR purification Kit (28104, QIAGEN) as per the manufacturer's instructions, with the exception that all DNA was eluted with sterile DNase and RNase free water

3.4.11.6 Restriction Digestion

PCR fragments and the target plasmid were digested with an appropriate restriction enzyme(s). All restriction enzymes were purchased from Promega unless otherwise stated. For digestion, a mixture reaction was prepared to a

final volume 40 µl. The following reagents were added: 4 µl of either buffer B or acetylated BSA (1µg/ml) and 2 µl of each relevant enzyme. Either 25 or 4 µl of template (about 25 ng) was added to purified PCR products or vector reaction.

3.4.11.7 DNA Ligation

DNA ligations were performed using clean DNA and a 1:6 ratio of vector to insert. Reactions were carried out using 1µl of T4 ligase (400000 U/ml, M0202S, Biolabs) and 2 µl of l 10× T4 buffer (B0202S, Biolabs) in a final volume of 20 µl. Control ligations containing no insert were also conducted where appropriate.

3.4.12 Transformation

3.4.12.1 Preparation of Electrocompetent E. coli Cells

2 ml of an overnight bacterial culture (*E. coli DH5α*) was added to 200 ml 2xYT broth and incubated at 37 °C and 200 rpm to an optical density at 600 nm of 0.6-0.9, which is mid-log phase. Then, the cells were harvested at 4000 rpm for 10 min at 4 °C, washed twice in ice-cold sterile H_2O and 10% (v/v) glycerol buffer respectively After that, the cell pellets were re-suspended to a concentration of approximately 10^{10} CFU/mL and aliquots flash frozen in liquid nitrogen and stored at -80 °C until use.

3.4.12.2 Electroporation of Electrocompetent E. coli cCells

Electroporation was performed in 0.2 cm electrode gap Gene Pulser cuvettes (BioRad, UK) containing 50 µl of competent cells. An electroporation pulse of 2.5 kV (200 Ω) was delivered using the BioRad Gene Pulsar connected to a BioRad pulse controller (BioRad, UK). 1 ml of LB broth was immediately added to the cells which were then incubated at 37 °C for 1:30 h in the absence of antibiotics.

3.4.12.3 Blue/white Screening

Transformed cells (*E. coli DH5α* has a lacZα deletion) containing inserted plasmids were screened by plating onto LB agar containing 100 µl/ml ampicillin in the presence of 20 µg/ml 5-bromo-chloro-3 indoyl β-D-galactoside (X-Gal) and 0.1 mM of isopropylthio-β-D-galactoside (IPTG) and grown overnight at 37 °C.

3.4.12.4 Plasmid-Insert Extraction

The recombinant plasmid was extracted from bacterial cultures using Sigma GenElute as manufacturer's guidelines. The DNA yield was then measured using a Nanodrop spectrophotometer.

3.4.12.5 Sequencing Determination

The amplified fragments were sequenced in the DNA Sequencing Laboratory, Queens Medical Centre, University of Nottingham, using the Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit and 3130xl

Genetic Analyzer. The results were analysed using Chromas software. For sequence alignment of the common parts, ClustalW software program was used (Larkin *et al.*, 2007).

3.4.12.6 Gel Extraction

a) Gel Electrophoresis

PCR products were double digested with the relevant restriction enzymes in the presence of 10x B buffer (R002A, Promega). The reaction was carried out for 4 h at a final volume 40 µl. Then, DNA samples were mixed with 6X DNA loading buffer and loaded into 0.8% (w/v) agarose gel in 1X Tris-acetic acid-EDTA (TAE) running buffer supplemented with ethidium bromide to a final concentration of 10 µg/ml. To determine the actual size of DNA fragments, 1 µg (10 µl) of 1 kb DNA ladder (Invitrogen, UK) was loaded on the agarose gel. The gels were run in (1x) TAE buffer in a DNA electrophoresis tank at 100 volt for 40 min.

b) Purification of DNA from Agarose Gels

DNA fragments were visualised and excised using a UV transilluminator (UVP, USA). Then, they were purified from the gel slices using a Qiagen Gel Extraction Kit (Qiagen, UK) according to the manufacturer's protocol. The gels pieces obtained were measured and the yielded weight (in mg) was considered as an equivalent one volume in µl. Briefly, a mixture of 3 volumes of QG and 1 volume of gel slice was melted at 50 °C for 10 min with repeated vortexing to ensure the gel was completely dissolved. One gel volume of

isopropanol (Fisher Scientifics, UK) was then added and applied to a Qiaquick column and centrifuged for 1 min. Later, the column was washed two times with 500 µl QG buffer and 750 µl PE buffer respectively and centrifuged. After discarding the flow through, the column was centrifuged for a further 1 min and placed in a clean microcentrifuge tube. DNA was eluted with 50 µl sterile distilled H_2O and centrifuged for an additional 1 min.

3.4.13 Construction of pME3087Δ*toxA*

3.4.13.1 De-phosphorylation of pME3087 plasmid

To decrease vector background, the digested vectors were dephosphorylated using 1 µl Antarctic phosphatase (M0289S, Biolabs) in 10x buffer to a final concentration 1X(B02890, Biolabs) and incubated for 30 min at 37 °C.

3.4.13.2 Insert Ligation

The cloned fragments were fused and subsequently, re-cloned into suicide plasmid, pME3087 using T4 ligase, as described previously. The PCR primers were designed in a way that provides an XbaI site between the joined fragments as well as KpnI and HindIII restriction sites at the two ends. For this purpose, the vector, upstream and downstream fragment were digested using relevant restriction enzymes, as previously described. For ligation, the vector: insert ratio used was calculated using the following equation:

$$\frac{50\ ng}{insert\ yield\ (ng)} = \text{µl of the insert required for the ligation reaction.}$$

This equation was used to calculate the insert from both upstream and downstream fragments required for the ligation for a final volume reaction 10 µl , and the results are shown as follows:

$\frac{50\ ng}{19.80\ ng}$ = 2.5 µl (for upstream fragment).

$\frac{50\ ng}{11.91\ ng}$ = 4.2 µl (for downstream stream fragment).

For monitoring purposes, a vector-only ligation reaction was run in parallel to check for the levels of background ligation (due to undigested and/or re-ligated vector molecules). The ligation reaction mixture included 1.3 µl of the suicide vector, 1 unit of T4 DNA ligase and 1x ligation buffer.

3.4.13.3 Transformation of pME3087ΔtoxA into E. coli S17-1 λ pir Competent Cells

Using electroporation method, the recombinant plasmid was transformed into *E. coli S17-1 λ pir* competent cells. Serial dilutions of cell suspension were plated on selective media, LB agar containing 25µg/ml tetracycline and incubated overnight at 37 °C.

3.4.14 Gene replacement in *P. aeruginosa*

3.4.14.1 Conjugation of pME3087ΔtoxAplasmid into P. aeruginosa

Both donor and recipient cells were grown o/n in 3 ml LB broth. PAO1-L recipient cells were grown at 24°C to inactive the restriction system while *E. coli* donor cells were grown at 37°C supplemented with 25µg/ml tetracycline. Then, 1ml of each culture were mixed and centrifuged at 13000 rpm for 5 min.

Pellets were re-suspended in 100µl LB broth. Conjugations were achieved by spotting the mixed bacteria onto an LB agar plate and incubating at 37°C for 4 h. Cells from the plate were then harvested, re-suspended in 1 ml of LB broth and plated onto LB agar plates containing 150µl/ml tetracycline and 2mg/ml nalidixic acid to select for *P. aeruginosa* transconjugants. Plates were then incubated between 24 and 48 h at 37°C. Donor and recipient cells were plated separately on the same medium. Colonies of transconjugants were grown overnight in LB broth at 37°C. A fresh culture was then inoculated in LB at 1:100 for 1 h with agitation (200 rpm) at 37°C. Tetracycline was added to a final concentration 20µl/ml (bacteriostatic concentration) and incubated under the previous conditions. After that, 2000 µg/ml of carbencillin was added to kill the growing cells and incubated for 4 h under the same conditions. The growing cells were centrifuged and re-suspended in 1ml LB broth. 100µl of serial dilutions was plated onto LB agar with antibiotics and incubated overnight at 37°C. The grown colonies were tested for loss of tetracycline resistance by plating onto LB agar with and without 150 µl/ml tetracycline. The mutant candidates were screened by PCR and sequencing as described before.

3.4.15 Western blot analysis of PAO1-LΔ*toxA* mutant strain

Equivalent bacterial supernatants were processed and electrophoresed (see Section 2.9). In this study, PAK and PAK.NT (Voulhoux *et al.*, 2000) strains (kindly provided by Stephan Heep) were used as positive and negative controls respectively (Voulhoux *et al.*, 2000).

3.4.16 Phenotypic characterisation of PAO1-LΔtoxA mutant strain

3.4.16.1 Monitoring bacterial growth through optical density measurements

Growth of bacterial cultures was determined under static conditions using a microplate reader (Infinite® M1000 PRO, TECAN, UK). In this work, an inoculum from an overnight bacterial culture was prepared in 25 ml LB broth in a 250 ml conical flask and incubated overnight at 37 °C with agitation (200 rpm). Then, the optical density (OD_{600}) of the liquid cultures was adjusted using a spectrophotometer (BioMate 3s, Thermo Scientific, UK) to 1% in 10 ml LB medium and grown in 96-well plates by adding 200 µl per well (in triplicates) and OD_{600} was read every 30 min for 24 h at 37 °C. Data were then analysed using Microsoft Excel 2010.

3.4.16.2 Swarming motility assay

Swarming assay was undertaken on polystyrene Petri dishes (90 mm in diameter) using swarming agar prepared according to a previously published study (Rashid and Kornberg, 2000). This is composed of 0.5% of Bacto agar (Difco BD, USA) and nutrient broth No.2 (Oxoid, USA). Filtered D-glucose (Sigma-Aldrich, UK) in sterilised distilled water was added to the autoclaved swarm medium to a final concentration of 0.5% (w/v).

Briefly, the optical density (OD_{600}) of an overnight bacterial growth in LB broth was adjusted to 1.0. Then, 5 µl of the diluted bacterial broth cultures was placed on the centre of the agar surface to allow the visualization of motility across the plate (Tremblay *et al.*, 2007, O'May and Tufenkji, 2011). The plates

were subsequently incubated at 37 °C for 16 hours and swarming images were obtained by placing plates on a black background with indirect illumination from below.

3.4.16.3 Twitching motility assay

Twitching motility was performed on polystyrene Petri dishes (90 mm in diameter) containing 1% LB agar. In this assay, a colony of an overnight bacterial culture was collected and inoculated on the centre of the agar by deep stabbing of a sterilised toothpick to the bottom of the Petri dish (Semmler *et al.*, 1999). The plates were then incubated at 37°C in saturated humidity for the indicated time. The mobility was quantified by measuring the diameter of the twitching zone (Alm and Mattick, 1995).

3.4.16.4 Protease production assay

The proteolytic activity of proteases secreted by *P. aeruginosa* is associated with a number of chronic infections due to their ability to impair tissue integrity and abrogate immune defences (Oldak and Trafny, 2005).

Briefly, 3 ml of azocasein (5 mg/ml) was added to 300 µl filtered-bacterial supernatant and incubated for 15 min at 37 ºC with agitation. Trichloroacetic was then added to stop the reaction. After centrifugation, the optical density of clear supernatants was read at $OD_{400.}$

3.5 Statistical analysis

For statistical analysis, significance was determined by one way ANOVA and post-hoc Tukey or Dunnett tests. $P<0.05$ was considered statistically significant. Actual values are indicated on individual figure legends.

3.6 Results

3.6.1 The *ΔtoxA* strain of PAO1-L was successfully constructed

A ToxA deficient strain in PAO1-L (Δ*ToxA*) background was created by allelic exchange of *E. coli* carrying Δ*toxA*- recombinant-*S17-1 λ-pir* vector with PAO1-L. The mutagenesis strategy involved using two pairs of primers in order to amplify the up and downstream regions of the target gene. Agarose gel images showed the expression of the expected band size in the mutant strains which is about 1200bp.

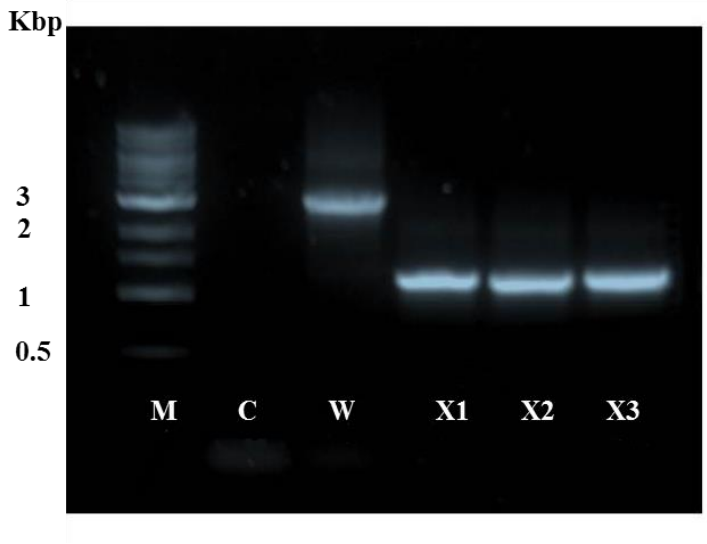

Figure 3.4: Agarose gel analysis of outer PCR screening of the Δ*toxA* mutant strain.
In this reaction, the candidates were screened for *ToxA* deletion using a set of primers which amplifies a larger DNA including the target gene. M= 1 kb DNA marker ladder (Sigma), W= PAO1-L (wild strain), X1-X3=expected mutant strains. C= negative control (without template) (expected mutant band around 1200bp).

PAO1-LΔ*toxA* was also analysed by western blot for ETA production as describe in Material and Method (chapter 2). The result (Figure 3.5) indicated that ETA was not detectable in PAO1-L Δ*tox*A supernatant compared to the wild type (PAO1-L) which suggest that that the mutation in PAO1-L Δ*tox*A

was correct. ETA production in the bacterial supernatant was also measured in PAO1-LΔ*rhIA*, PAO1-LΔ*rhIC* and PAO1-LΔ*toxR* strains. The result showed that there was a high expression of ETA in the first two mutants but not in the former one. A supernatant from either PAK or PAK.NT (Voulhoux *et al.*, 2000) was used to confirm the result as a positive and negative control respectively.

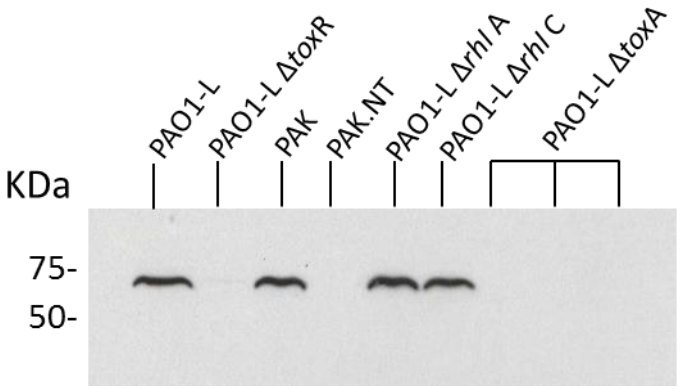

Figure 3.5: Western blot analysis of PAO1-LΔ*toxA* mutant strain.
ETA production was abolished in PAO1-LΔ*tox*A mutant supernatant compared to PAO1-L. ETA was highly expressed in PAO1-LΔ*rhI*A and PAO1-LΔ*rhI*C mutants while severely reduced in PAO1-LΔ*toxR* supernatant. PAK and PAK.NT strains were used as positive and negative controls respectively. Data obtained from a single independent experiment.

3.6.2 Phenotypic characterisation of PAO1-LΔ*toxA* in comparison with PAO1-L

3.6.2.1 Identical growth rate of PAO1-L and its isogenic toxA deficient strain under static conditions

In an attempt to determine whether the deletion of *toxA* gene might affect the growth rate of *toxA* deficient strain compared to PAO1-L wild type strain growth of each strain was assessed. The growth curve of each strain was

obtained by measuring the optical density (OD_{600}) every 30 min at 37 ºC for 24h under static conditions (Figure 3.6).

Based on the growth curves observed, the deletion of the *toxA* gene does not have a negative impact on *P. aeruginosa* growth under static conditions suggesting that *toxA* gene mediates ETA production and promotes bacterial cytotoxicity rather than bacterial growth.

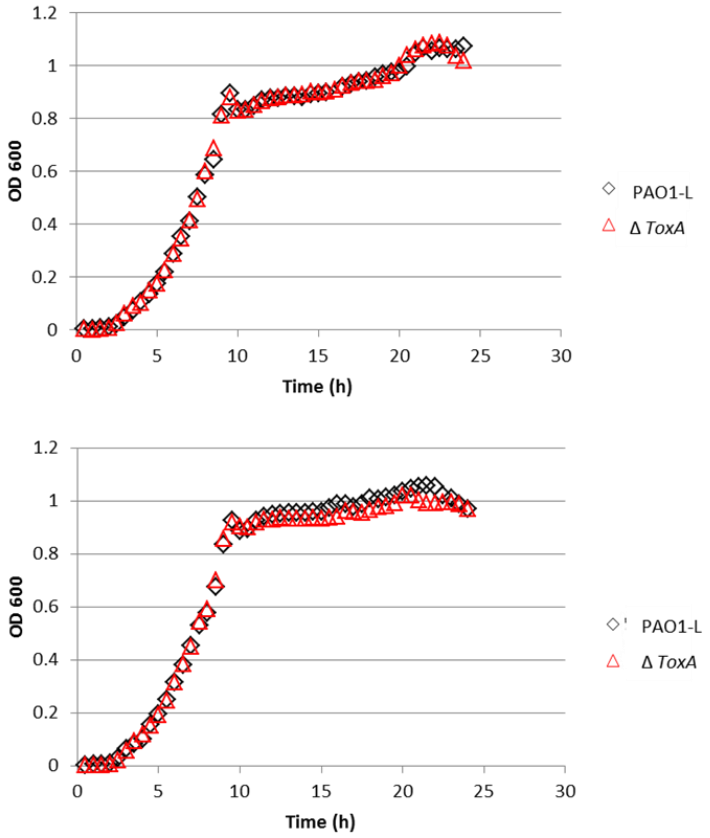

Figure 3.6: Similar growth of *P. aeruginosa* PAO1-L (WT) and PAO1-LΔ*toxA* in LB broth under static conditions.
PAO1-L wild type and ΔtoxA deficient strain in PAO1-L background growth curve were measured by inoculating a single colony of an overnight culture in 5ml LB broth at 37oC, 200 rpm. Then, the OD600 was adjusted to 1.0 in 10 ml fresh LB medium and grown in 96 well-plate by adding 200 µl bacterial growth per well in triplicates. The growth curve was obtained by measuring OD600 every 30 min for 24 h. Data presented are from two independent experiments.

3.6.2.2 Lack of toxA does not alter P. aeruginosa motility

P. aeruginosa exhibits different types of motilities such as swarming and twitching (Tremblay *et al.*, 2007). Swarming is unique motility behaviour comprises prompt and organised movement employed by hyper-flagellated *P. aeruginosa* cells (Kohler *et al.*, 2000). Furthermore, the production of biosurfactants by *P. aeruginosa* facilitates this motile behaviour by reducing the surface tension and liquefying the solid surface beneath the motile cells. Rhamnolipids and 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs), are considered vital exoproducts released by *P. aeruginosa* and behave as a wetting substance that promotes in a coordinated manner bacterial migration and colonisation over solid surfaces (Soberon-Chavez *et al.*, 2005, Maier and Soberon-Chavez, 2000, Deziel *et al.*, 2003, Caiazza *et al.*, 2005).

P. aeruginosa can also spread rapidly across solid surfaces based on the action of specific external structures known as type IV pili (Burrows, 2012, Kearns *et al.*, 2001). This surface-associated component provides the bacterium with motility leading to bacterial invasion throughout host tissues (Alarcon *et al.*, 2009).

To investigate whether the deletion of *toxA* gene would down regulate motility performance by *P. aeruginosa*, Swarming (Figure 3.7) and twitching assays (Figure 3.8) were done in both PAO1-L and PAO1-L*ΔtoxA.* The data revealed that bacterial movements including swarming and twitching did not decrease in the *toxA*-deficient strain compared to wild type and neither flagella and type IV pili gene expression are controlled or modulated by *toxA* gene (Tremblay and Deziel, 2010).

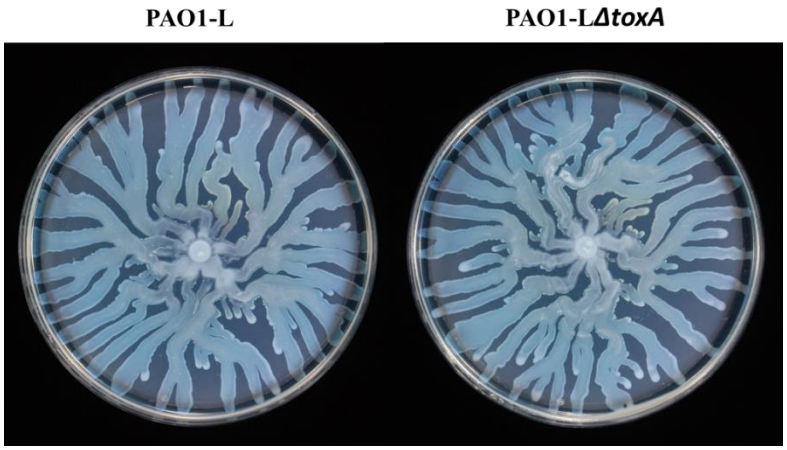

Figure 3.7: Swarming motility was not altered in *toxA* deficient strain compared to wild type parental strain.
PAO1-LΔ*toxA* and its isogenic wild type strain were grown on semi-solid swarming medium (0.5% agar) for 16 h at 37 ºC. The results showed that swarming motility is unaffected by mutation of *toxA* in the Lausanne background compared to its wild type. Images are representative from two independent experiments showing identical trends in motility**.**

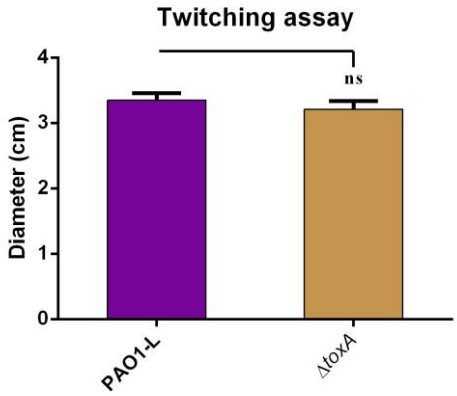

Figure 3.8: Twitching motility was not altered in *toxA* mutant strain compared to wild type parental strain.
PAO1-L and its isogenic *toxA* mutant strains were plated in the centre of 1% LB agar by surface stabbing using toothpick and incubated at 37 ºC with saturated humidity for 24 h. The data revealed that there is no variation in twitching motility between PAO1-L and PAO1-L*toxA* strains. Results are from three independent experiments showing the mean and standard deviations. Samples in each experiment were conducted in quadruplicates. Statistical significance was calculated by unpaired Student's t test.

3.6.2.3 Production of protease does not change in PAO1-LΔtoxA compared to its wild type parental strain

To examine whether the production of proteases in bacterial supernatants would be influenced by mutation in the *toxA* gene, PAO1-L and PAO1-L*ΔtoxA* were inoculated in LB broth and incubated at 37 ºC for 24h. Bacterial growth cultures were then centrifuged, supernatants filtered and mixed with the protease reagent for 15 min. The graph showed that the levels of protease produced by *P. aeruginosa* slightly decreased after the deletion of Δ*toxA* gene compared with its parent strain (Figure 3.9).

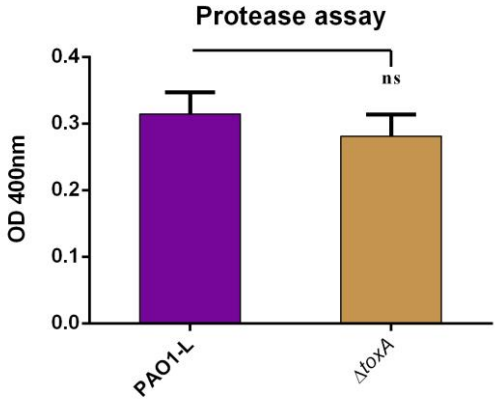

Figure 3.9: Protease production in bacterial supernatant is slightly decreased in *toxA* deficient strain compared with its parental strain
Overnight bacterial growth cultures of PAO1-L and PAO1-L*ΔtoxA* in LB broth were centrifuged and filtered. The levels of protease in bacterial supernatants were then detected by adding 900µl protease reagent to 100 µl filtered culture. After 15 min incubation at 37 ºC, the reaction was stopped using trichloroacetic, the reaction was then centrifuged and the optical density was measured at OD_{600}. Results are from two independent experiments showing the mean and standard deviations. Statistical significance was calculated by unpaired Student's t test.

3.7 Discussion

This study involved the construction and characterization of a *toxA* mutant strain in the Lausanne background in an attempt to determine the role of ETA in *P. aeruginosa* cytotoxicity. The mutagenesis strategy involved the integration of the external fragments that are homologous to the *toxA* gene cloned into a non-replicating plasmid and integration into the bacterial chromosome by double crossover recombination resulting in replacement of the original wild-type gene by the new construct and eventually abrogation the ETA production.

ToxA is a structural gene that encodes a 66 kDa ETA protein which is secreted to the surrounding environments by a type II secretion system (Ochsner *et al.*, 1996, Davinic *et al.*, 2009). ETA is considered the most toxic factor secreted by *P. aeruginosa* (Hamood *et al.*, 1996b) as it possess ADP-ribosylation activity which catalyses the transfer of ADP-ribose from NAD+ to elongation factor 2 leading to inhibition protein synthesis and eventually cell death (Chaudhary *et al.*, 1990).

In this chapter, *toxA* deficient strain in the Lausanne was constructed successfully and the expression of ETA was completely abolished when the *toxA* gene is deleted from the bacterial genome. The phenotype of *toxA* mutant strain was similar to that of the wild type strain and showed equivalent growth pattern after growing in LB broth. Further , both PAO1-L and PAO1-LΔ *toxA* strains displayed similar motility as they swarmed identically in swarming agar and there was no difference in the diameter of the twitching motility zones after incubation at 37 º C for the indicated time. The statistical analysis of the

protease production showed that there is no significant variance in protease levels after mutation in the Δ*toxA* gene compared to the parental strain.

Based on this, it seems that *toxA* gene is directly involved in ETA production by *P. aeruginosa* which is crucial for bacterial cytotoxicity (Gaines *et al.*, 2007) and is under the control of the regulatory element *toxR* (Hedstrom *et al.*, 1986). Deletion of this gene did not cause differences in the bacterial behaviour in comparison with the wild type strain. Using this strain in further studies might provide a better understanding of how the lack of ETA production might affect the cytotoxic impact of *P. aeruginosa* on monocyte viability and the proliferative response of T lymphocytes as seen in the wild type strain (see chapter 3). Identifying the key factor and its mechanism of action will allow us to establish key contributors to the pathogenesis of *P. aeruginosa* and elucidate the molecules basis of its ability to modulate the immune system.

In order to ensure that mutant phenotype does not have secondary mutations that may have arisen during the creation of the mutant strain, confirmation through the generation of the complementation strain is required by re-introducing *toxA* gene and its upstream and downstream flanking regions into the mutant strain. However, the complementation has not been done because lack of ETA production did not lead to the optimal recovery of T cell proliferation in response to Δ*toxA* supernatant as predicted (see chapter 4).

In conclusion we have generated a *ToxA*-deficient strain in the *P. aeruginosa*-Lausanne background. As expected, ETA did not contribute to bacterial growth or motility. This mutant strain will enable direct investigation of the contribution of ETA to the effect of *P. aeruginosa* secretions on immune cells in the context of other *P. aeruginosa*-secreted products.

4. The impact of Exotoxin A (ETA) on immune cells

4.1 Introduction

The strategy employed in the present study was to assess whether the presence of ETA in bacterial supernatant is a major pathogenic determinant which dominates the cytotoxicity exhibited by *P. aeruginosa* on immune cells. In order to do this, PBMCs from healthy individuals were stimulated *in vitro* in the presence of supernatants from an ETA-deficient strain (see Chapter 3) and the corresponding exotoxin A-producing parent strain. Other mutant strains in the PAO1 Lausanne background such as a strain deficient in rhamnolipid production were also tested. The presence of non-specific stimuli is essential to provide a sufficient signal to drive cell division (Godoy-Ramirez *et al.*, 2004).

As stated in chapter 2, the proliferative response of SEB-stimulated T cells in PBMCs cultures was significantly reduced in the presence of PAO1-L supernatants. However, T cell expansion was just slightly decreased in the presence of PAO1-N supernatants under the same conditions. Interestingly, the down regulation of T cell proliferation by *P. aeruginosa* is mediated by the cytotoxic impact of this organism on APCs which ultimately reduces T cell division (Issekutz and Stoltz, 1985).

The transcriptional regulator *toxR* gene is associated with the production of ETA by *P. aeruginosa* and is an important missing element in the PAO1-N strain (Hedstrom *et al.*, 1986, Gaines *et al.*, 2007) A *toxA* in-frame deletion

mutant in PAO1-L was constructed (Chapter 3) and employed as a tool to evaluate the contribution of ETA to the effects of PAO1 supernatants on human immune cells.

T cells play a decisive role in initiating and shaping immune responses (Isomaki *et al.*, 2005). In an attempt to understand the T cell responses against bacterial infection *in vitro*, non-specific stimuli such as mitogens and superantigens have been extensively used. These include the superantigen SEB as described in chapter 2 and the mitogen phytohaemagglutinin (PHA) (Herman *et al.*, 1991, Greaves *et al.*, 1974, Ceuppens *et al.*, 1988).

PHA is a potent polyclonal activator of a wide number of immune cells (Greaves *et al.*, 1974, Christmas *et al.*, 2006, Kesherwani and Sodhi, 2007). It has been proposed that T-cell clonal expansion can be triggered by PHA even if T cells are unable to react sufficiently to MHC-peptide complexes via the crosslinking of surface glycoproteins, including the TCR, though the ligation of their carbohydrate moieties (Movafagh *et al.*, 2011, Ceuppens *et al.*, 1988). However, the adequate activation and proliferation of PHA-stimulated T cells require additional key molecules produced by accessory cells in the microenvironments such as IL-6 and IL-2 (Ceuppens *et al.*, 1988, Greaves *et al.*, 1974, Katzen *et al.*, 1985).

In the present study, staining with Annexin-V is used to specifically identify apoptotic monocytes upon stimulation with bacterial supernatants in order to gain further insight into the role of the secreted ETA in *P. aeruginosa* cytotoxicity (Appelt *et al.*, 2004, Lund *et al.*, 2009, Bengtsson *et al.*, 2004, Collins, 2002). Staurosporine was used as inducer of apoptosis in monocytes due to its ability to cleavage and activate the caspase-3 signalling pathway and

induce apoptotic cell death (Zhang *et al.*, 2004, Voss *et al.*, 2005). Staurosporine is an alkaloid produced by *Streptomyces staurospores* and it is considered as a potential suppressor of protein kinases C (Chae *et al.*, 2000). Apoptosis, also called programmed cell death, is a form of cell death first described by Kerr and his colleagues (Kerr *et al.*, 1972). This pathway is characterised by the activation of a group of cysteine proteases termed caspases that results in a massive changes in morphological features of the dying cells such as cell shrinkage, nuclear chromatin condensation and extensive bleb formation which consequently become apoptotic bodies that are eliminated by phagocytic cells (Kerr *et al.*, 1972, Kerr, 2002, Paweletz, 2001, Restifo, 2000, Chae *et al.*, 2000, Elmore, 2007). DMSO is a toxic solvent which was also employed in this study. DMSO may cause cytotoxicity in monocytes that can be detected using Annexin-V staining (Galvao *et al.*, 2014). It has been reported that DMSO can interact with the phospholipid membrane and at a high concentration and long-term exposure can dissolve lipid molecules leading to pore formation, disintegration of the membrane structure and irreversible cell damage (Da Violante *et al.*, 2002, Gurtovenko and Anwar, 2007, Marrink *et al.*, 2009).

4.2 Hypothesis

ETA is the most toxic of a wide array of the extracellular proteins secreted by *P. aeruginosa*. It may play a crucial role in the suppression the immune responses due to its negative impact on APCs viability and ultimately down regulation of the proliferative response and clonal expansion of T lymphocytes.

4.3 Aims

To determine if the presence of ETA is fundamental for *P. aeruginosa* pathogenicity by evaluating the effect of Pseudomonas supernatants on monocyte viability and T cell proliferation in the presence of supernatants from Δ*toxA* PAO1 deficient strain or its parental strain.

4.4 Materials and Methods

4.4.1 Analysis of the effect of *P. aeruginosa* supernatants on monocyte viability

Monocytes were prepared as described in section 2.4.7, re-suspended in RPMI complete medium supplemented with 50 ng/ml rhM-CSF at a density of 1.7 X 10^6 /ml and plated in duplicate onto 12 mm coverslips in 24 well plate at a density of 3.5 x 10^5 per well in a final volume of 300 µl. Monocytes were treated with supernatants (20 %, prepared in Costar flasks) from PAO1-L, PAO1-LΔ*toxR* and PAO1-LΔ*toxA*, and incubated at 37°C, 5% CO2 for 16 hrs. Negative control was untreated monocytes while positive controls were monocytes treated with either Dimethyl Sulfoxide (DMSO) (1:100) (Sigma D8418) or Staurosporine (5µM, Sigma S4400, diluted in DMSO).

The formation of apoptotic bodies from monocytes was determined by flow cytometry using Annexin-V-FITC based kit (Serotec Inc., ANNEX300F) with a slight modification to the manufacturer's instructions. Briefly, culture supernatants were transferred to FACs tubes, washed with PBS and re-suspended in 195 µl binding buffer. Later, the cells were labelled with 7µl Annexin-V-FITC for 15 minutes in the dark at room temperature. The labelled cells were then washed with 200 µl of binding buffer, centrifuged (350 x g, 10 minutes), re-suspended in 500 µl binding buffer and analysed using a Beckman Coulter FC500 flow cytometer.

For microscopic examination of adherent cells, the coverslips were washed twice with cold PBS, fixed with 4% formalin in PBS at 4°C for 10 min, stained using the Hemacolor staining kit (Merck Millipore) as per the manufacturer's

instructions, mounted and assessed using Nikon Diaphot 300 inverted microscope using spot software.

4.4.2 Analysis of the effect of *P. aeruginosa* supernatants on T cell proliferation

Fresh blood from healthy donors was used as a source of immune cells. PBMCs were purified as described in section 2.4.1 and re-suspended in RPMI complete medium. PBMCs were then labelled with CFSE, washed, re-suspended in the same medium at 2.1 X 10^6 PBMCs/ml and plated in a sterile 96 well plate U shaped bottom (see section 2.4.3).

PBMCs were treated with PAO1 supernatants (prepared as described in Section 2.2) (20% (v/v)) from PAO1-L, PAO1-LΔ*toxR*, PAO1-LΔ*toxA*, PAO1-LΔ*Bif* and PAO1-LΔ*rhlA* in the presence of either 10ng/ml of SEB or the mitogen phytohaemagglutinin (PHA, 2%, Invitrogen, UK),) in a final volume of 100 µl. PAO1-LΔBif and PAO1-LΔrhlA strains had been generated previously by members of the Quorum Sensing Group, University of Nottingham.

After 5 days incubation at 37°C, 5% CO_2, PBMCs were transferred to FACS tubes, washed, and labelled for T cell surface markers with conjugated primary antibody and appropriate isotype-matched controls: CD3-PC5 (Beckman-Coulter, IM2635U) alone or in combination with either CD4-ECD (Beckman-Coulter, 6604727) or CD8-ECD (Beckman-Coulter, 737659) as described in Section 2.4.3.

Samples were analysed using a Beckman Coulter FC500 flow cytometer within one week of staining and 40,000 events were collected depending upon the initial cell number. Data were processed using Weasel v3.0.1.

4.5 Results

4.5.1 The presence of ETA in PAO1 supernatants has a negative impact on monocyte viability

As the objective of this study was to assess the importance of ETA in *P. aeruginosa* cytotoxicity, a Δ*toxA* deficient strain in the highly virulent PAO1-L background was generated. This approach would determine whether ETA dominates the cytotoxic effect of PAO1-L supernatants on monocytes. To achieve this objective, supernatants from PAO1-L, Δ*toxA* and Δ*toxR* were prepared and tested for their effect on monocyte viability using Annexin staining.

Flow cytometric analysis revealed that 16 hrs exposure to either staurosporine or PAO1-L supernatant resulted in significant reduction of cell viability. The number of viable monocytes also decreased upon stimulation with PAO1-LΔ*toxR* (less ETA production), PAO1-LΔ*toxA* (ETA-) or DMSO at the required time point but this effect was not statistically significant. Interestingly, even untreated monocytes were poorly viable. Reduced monocyte viability could be attributed to the fact that monocytes are fragile and appeared to be donor-dependent.

The cytotoxicity of ETA on human monocytes was confirmed by the microscopic examination using Hemacolor Staining Kit. There was a marked loss of adherent monocytes after incubation with 20% PAO1-L supernatant (ETA+) compared to either PAO1-LΔ*toxR* (low ETA production) or PAO1-LΔ*toxA* (ETA-). This observation suggests that the ability of PAO1-L

supernatants to impact on monocytes viability is dependent on the presence of ETA (Figure 4.1).

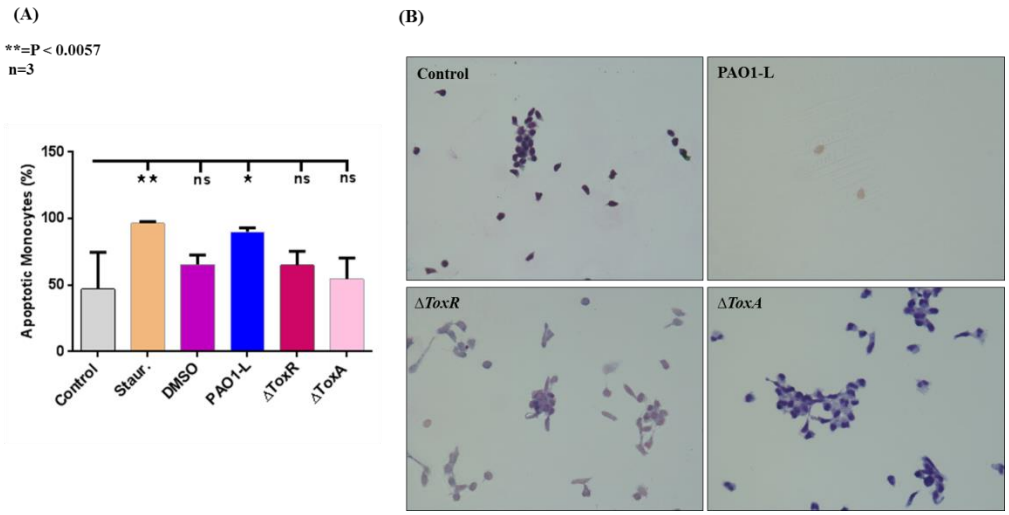

Figure 4.1: Altered monocyte viability after exposure to PAO1-L supernatant in comparison to supernatants from PAO1-LΔ*toxR* or PAO1-LΔ*toxA*.
A & B. Monocytes were treated with 20% PAO1-L, PAO1-LΔ*toxR* or PAO1-LΔ*toxA* supernatant (prepared in Costar flasks) for 16 hrs. As a positive control, monocytes were treated with staurosporine (5µM). DMSO was included in this study as because it was used to dilute stausporine. Untreated monocytes were used as a negative control. To facilitate comparison among experiments apoptotic monocytes in response to PAO1 supernatants were normalised to the level of staurosporine-induced cell death (100% death). One-way ANOVA and post-hoc Dunnett multiple comparison tests were used to calculate the significance. Data were obtained from three independent experiments.

4.5.2 The presence of ETA in PAO1 supernatant has a negative impact on T cell proliferation

In order to determine if the down regulation of T cell proliferation depended on the negative impact of ETA on the APC present in the PBMC cultures, the T cell proliferative response to SEB stimulation was evaluated in the presence or absence of ETA in PAO1 supernatants. PBMCS were purified, labelled with CFSE (see Section 2.4.3), stimulated with SEB (10 ng/ml) in the presence of 20% PAO1 supernatants (prepared in Costar flask) including: PAO1-L

(ETA+), PAO1-LΔ*toxR* (low ETA) or PAO1-LΔ*toxA* (ETA-) and incubated for 5 days. PBMCs were labelled with T cell surface markers and the number of the proliferated T cell was analysed by flow cytometry.

Preliminary observations revealed that upon stimulation with SEB the number of proliferated CD3+ T cells was drastically reduced in the presence of PAO1-L supernatant while supernatants obtained from either PAO1-LΔ*toxR* or PAO1-LΔ*toxA* strain exhibited a minor effect (Figure 4.2). The findings confirm the previous observations that the effect of PAO1-L on T cell division correlates with the activity of *toxR* regulatory gene.

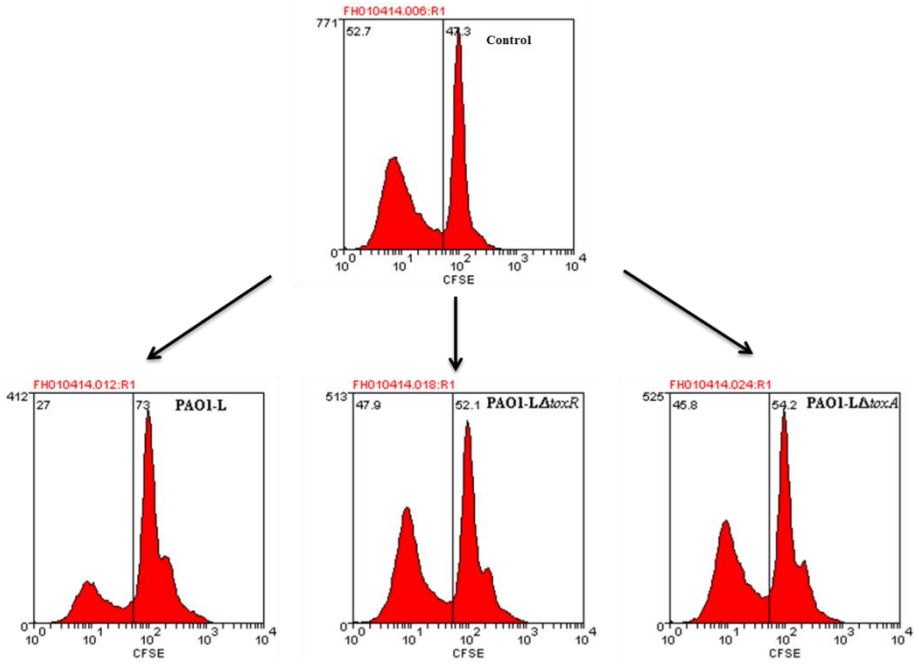

Figure 4.2: The proliferative response of CD3 T lymphocyte in the presence or absence of ETA in PAO1 supernatants.
CFSE dilution in the presence of 10ng/ml SEB alone (control) or in combination with of PAO1 supernatants (20% (v/v)) including PAO1-L, PAO1-LΔ*toxR* and PAO1-LΔ*toxA* for 5 days. This data is from a single experiment.

Although the SEB was the main inducer used to promote T cell clonal expansion, it was noticed that the number of the proliferated T cells in response to SEB began to diminish. New preparations of SEB and different

concentrations (10, 20, 30) ng/ml were tested but the robust T cell proliferation consistently observed previously (see chapter 2) was never recovered (Figure 4.3). The cause for this poor SEB-induced T cell proliferation is still unknown.

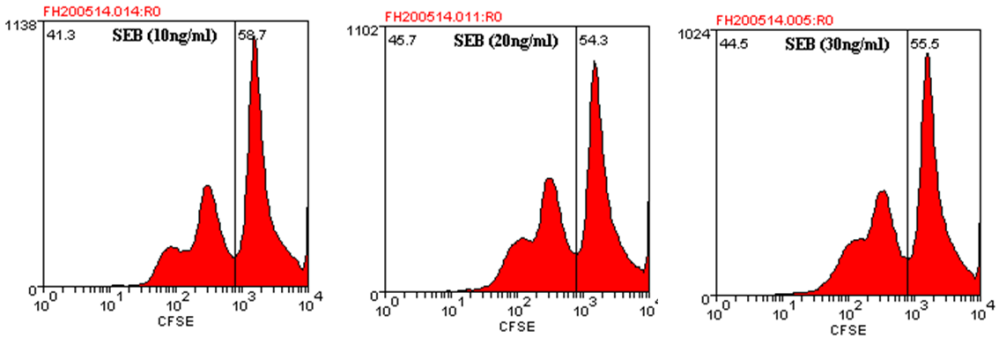

Figure 4.3: Testing the proliferative response of PBMCs upon stimulation with different SEB preparations and doses.
Purified PBMCs were plated in a sterile U-shape bottom 96-well plate (1.5 X 10^5 PBMCs in 70µl),) and treated with different SEB concentrations prepared in RPMI complete media in a final volume of 100 µl for 5 days. Data were obtained from a single experiment.

To determine if results observed with SEB could be reproduced with a different stimulus, PBMC cultures were stimulated with the mitogen PHA (2%) in the presence and absence of PAO1 supernatants (20% (v/v)).

According to the flow cytometric analysis (n=3) (Figure 4.4A & B), a marked decrease in either PHA-induced CD3+ or PHA-induced CD3+/CD4+ T cell proliferation was observed in the presence of PAO1-L supernatant (20%, v/v) compared to PHA stimulation alone. This effect was also observed in the case of CD3+/CD8+ T cells (Figure 4.3C) but the difference was not significant. Supernatants from PAO1-LΔ*toxR* and PAO1-LΔ*toxA* had a minor effect. Of particular interest for this study, full T cell proliferation was not recovered in the presence of PAO1-LΔ*toxA* supernatants. Interestingly PAO1-N supernatants, as in the case of SEB-induced proliferation, did not affect PHA-induced T cell proliferation.

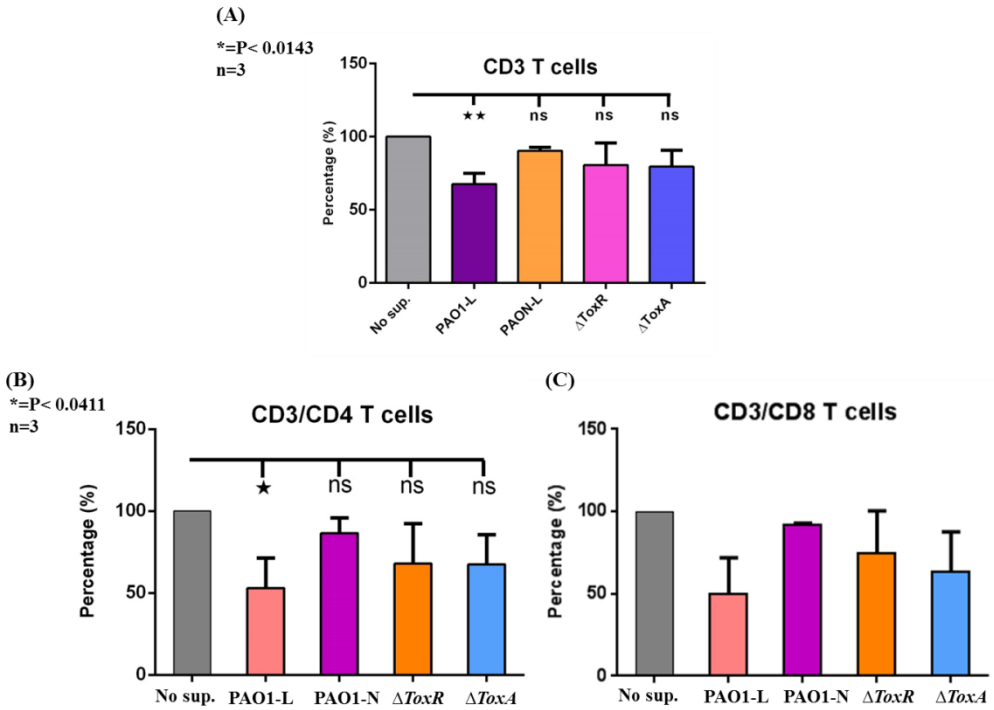

Figure 4.4: Comparison the proliferative response of PHA-induced T cells in the presence of different PAO1 supernatants.
CFSE-labelled PBMCs were stimulated with PHA (2%) alone or in combination with PAO1-L, PAO1-N, PAO1-LΔ*toxR* or PAO1-LΔ*toxA* supernatant (20%, Costar flask). PHA-treated T cells were used as a positive control. Graphs show CD3 **(A),** CD3/CD4 **(B)** and CD3/CD8 **(C)** T cell proliferation in response to PAO1 supernatants normalised to the level of PHA-induced proliferation. One-way ANOVA and post-hoc Dunnett multiple comparison tests were used to calculate the significance. Data were obtained from three independent experiments.

Based on these observations, further studies were performed using preparations of supernatants from PAO1-LΔ*Bif* and PAO1-LΔ*rhlA* mutant strains. A rhamnolipid-deficient mutant strain in the PAO1-L background (PAO1-LΔ*rhlA*) was included because rhamnolipid is a potent bio-surfactant with a wide range of potential roles during *P. aeruginosa* infection (Beal and Betts, 2000, Caiazza *et al.*, 2005). Furthermore, the Quorum Sensing group found that the expression of this surfactant is controlled by *toxR*, suggesting that rhamnolipids might be involved in the attenuation of PAO1-LΔ*toxR* cytotoxicity. The *Bif* gene is another genetic element missing in PAO1-N (the

role unknown) and it was used in this study as PAO1-N strain is less cytotoxic than PAO1-L and it is possible *Bif* might contribute in PAO1-L pathogenicity.

Our data demonstrated a significant decline in the percentage of the proliferated CD3 T cells after stimulation with PAO1-L, PAO1L-LΔ*rhl* or PAO1-LΔ*Bif* supernatants. However, the negative effect was reduced in the case of PAO1L-LΔ*rhl* (31% inhibition) compared to either PAO1-L or PAO1-LΔ*Bif* strains which caused approximately 50 % and 54.3% inhibition respectively (Figure 4.5).

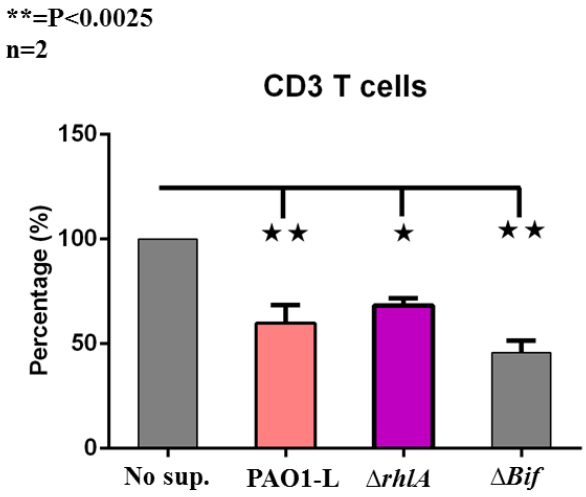

Figure 4.5: Comparison of the inhibitory effect of different PAO1 supernatants on PHA-treated CD3T cells.
CFSE-labelled PBMCs were treated with PHA (2%) alone or in the presence of PAO1-L, PAO1L-LΔ*rhl* or PAO1-LΔ*Bif* supernatant (20%, Costar flask) for 5 days (two independent experiments) and normalised to the level of PHA-induced proliferation. Positive control was treated with PHA alone. Statistical significance was calculated by one-way ANOVA and post-hoc Dunnett multiple comparisons test.

4.6 Discussion

The present study aimed to investigate the role of ETA secreted by *P. aeruginosa* on monocyte viability and consequently the proliferative response of T lymphocytes. Although a number of studies have previously demonstrated the impact of purified ETA from *P. aeruginosa* on immune cells (Michalkiewicz *et al.*, 1999, Michalkiewicz *et al.*, 1998, Patzer *et al.*, 1989b, Holt and Misfeldt, 1984, Pollack, 1983, Laithwaite *et al.*, 1999), there are limited studies that assess the importance of ETA among other effector molecules in bacterial supernatants using an unbiased approach .

ETA is an important virulence factor secreted by *P. aeruginosa* (Liu, 1973, Liu *et al.*, 1973). ETA-producing strains are more toxic than non-producing counterparts, and the cytotoxicity of these strains could be attenuated by neutralising this toxin using α-ETA antibody (Kohzuki *et al.*, 1993). Furthermore, samples obtained from CF patients exhibited elevated titres of α-ETA antibody, probably due to frequent exposure to the ETA antigen. Thus, the high level of α-ETA associates with a high rate of mortality in CF patients (Jaffar-Bandjee *et al.*, 1995, Klinger *et al.*, 1978, Moss *et al.*, 1986, Gallant *et al.*, 2000).

In this study, the superantigen SEB and the mitogen PHA were used to trigger T cell proliferation. SEB is produced by *S. aureus* and can activate up to 20-30% T cells mainly CD4+ T cells (Herman *et al.*, 1991). While PHA, an extract from the red bean *Phaseolus vulgaris*, is widely used to support T cell clones. PHA activity largely depends upon external signals provided by other cells such as monocytes in the surrounding milieu (Movafagh *et al.*, 2011, Ceuppens *et al.*, 1988, Katzen *et al.*, 1985).

Our data showed an increased generation of apoptotic monocytes upon stimulation with PAO1-L supernatant and indicate that *Pseudomonas aeruginosa* cytotoxicity on monocytes correlates with high expression of ETA as both PAO1-L*toxR* and PAO1-L*toxA* are less toxic than the wild type. This would be in agreement with previous work demonstrating that expression of the ETA receptor CD91, is highly restricted to monocytes (Moestrup *et al.*, 1992). Furthermore, this toxin is able to initiate apoptotic signalling cascade in a wide range of eukaryotic cells such as hepatocytes and mast cells leading to cell death by triggering production of TNFα by macrophages, and the activation of either perforin, Fas or caspase pathway (Schumann *et al.*, 1998, Park *et al.*, 2010, Jenkins *et al.*, 2004, Usher *et al.*, 2002). However, our data revealed that the untreated monocytes showed variations in the viability even in the presence of M-CSF which might be dependent on the donor. Furthermore, an attempt to increase monocyte survival was being carried out in our group by another PhD student, using other media and different amounts of serum (personal communication) but not consistent results were observed.

Initial studies into the effect of PAO1-L supernatant on T cell proliferation revealed a negative impact on the proliferative response of T cell lymphocytes (Figure 4.2). However, the SEB ability to stimulate T cell expansion was lost later during the project with the reason for this still unknown. To overcome this problem, PHA was employed in this study as a promoter of T cell division (Movafagh *et al.*, 2011).

According to work by Michałkiewicz et al, ETA caused inhibition of PHA-treated lymphocyte proliferation in a dose-dependent manner (Michalkiewicz *et al.*, 1989). Furthermore, the toxic effect was dependent on a sequence of

events starting with the internalisation of this toxin by APCs, mainly monocytes, induction of cell death and eventually suppression of T cell division (Patzer *et al.,* 1989a, Katzen *et al.*, 1985, Issekutz and Stoltz, 1985). These findings suggest that the presence of monocytes is crucial to mediate the inhibitory effect of ETA on T cell expansion (Issekutz and Stoltz, 1985). However, this study suggests that although ETA is essential for monocyte toxicity in response to *P. aeruginosa* supernatants, it is only partially responsible for the reduction in PHA-induced T cell proliferation caused by PAO1-L supernatants. Full T cell proliferation in response to PHA was not fully recovered in the absence of *toxA, rhlA* or *toxR*; in all instances there is less T cell proliferation compared to control cultures but levels of proliferation are higher than when wild type supernatants were used.

PAO1-N supernatants did not affect PHA-induced T cell proliferation. *Bif* is one of genetic differences between PAO1-N and PAO1-L but does not seem to play a role in this effect as Δ*bif* supernatants inhibited PHA-induced T cell proliferation as strongly as wild type PAO1-L supernatants.

These results may be partly explained because full PHA-induced T cell proliferation needs soluble factors such as IL-2 supplemented by accessory cells and the viability of monocytes is not crucial (Ceuppens *et al.*, 1988, Greaves *et al.*, 1974, Katzen *et al.*, 1985). Thus, the response of PHA-treated PBMCs cultures showed the same trend as SEB-treated cultures although the contribution of ETA is less dominant Previous work employed a wide number of *P. aeruginosa* strains such as PA-103 (Bjorn *et al.*, 1978, Raivio *et al.*, 1994, Lory, 1986, Brandhuber *et al.*, 1988), PA- 4384 (Gallant *et al.*, 2000) and

PAO1 (Barton *et al.*, 1996, Hassett *et al.*, 1996, Wretlind *et al.*, 1987, Pillar and Hobden, 2002) in both in-vitro and in-vivo studies.

In conclusion, this work indicates that ETA dominates the cytotoxic effect of PAO1-L on monocytes but how this translates to an effect on T cell proliferation might depend on the agent used to stimulate the cultures.

5. Cross-kingdom interactions: *Candida albicans* and *P. aeruginosa*

5.1 Introduction

Microbial communities associated with infections comprise a mixed population of microorganisms that are derived from miscellaneous ecological niches and compete for nutritional resources and space in their habitat (Hibbing *et al.*, 2010). *P. aeruginosa* and *Candida albicans* are the most frequent pathogens co-isolated from tracheobronchial samples obtained from CF and intensive care unit patients (Delisle *et al.*, 2008, Mear *et al.*, 2013).

C. albicans is a commensal fungus of humans that inhabits different body sites of most healthy individuals, such as the skin and oral cavity (Bernhardt *et al.*, 2001, Rosenbach *et al.*, 2010, Thein *et al.*, 2006, Mayer *et al.*, 2013). While it is a member of the human microflora, *C. albicans* is an opportunistic pathogen with the potential to cause life-threatening systemic infections, particularly in CF immuno-compromised patients (Ruhnke, 2006).

The pathogenicity of *C. albicans* relies on a wide array of fitness traits (Mayer *et al.*, 2013, Brown *et al.*, 2014, Brown *et al.*, 2007, Butler *et al.*, 2009, Nikolaou *et al.*, 2009). For instance, *C. albicans* is a polymorphic fungus displaying reversible morphogenetic transitions between yeast, pseudohyphal and hyphal growth forms (Vautier *et al.*, 2015, Mayer *et al.*, 2013). It has been proposed that the morphological plasticity of *C. albicans* is a fundamental virulence factor that facilitates pathogenesis (Jacobsen *et al.*, 2012).

The first stage of *C. albicans* infection is mediated by the yeast form and it is facilitated by failure of host defences; hyphal formation appears to be important in fungal invasion throughout the host tissues and causes opportunistic infections (Saville *et al.*, 2003, Berman and Sudbery, 2002, Sherwood *et al.*, 1992, Brand, 2012, Gow *et al.*, 2002). In this context, *C. albicans* hyphae can invade host cells by either endocytosis or active penetration. Endocytosis depends on the expression of proteins on the outer surface (termed adhesins) which ligate with defined host cell ligands, i.e. E-cadherin on epithelial cells and N-cadherin on endothelial cells, eventually inducing endocytosis (Phan *et al.*, 2007, Phan *et al.*, 2005, Mayer *et al.*, 2013). In contrast, factors that are required for the hyphae penetration route are still largely unknown (Mayer *et al.*, 2013).

It has been found that a variety of environmental signals can control *C. albicans* morphology. For example: in acidic environs where the pH is less than 6, yeast cells dominate *C. albicans* growth; whilst with neutral to alkali pH, i.e. greater than 7, result in morphological transitions into the hyphal growth form (Douglas, 1988). It has been proposed that after hyphal invasion of the host, *C. albicans* can produce a plethora of secreted products with hydrolytic activity, such as proteases, phospholipases and lipases, which contribute to both effective penetration of the host tissues and nutrient acquisition from the surrounding environment (Wachtler *et al.*, 2012, Naglik *et al.*, 2003). However, *C. albicans* cells can also communicate via the production of farnesol, a QS molecule, which when above threshold concentrations is able to block the morphologic transition from yeast to hyphae (Ramage *et al.*, 2002, Hornby *et al.*, 2001, Lu *et al.*, 2014).

Furthermore, the filamentous form of the hyphae growth is known to play a significant role in biofilm establishment, which is considered to be a primary feature of *C. albicans* pathogenicity, and is associated with an increased resistance to anti-fungal medications (Brand, 2012). Mature biofilms comprise a dense network of yeasts and hyphae embedded within an exopolymeric material (Ramage *et al.*, 2001). Thus, *C. albicans* represents the fourth cause of hospital-acquired bloodstream infections and death in a majority of infected individuals (Brown *et al.*, 2014). In addition, extensive studies have demonstrated that *C. albicans* is able to adjust to physiological changes in the surrounding microenvironments by utilizing specific strategies dependent on the recruitment of particular signalling pathways which lead to stress adaptation within the local host (Chauhan *et al.*, 2006, Alonso-Monge *et al.*, 2009, Brown *et al.*, 2009, Miramon *et al.*, 2012). These strategies are mediated by the activation of a battery of genes and signalling pathways which induces the transcription of a target protein such as heat shock proteins (HSPs) and mitogen-activated protein (MAP) kinase pathway that promote *C. albicans* survival under stress conditions (Brown *et al.*, 2014, Mayer *et al.*, 2013).

Bacterial-fungal interactions are inherently of significant importance from both environmental and medical viewpoints. *P. aeruginosa* and *C. albicans* are typical opportunistic respiratory tracts organisms and their pathogenic interactions have been reported in several studies (Delisle *et al.*, 2008, Mear *et al.*, 2013). However, the nature of the relationship between these two pathogens remains unclear.

A number of studies showed that the interaction between *P. aeruginosa* and *C. albicans* is likely to be antagonistic both *in vivo* and *in vivo* (Kerr, 1994, Hogan

and Kolter, 2002, El-Azizi *et al.*, 2004, Kerr *et al.*, 1999). It has also been demonstrated that *P. aeruginosa* has the ability to kill *C. albicans* via the formation of a compact biofilm on the hyphae growth form, whilst the yeast form is resistant to *P. aeruginosa* killing, as the bacterial cells are unable to adhere to the yeast-form *C. albicans* cells (Hogan and Kolter, 2002). The selective binding of *P. aeruginosa* to the filamentous form of *C. albicans* correlates well with variations in the cell wall components of the yeast and hyphae growth forms (Chaffin *et al.*, 1998). In general, the cell wall of *C. albicans* is mainly composed of carbohydrates. In this context, work by Ovchinnikova and co-workers found that a chitin-binding protein expressed by *P. aeruginosa* has a fundamental role in the physical binding to *C. albicans* hyphae and a chitin-binding protein deficient strain displayed markedly less adhesion ability compared with the wild type (Ovchinnikova *et al.*, 2013).

Furthermore, it has been reported that the yeast *C. albicans* is common in commensal interactions whilst the hyphal mode of growth has been implicated in virulence. The 3-oxo-C12-HSL QS molecule produced by *P. aeruginosa* is able to repress *C. albicans* filamentation process which is a prerequisite for fungal pathogenicity (Hogan *et al.*, 2004). In addition, different phenazines produced by *P. aeruginosa*, such as pyocyanin and 1-hydroxyphenazine also have a negative influence on *C. albicans* viability (Kerr, 1994, Kerr *et al.*, 1999, Gibson *et al.*, 2009).

It has been proposed that both organisms might compete for host cell receptors: carbohydrates that occur as ‘mannoproteins’ are expressed as thin filamentous surface structure on the fungal cells and it is likely that lectins expressed by *P. aeruginosa* might bind to these carbohydrates, inhibiting *C. albicans* ability to

ligate with corresponding substrates and the subsequent colonisation and the establishment of a fungal infection (Chaffin *et al.*, 1998, Yu *et al.*, 1994b). Furthermore, these appendages are composed of 85% carbohydrate and 15% protein (Yu *et al.*, 1994a) and is essential for *C. albicans* adherence to glycolipids, such as asialo-GM1, on the host cell surface such as epithelial cells (Yu *et al.*, 1994b, Foldvari *et al.*, 2005, Vitkov *et al.*, 2002).

Interestingly, a recent study, by Purschke and co-workers, revealed the production of multiple virulence factors by *P. aeruginosa* is significantly increased during co-culture with *C. albicans*; for example ETA, which has a potential role in bacterial pathogenicity and immuno-suppression within the eukaryotic host (Purschke *et al.*, 2012).

5.2 Hypothesis

P. aeruginosa and *C. albicans* commonly exist together in mixed infections *in-vivo* with an antagonistic relationship. Exotoxin A (ETA) might provide an advantage *to P. aeruginosa* in competition with *C. albicans* as ETA production is up-regulated in the presence of *C. albicans.*

5.3 Aims

The purpose of this study was to develop an assay to study the impact of *P. aeruginosa* secreted products, predominantly ETA, on the growth mode of *C. albicans*. ETA is the most damaging virulence factor produced by *P. aeruginosa* and is responsible for cell death by blocking protein translation in eukaryotic cells.

5.4 Materials and Methods

5.4.1 Analysis of the impact of different amounts of PAO1 supernatants on *C. albicans* growth

In an attempt to assess the impact of *P. aeruginosa* secretions on *C. albicans* growth pattern, different concentrations of PAO1 supernatant (20, 40 and 80%) prepared in X-Vivo 15 using Costar flasks were used (see chapter 2, section (2.2)). In this work PAO1-L and ETA-deficient (PAO1-LΔ*toxA*) strains were employed. To determine whether other key molecules secreted by *P. aeruginosa* could affect *C. albicans* growth, a number of mutant strains in the PAO1-Lausanne background were also used in this study including: pyocyanin-deficient (Δ*phenazine*), 58-Kb chromosomal deletion (PAAMP1), *toxR*-deficient (Δ*toxR*), rhamnolipid-deficient (Δ*rhlA*) and *Bif*-deficient (Δ*Bif*). Single colonies of *C. albicans* (SC53) were cultured overnight (kindly provided by Simon Avery, UoN) on Yeast Extract Peptone Dextrose agar (YEPD) (Yeast extract (10g/L) OXOID, Bacteriological peptone (20g/L) OXOID, Agar (20g/L) Sigma). Single colonies were used to inoculate 10 ml YEPD broth (20% glucose), and incubated for 18 hrs at 37ºC with agitation (200 rpm). The following day, the growth was diluted to OD_{600} =0.5 and a new culture was re-started in 10 ml YEPD and incubated for a further 4 hrs under the same conditions. At the required time, the optical density (OD_{600}) of the culture was adjusted to 2 in the same medium. Aliquots (15 µl) of the culture were then transferred to 48-well plates (Greiner Bio-One, Monroe, NC) in duplicate and adjusted to a final volume of 300 µl and OD_{600}=0.1 as follows:

Control: 45 µl YEPD broth (2.33x) + 240 µl X-Vivo 15.

80% PAO1 supernatant: 45 µl YEPD broth (2.33x) + 240 µl sup.

40% PAO1 supernatant: 45 µl YEPD broth (2.33x) + 120 µl sup. + 120 µl X-Vivo 15

20% PAO1 supernatant: 45 µl YEPD broth (2.33x) + 60 µl sup. + 180 µl X-Vivo 15.

The plate was then incubated at 37ºC for 24 hrs with shaking in a Bioteck PowerWave XS microplate spectrophotometer.

5.4.2 Microscopic examination of *C. albicans* growth in response to PAO1 supernatants

For microscopic examination, 200 µl of *C. albicans* growth from each condition was harvested in Eppendorf tubes, washed twice with PBS, re-suspended in 5 µl PBS, placed on a glass slide and analysed by collecting 33 random images for each condition using 1000 x magnification. Statistical analysis was done by counting number of squares containing hyphae out of 99 total squares in 33 random images per condition. One-way ANOVA and post-hoc Dunnett multiple comparison tests were used to calculate degree of significance.

5.5 Results

5.5.1 *P. aeruginosa* alters the growth of *C. albicans* under defined experimental conditions

Microbial communities associated with infections such as pneumonia and CF often consist of pathogens from various microbiota such as *C. albicans* and *P. aeruginosa* (Hibbing *et al.*, 2010). Thus, this study investigated the growth of *C. albicans* in the presence of different amounts of *P. aeruginosa* secreted products. *C. albicans* was grown and plated on 48 well-plates as described above. Different amount of bacterial supernatants prepared from PAO1-L and its mutant strains were then added. The optical density of the grown *C. albicans* was measured every 30 min for 24 hrs.

Based on the growth curves observed (Figure 5.1) PAO1-L supernatants reduced the growth of *C. albicans* in a dose dependent manner and this effect is independent of the production of ETA, rhamnolipid, pyocyanin, or the activity of either the *Bif* or *toxR* genes; *toxR* promotes the expression of both ETA and rhamnolipid. However, in the presence of supernatants from the PAAMP1 strain (mutant with 58 Kb deletion), *C. albicans* appears to grow as in control wells.

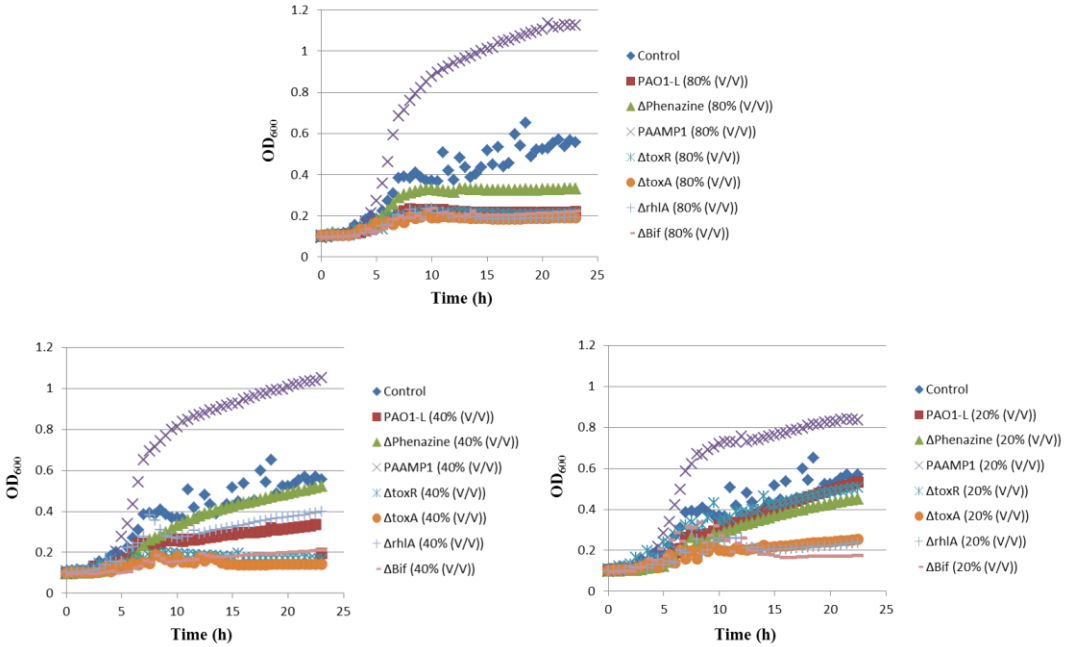

Figure 5.1: *C. albicans* growth changed upon incubation with different amounts of *P. aeruginosa* supernatants.
A single colony of *C. albicans* was grown in YEPD broth for 18 hrs at 37ºC with agitation. The OD_{600} was then adjusted to 0.5 and incubated for an extra 4 hrs under the same conditions. Aliquots of *C. albicans* growth (OD_{600}=2) were transferred to 48-well plates and treated with 80%, 40% and 20 % (v/v) supernatants from different strains including: from PAO1-L, Δ*phenazines* PAAMP1, PAO1-*LΔtoxR.* PAO1-*LΔtoxA*, PAO1-*LΔrhlA* and PAO1-*LΔBif* to a final OD_{600}=0.1. Control was treated with X-vivo-15. Data presented are from one representative experiment of 5.

Remarkably, the growth of untreated *C. albicans* (control) varied between experiments. (Figure 5.2). These variations might be associated with changes in a number of parameters used in this study such as culture age and medium preparation. In spite of this robust growth of *C. abicans* was consistently observed in the presence of PAAMP1 supernatants compared to wt and other mutant strains.

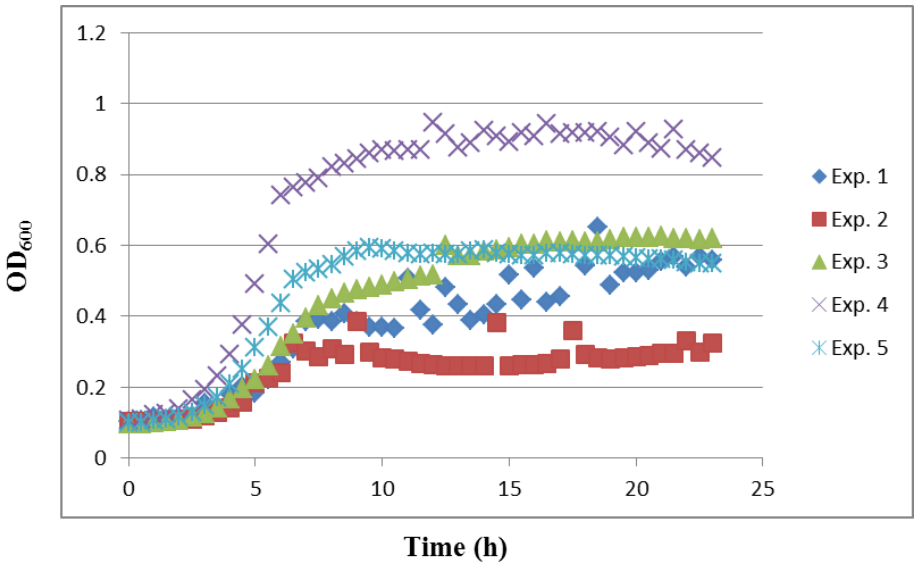

Figure 5.2: The growth of untreated *C. albicans* (control) varied among experiments.
A single colony of *C. albicans* was grown in YEPD broth for 18 hrs at 37ºC with agitation. The OD_{600} was then adjusted to 0.5 and incubated for an extra 4 hrs under the same conditions. Aliquots of *C. albicans* growth (OD_{600}=2) were transferred to 48-well plates and cultured in the presence of 80% X-Vivo 15.

5.5.2 Extensive formation of hyphae is associated with the variations in *C. albicans* growth

The induction of hyphal growth is proposed to be beneficial for bacterial predominance in the microenvironments as *P. aeruginosa* is able to bind to the filamentous cells and kill *C. albicans* (Hogan and Kolter, 2002). The next stage of the study was to determine whether the observed change in *C. albicans* growth was related to changes in morphology, as *C. albicans* has the ability to grow to either as budding yeast or hyphae (Vautier et al., 2015, Mayer et al., 2013). Microscopic analysis of *C. albicans* growth (Figure 5.3) revealed that in the presence of either PAO1-L or PAO1-LΔ*toxR* supernatants there was a morphological shift to hyphal growth. Conversely, *C. albicans* treated with supernatants obtained from PAAMP1 typically grew as yeasts with hyphae

rarely observed in these cultures. Morphological switching to hyphal growth is consistent with the proposal that *P. aeruginosa* might dominate in a complex microbial community, as bacterial cells exhibit anti-fungal activity via their ability to attach and kill *C. albicans* filamentous form (Shareck and Belhumeur, 2011).

These observations suggest that the genetic element which is responsible for either the expression or regulation of the molecule that promotes hyphal growth might be located in 58 Kb region lacking in the PAAMP1 mutant.

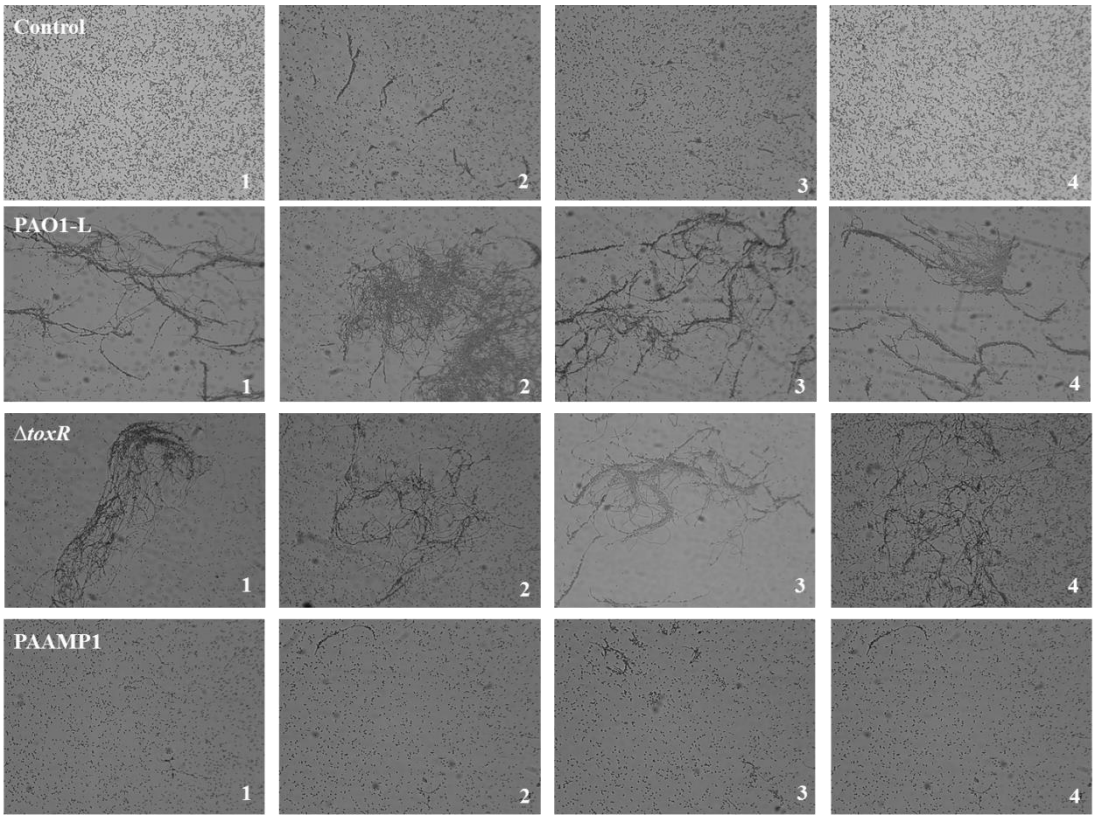

Figure 5.3: Alterations in *C. albicans* growth associate with an increased hyphae formation.
C. albicans was grown in YEPD broth for 18 hrs at 37ºC with agitation. The growth was then diluted to OD_{600} =0.5 and incubated for further 4 hrs under the same conditions. 15 µl aliquots of *C. albicans* growth (OD_{600}=2) were then plated on 48-well plates and stimulated with 80% (v/v) supernatants from PAO1-L, PAO1-*LΔtoxR* and PAAAMP1. The growth was then transferred into Eppendorf tubes and washed twice with PBS and analysed using a GX microscope L3201-LED. Control was untreated with X-vivo 15. Magnification = 1000x

Further statistical analysis of hyphal growth (Figure 5.4) revealed that the number of hyphae significantly increased upon stimulation with either PAO1-L or PAO1-LΔ*toxR* supernatants compared to untreated control. However, there is no significant difference in hyphal growth between untreated and PAAMP1-treated fungus, suggesting that the impaired bacterial ability of *P. aeruginosa* to induce hyphal formation in *C. albicans* is due to the 58 Kb deletion in the PAAMP1 strain.

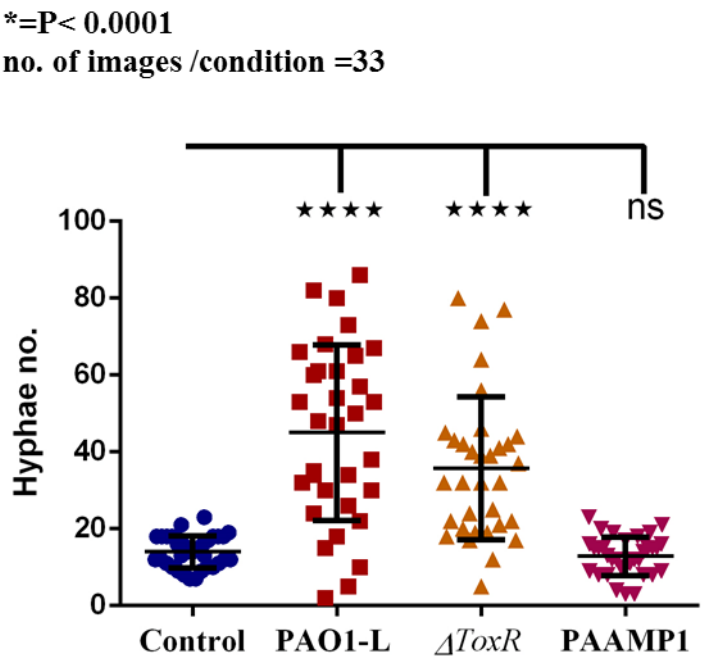

Figure 5.4: 58 Kb deletion in *P. aeruginosa* repressed bacterial ability to promote hyphal growth in *C. albicans.*
C. albicans was grown on 48 well plate and treated with bacterial supernatants (80% (v/v)) obtained from PAO1-L, PAO1-L*ΔtoxR* and PAAAMP1 (has 58 Kb deletion in its chromosome) and incubated for 24 hrs at 37ºC with agitation (200 rpm). Control was treated with X-vivo 15. The cultures were then harvested, washed twice with PBS and examined using GX microscope L3201-LED. The number of hyphae was calculated by counting squares containing hyphae out of 99 total squares in 33 random images per condition. One-way ANOVA and post-hoc Dunnett's multiple comparison tests were used to calculate significance. Magnification = 1000x.

5.6 Discussion

It is well established that a microbial communities are very sophisticated and comprise a diversity of micro-organisms, including commensals like *C. albicans* which encounter opportunistic pathogens such as *P. aeruginosa* (Shareck and Belhumeur, 2011, Pierce, 2005). *C. albicans* is a common member of human microbiota, however, in susceptible individuals it is responsible for a wide range of infections (Mayer *et al.*, 2013, Lopez-Ribot, 2005). Futhermore, *C. albicans* and *P. aeruginosa* are often co-isolated from lung samples obtained from CF patients (Navarro *et al.*, 2001, Bakare *et al.*, 2003, Valenza *et al.*, 2008).

The ability of *C. albicans* to exist in different morphological forms such as yeasts and hyphae contributes to its virulence (Vautier *et al.*, 2015, Mayer *et al.*, 2013). Whilst the morphological switch of *C. albicans* from yeast to hyphae is crucial for fungal pathogenicity, a wide spectrum of ecological factors may control this transition (Biswas *et al.*, 2007, Whiteway and Bachewich, 2007). Furthermore, *P. aeruginosa* attaches to and grows on the outer surface of hyphae resulting in the production of biofilms and the simultaneous elimination of *C. albicans* (Hogan and Kolter, 2002). It has been also demonstrated that *P. aeruginosa* exoproducts such as pyocyanins have a cytotoxic effect on *C. albicans* hyphae resulting in cell death (Kerr, 1994, Kerr *et al.*, 1999, Gibson *et al.*, 2009).

It has been proposed that in microbial communities, *P. aeruginosa* employs different strategies to promote its survival and dominance within these ecosystems. Thus, it was hypothesised that ETA may be an important signal produced by *P. aeruginosa* that might be responsible for the attenuation of *C.*

albicans growth, due to its ability to block protein synthesis in eukaryotic cells ultimately leading to cell death (Siegall *et al.*, 1989, Webb *et al.*, 2008). In order to determine the key molecule which mediates the *P. aeruginosa* effect on *C. albicans*, an ETA deficient strain and a number of other mutants in Lausanne background were used.

Our data revealed that *P. aeruginosa* supernatants altered *C. albicans* growth in a dose dependent manner (Figure 5.1). Furthermore, the microscopic images demonstrated that the changes in *C. albicans* growth correlate with increased hyphae formation (Fox *et al.*, 2013). Together, this suggests that *P. aeruginosa* secreted factors can shape and induce morphological transition of *C. albicans* from yeast to hyphae. Switching *C. albicans* to hyphal growth might be beneficial for *P. aeruginosa* as it preferentially attaches to and kills the hyphal form (Fox *et al.*, 2013, Hogan and Kolter, 2002).

Our results are in agreement with studies that showed *P. aeruginosa* remarkably attenuates *C. albicans* growth *in vitro* and in thermal injury patients (Gupta *et al.*, 2005, Kerr, 1994). In addition, using anti-bacterial agents to suppress *P. aeruginosa* infections in CF patients can result in an increased growth of *C. albicans* (Burns *et al.*, 1999).

However, the yeast form dominates *C. albicans* growth following stimulation with supernatants from the PAAMP1 mutant strain which contains a 58 Kb deletion in its genome. Genome analysis of the 58 Kb region showed that it comprises a wide number of potential genes which regulate the expression of remarkable virulence leading to increased pathogenicity of *P. aeruginosa* in the infected host (Llamas *et al.*, 2009). For instance, it contains a battery of genes

such as *hxc* (*U, P, X, Y, Z, and Q*) which are type II secretion system protein involved in alkaline phosphatase secretion (Llamas *et al.*, 2009).

Thus, it could be concluded that enhanced hyphal formation in *C. albicans*, promoted by *P. aeruginosa* secreted products does not correlate with the production of pyocyanins, ETA, rhamnolipids, or the activity of the regulatory genes *toxR* and *Bif*. However, the potential gene that encodes or regulates the active agent which is associated with hyphal induction in *C. albicans* might be located within a 58 Kb region and the deletion of this gene in the bacterial chromosome abrogate the ability of *P. aeruginosa* supernatants to switch the morphology of *C. albicans* from year to hyphae form.

6. General Discussion and Future perspectives

P. aeruginosa is a Gram-negative opportunistic pathogen that can interfere with immune defences and cause severe infections with a high death rate in susceptible individuals, like those with CF (Rogers *et al.*, 2005, Hauser *et al.*, 2011, Hart and Winstanley, 2002, Pedersen, 1992, Ciofu *et al.*, 2005, Al-Aloul *et al.*, 2004, Salunkhe *et al.*, 2005, Harrison, 2007). *P. aeruginosa* was one of the first bacterium associated with nosocomial infections; it displays multidrug resistance, leading to a significant increase in morbidity and mortality in infected patients (Obritsch *et al.*, 2005, Aloush *et al.*, 2006, Zavascki *et al.*, 2006, Lucena *et al.*, 2014, Defez *et al.*, 2004). The pathogenicity of *P. aeruginosa* is dependent on the expression of an array of potent virulence determinants that facilitate bacterial colonisation, invasion and eventually, the successful establishment of infections (Ben Haj Khalifa *et al.*, 2011, Hauser, 2011, Strateva and Mitov, 2011, de Bentzmann *et al.*, 2000). Secreted or surface-localised microbial agents are able to damage or negatively affect multiple cell types. For instance, ETA inhibits protein synthesis in monocytes, and loss of cell integrity may provide a mechanism by which *P. aeruginosa*, together with other microbial agents, establish infections in infected patients.

Multiple immune cell types play a fundamental role in orchestrating immune responses against *P. aeruginosa* infections. T lymphocytes play a cardinal role in shaping the immune system response; their activation rely on several factors such as the ligation between T cell receptors and peptide-MHC at the surface of APC (signal 1), co-stimulatory molecules (signal 2) and cytokine milieu

(signal 3) (Lanzavecchia, 1996, Banchereau and Steinman, 1998, Zietara *et al.*, 2014, Zhu and Paul, 2008, Magombedze *et al.*, 2013, Dong, 2006). There is evidence that T cells have the ability to generate a protective cell-mediated immunity against *P. aeruginosa* via clonal expansion, which increases cell numbers and the production of vital immuno-modulatory cytokines, such as pro-inflammatory IL-17A (Bayes *et al.*, 2014, Xu *et al.*, 2014, Priebe *et al.*, 2008). At the same time, the expression of surface receptors, such as TLR4, TLR5 and the mannose receptor on APCs such as monocytes and macrophages, also have a significant role in the recognition of *P. aeruginosa* and the subsequent generation of the pro-inflammatory response to infection (Xaplanteri *et al.*, 2009, Sun *et al.*, 2010, McIsaac *et al.*, 2012a).

The goal of this study was, to investigate the impact of *P. aeruginosa* secretome on T cells, in terms of cell expansion and viability, and to subsequently determine the key elements responsible for any observed effect. The aim was to use bacterial supernatants as a source of secreted virulence factors and PBMCs from healthy donors as a model system in which to study the human immune response (Clay *et al.*, 2001). It was considered that using PBMCs as a source of T cells would provide a more physiological model, which may better mimic the immune system, by taking into consideration the intercellular communication among various immune cells through soluble factors and cell-cell contacts (Sullivan et al., 2000, Schildberger et al., 2013, van't Wout et al., 2008). Furthermore, identifying such factors and their mechanism(s) of action would potentially enable the establishment of the key contributors to the pathogenesis of *P. aeruginosa* and provide a better

understanding of the molecular basis of its ability to modulate the immune system.

The immuno-modulatory effect of some *P. aeruginosa* exo-products on individual cell types has been extensively studied. For instance, it has been demonstrated that the production of pyocyanin induces neutrophil death via the apoptosis pathway *in vitro* (Prince *et al.*, 2008, Lau *et al.*, 2004). Furthermore, the expression of ETA can trigger non-apoptotic death in epithelial cells (Plotkowski *et al.*, 2002) and exert a negative impact on the phagocytic ability of both murine and human PMNs leading to the establishment of *P. aeruginosa* infection (Miyazaki *et al.*, 1995). Rhamnolipids impair the integrity of human epithelial cells and promote the infiltration of the *P. aeruginosa* into tissues (Zulianello *et al.*, 2006).

The PAO1 strain is the most common *P. aeruginosa* laboratory strain and is widely used in *P. aeruginosa* research (Klockgether *et al.*, 2010). Two sub-lines of PAO1 were employed throughout this study; PAO1 subline Nottingham (PAO1-N), and subline Lausanne (PAO1-L). Work by Klockgether and co-workers has shown that different sub-lines of PAO1 exhibit genomic variation from the original PAO1 obtained by Bruce Holloway in 1955 and from each other (Holloway, 1955, Klockgether *et al.*, 2010).

The PAO1 sub-line Lausanne (PAO1-L) has genetics similar to Holloway's first isolate and was used for this study. Work by the Quorum Sensing Group (Nottingham University) has revealed that the PAO1 strain used in the Nottingham laboratory (PAO1-N), among other changes, has a large chromosomal deletion (58 kb), including the *toxR* gene, which may reduce the virulence of this strain. The same group demonstrated that the transcriptional

product of the *toxR* gene is involved in both ETA (Hedstrom *et al.*, 1986) and Rhamnolipid production (unpublished work). The genetic variations between PAO1-N and PAO1-L provided an ideal tool to investigate the contribution of *toxR*-dependent secreted factors and other genetic elements missing in the PAO1-N genome to the ability of the *P. aeruginosa* secretome to modulate the behaviour of immune cells. Therefore, the T cell proliferative responses in the presence of PAO1-L or PAO1-N supernatants were compared (Chapter 2). Two types of polyclonal activators were initially used to stimulate T cells, the superantigen SEB and α-CD3 and α-CD28 antibodies. SEB is a powerful mitogen for both murine and human T cells. SEB is produced by *S. aureus* and activates T cells via the association of the Vβ chain of the T-cell receptor (TCR) with HLA class II molecules on APCs (El Hage *et al.*, 2010, Bisset *et al.*, 1993, Marrack and Kappler, 1990). To prepare bacterial supernatants, X-Vivo 15 (Lonza, UK), which is a serum-free tissue culture medium, was employed in this study in order to avoid any substances in the serum that might interfere with the bacterial behaviour. Furthermore, X-Vivo 15 appeared to be appropriate for the culture of *P. aeruginosa* as it supports bacterial growth satisfactorily (Singh *et al.*, 2015).

As T cells play a fundamental role in the development of the immune response to *P. aeruginosa* in both mice (Chapman *et al.*, 2009, Roy *et al.*, 2010) and humans (Hedstrom *et al.*, 1986, Iglewski and Kabat, 1975), T cell proliferation was evaluated in the presence of PAO1 supernatants (Chapter 2).

Our data showed a drastic reduction in the percentage of SEB-induced T cell division (total CD3+ and CD3+/CD4+ and CD3+CD8+ subsets) in to the presence of 20% PAO1-L supernatant while PAO1-N has a minor effect on this

proliferative response. α-CD3 and α-CD28 antibody were used to activate T cells in an APC-independent manner to assess whether the bacterial influence on T cell proliferation is mediated by direct interference with T cell function (Chapter 2). As the co-stimulatory receptor CD28, expressed by T lymphocytes, plays a cardinal role in T cell activation by providing an additional signal to TCR-associated CD3 (Damle *et al.*, 1983, Appleman *et al.*, 2000, Sharpe and Freeman, 2002, Fraser *et al.*, 1991, Lindstein *et al.*, 1989), stimulation was performed in the presence and absence of α-CD28 antibody. Interestingly, our results demonstrated that there are no significant differences in the proliferation of α-CD3/CD28-stimulated T cell and their subpopulations in the presence of 20% of either PAO1-L or PAO1-N supernatants.

We tested if reduction in the number of proliferated T cells in the presence of PAO1-L supernatants, might be caused by the direct cytotoxic effect of *P. aeruginosa* supernatants on T cells (Bruno *et al.*, 2000, Epelman *et al.*, 2002, Bruno *et al.*, 1998, Mody *et al.*, 1995). Thus, Annexin-V was used to detect apoptotic bodies based on the presence of exposed phosphatidylserine (PS) (Brumatti *et al.*, 2008, Vermes *et al.*, 1995, Zhang *et al.*, 1997b) (Chapter 2). Results confirmed that PAO1-L supernatant has no lethal impact on T lymphocytes compared to staurosporine-treated cells suggesting that the *P. aeruginosa* supernatant might exert a direct negative effect on other cells and subsequently impair T cell proliferation.

In light of this, monocytes were the first candidate as they are APCs and give rise to DCs which are a professional APCs. These cells play a key role in processing and presenting foreign antigen on MHC molecules (Paquette *et al.*, 1998) which engages TCR on T cells and triggers cell proliferation and

activation (Smith-Garvin *et al.*, 2009, Steinman and Hemmi, 2006). Therefore, a further study was done to evaluate the impact of *P. aeruginosa* on monocyte viability using annexin V. However, we were unable to accurately determine the percentage of apoptotic monocytes, as most, if not all, the live cells (unstained cells) remained adhered to the bottom of the tissue culture plate (Chapter 2).

Thus, we employed LDH assay to identify another type of cell death termed necrosis (Chan *et al.*, 2013, Essmann *et al.*, 2003, Kennedy *et al.*, 2009, Kim *et al.*, 2013, Zong and Thompson, 2006) which is characterised by the cell membrane rupture and leaking of the intracellular contents to the surrounding milieu (Kroemer *et al.*, 2009). The release of the cytosolic enzyme, LDH, from the damaged cells is used as an indicator of necrotic cell formation (Chan *et al.*, 2013). However, our results showed that there was no significance difference in the concentration of LDH released from PAO1-L supernatants (+/-SEB)-treated monocytes compared to untreated counterparts at different time points (Chapter 2).

It was decided, therefore, to conduct the study using microscopic examination to visualise the impact of *P. aeruginosa* on monocyte viability. The initial data revealed that adherent cells within the PBMC cultures (monocytes) showed alterations in morphology and number after stimulation with PAO1-L supernatant in the absence and presence of SEB for 2 days compared to untreated cells (Chapter 2).

In this context, a subsequent study to evaluate the effect on purified monocytes of PAO1-L (20% (v/v)) supernatant was performed. Furthermore, a *ΔtoxR* strain constructed in the Lausanne background was also used to test the effect

of *toxR*-regulated secreted products on monocyte viability. Interestingly, the viability of monocytes was not affected by the presence of 20% of either PAO1-N or PAO1-L Δ*toxR* supernatant. However, after treatment with 20% PAO1-L supernatant, monocytes showed a reduction in cell size and number suggesting that the cytotoxicity exhibited by PAO1-L strain on monocyte viability is mediated by the activity of *toxR* regulatory gene.

ETA is considered the most toxic factor secreted by *P. aeruginosa* (Hamood *et al.*, 1996b) as it possesses ADP-ribosylation activity which catalyses the transfer of adenosine di-phosphate ribose (ADP-ribose) from Nicotinamide Adenine Dinucleotide (NAD+) to elongation factor 2 leading to inhibition of protein synthesis and eventually cell death (Chaudhary *et al.*, 1990). ETA is produced by many bacterial isolates from different diseases such as CF pulmonary, cancer and burn victims (Van Delden and Iglewski, 1998, Vasil *et al.*, 1986, Song *et al.*, 2000). Furthermore, this toxin appears to be a fundamental virulence determinant in CF isolates as their sera show a high accumulation of ETA-specific antibody likely due to the recurrent exposure to the ETA antigen. The high expression of ETA significantly correlates with a high rate of mortality in CF patients, suggesting that ETA contributes to disease severity (Jaffar-Bandjee *et al.*, 1995, Klinger *et al.*, 1978, Moss *et al.*, 1986). In addition purified ETA has been shown to down-regulate expression of co-stimulatory molecules including CD80, CD86, ICAM-1 and HLA-DR on IFN-γ-treated monocytes (Michalkiewicz *et al.*, 1999) and block T cell expansion (Michalkiewicz *et al.*, 1998). Therefore, the expression of ETA in PAO1 supernatants was investigated. Whilst 3.3 µg/ml of ETA was detected in PAO1-L supernatants, less than 166 ng/ml of ETA was detected in the

supernatant from the *toxR* mutant (Figure 2.14). This result indicates that the high amount of ETA in PAO1-L supernatant correlates with its cytotoxicity on monocytes and inhibitory effect on T cell proliferation. Also it supports the key role of *toxR* in the regulation of ETA expression.

In an attempt to confirm ETA as responsible for the effects obtained with the PAO1-L supernatants on T cell proliferation, we constructed a *toxA* deficient strain in the PAO1-L background (Chapter 3). *ToxA* is a structural gene encoding the 66 kDa ETA protein which is then secreted to the surrounding environment by a type II secretion system (Ochsner *et al.*, 1996, Davinic *et al.*, 2009). To construct an ETA-deficient strain in the PAO1-Lausanne background by generating an in-frame deletion, the upstream and downstream flanking regions of the gene of interest were cloned into the suicide vector pME3087 and conjugated from *E. coli* S17-1 λ*pir* into PAO1-L via homologous recombination using LB medium supplemented with 150 µg/ml tetracycline and 15 µg/ml nalidixic acid. Candidates were then screened for the deletion using PCR. Generation of the complementation strain by re-introducing *toxA* gene and its upstream and downstream flanking regions into the mutant strain would have been desirable. However, as the levels of T cell proliferation in response to SEB changed when the Δ*ToxA* supernatants were being tested (Chapter 4) it was decided not to pursue this aim. An alternative control would have been the addition to purified ETA to the Δ*ToxA* supernatants.

Our data demonstrated increased monocyte death upon stimulation with PAO1-L (20% (v/v)) supernatants suggesting that *P. aeruginosa* cytotoxicity on monocytes correlates with the high expression of ETA, as both PAO1-LΔ*toxR* and PAO1-LΔ*toxA* produce less toxic supernatants than the wild type. This is

in agreement with studies by other groups that demonstrate ETA receptor (CD91) expression is highly restricted to monocytes (Moestrup *et al.*, 1992). This would suggest that PAO1-L would preferentially cause monocyte cell death as its supernatant contains a high amount of ETA compared to other mutant counterparts. Initial studies into the effect of PAO1-L supernatant on T cell proliferation in response to SEB revealed a negative impact (Chapter 2). However, when testing the effect of Δ*toxA*-supernatants on T cell expansion, the stimulation with SEB (10 ng/ml) alone did not achieve the same proliferation response (Chapter 4) despite using different SEB batches and concentrations, the reason for which are still unknown. To overcome this problem, PHA was employed as a promoter of T cell division (Movafagh *et al.*, 2011).

Michałkiewicz et al, showed that ETA caused the inhibition of PHA-treated lymphocyte proliferation in a dose-dependent manner (Michalkiewicz *et al.*, 1989). Furthermore, this toxic effect is dependent on a sequence of events starting with the internalisation of this toxin by APCs, mainly monocytes, initiation of cell death and eventually suppression of T cell division (Patzer *et al.*, 1989a, Katzen *et al.*, 1985, Issekutz and Stoltz, 1985).

PHA is a powerful polyclonal mitogen of a variety of immune cells such as T via the ligation of surface glycoproteins, including the TCR with their carbohydrate moieties (Greaves *et al.*, 1974, Christmas *et al.*, 2006, Kesherwani and Sodhi, 2007, Movafagh *et al.*, 2011, Ceuppens *et al.*, 1988). However, T cell activation and expansion in the presence of PHA depends on the expression of potent molecules by accessory cells such as monocytes in the

surrounding milieu such as IL-6 and IL-2 (Ceuppens *et al.*, 1988, Greaves *et al.*, 1974, Katzen *et al.*, 1985).

However, the suppressor effect of PAO1-L supernatant was clear on only the PHA-treated $CD3^+/CD4^+$ T cell subset and no effect on either $CD3^+$ or $CD3^+/CD8^+$ T cells in comparison with either, PAO1-N and PAO1-L*ΔtoxR* strains with lower toxin production, or the PAO1-L*ΔtoxA* (ETA-) strain lacking ETA expression.

In order to confirm whether ETA dominates *P. aeruginosa* cytotoxicity and its function is not enhanced in the presence of other secreted factors, additional mutant strains in the Lausanne background that lack the expression of a defined element were tested. Thus, further studies were performed using supernatants from PAO1-L*ΔrhlA* and PAO1-L*ΔrBif* strains. Both strains exhibited high expression levels of ETA in their supernatants (Chapter 2). It has been shown that the *rhlA* gene is required for the production of rhamnolipid, a surfactant promoting swarming motility in *P. aeruginosa* (Zhu and Rock, 2008, Ochsner *et al.*, 1994, Déziel *et al.*, 2003). While the *Bif* gene (uncharacterised protein) is one of the genetic variations between the PAO1-N and PAO1-L sub-lines and may be a potential candidate for PAO1-L pathogenicity. The findings revealed that PAO1-L*ΔrhlA* and PAO1-L*ΔrBif* strains suppressed PHA-induced $CD3^+$ T cell proliferation compared to control PHA-treated cells. These data confirmed that the effect of *P. aeruginosa* on T cell proliferation is strongly influenced by the presence of multiple secreted virulence factors, including ETA, in *P. aeruginosa* supernatants.

Full PHA-mediated induction of T cell proliferation may need extra soluble factors, such as IL-2, supplemented by accessory cells in their milieu and the

viability of monocytes may not be as crucial to stimulate T cell proliferation (Ceuppens *et al.*, 1988, Greaves *et al.*, 1974, Katzen *et al.*, 1985). Thus, PHA- and SEB-treated T cells exhibited the same trend, but full T cell proliferation in response to PHA was not observed with the Δ*toxA* deficient strain probably due to the fact that monocytes contribute in different ways.

The novelty of this project arises from the fact that previous work with ETA were performed using purified ETA (Michalkiewicz *et al.*, 1999, Michalkiewicz *et al.*, 1998, Patzer *et al.*, 1989b, Holt and Misfeldt, 1984, Pollack, 1983, Laithwaite *et al.*, 1999). In this study, the impact of bacterial supernatants on a particular immune cell within PBMC cultures in the presence of absence of ETA is compared. Further, the concentration of ETA was consistent throughout this work and relatively low levels of ETA were used (20% (v/v)) while it varied among studies. For instance, ETA was injected in a total volume of 250 µl /25 g per mouse (Mühlen *et al.*, 2004, Schumann *et al.*, 1998, Schumann *et al.*, 2000). While (10–100) ng/ml (Michałkiewicz *et al.*, 1999, Michalkiewicz *et al.*, 1998) and (10-1000) ng/ml (Schultz *et al.*, 2000) of ETA were used for *in-vitro* studies. As mentioned above, data from our study demonstrated a significant attenuation in SEB-induced T cell proliferation in the presence of PAO1-L supernatant and this effect requires presence of monocytes and the activity of the *toxR* regulatory gene, which regulates the production of ETA. ETA appears to dominate SEB-induced T cell proliferation but not PHA-induced T cell proliferation, where other factors also play a role. We believe this work provides a better understanding of both the molecular basis of *P. aeruginosa* ability to modulate the immune system. However, much more work is required into the phenotypic analysis of the ETA

deficient strain by either generation a new plasmid to complement a *toxA* mutant or by adding ETA protein to restore the wild type phenotype. Furthermore, it has been revealed that CD91, which is a unique endocytic receptor in a number of cell types, is the principal receptor for ETA (Moestrup *et al.*, 1992, Walters and Berwin, 2005). Thus, further studies into the mechanism of CD91 receptor expressed by monocytes may yield interesting clues about its role in *P. aeruginosa* pathogenicity. This might be achieved by blocking the ligation of CD91receptor with ETA by neutralising α-C91 antibody and then evaluate the impact of *P. aeruginosa* on monocyte viability.

Associations between bacteria and fungi are of medical and environmental significance. They are found in diverse environments, such as the lungs of CF patients (Bauernfeind *et al.*, 1987). These interactions might alter the microbial pathogenicity or nutritional availabilities of one or both organisms towards the host (Frey-Klett *et al.*, 2011). *P. aeruginosa* and *C. albicans* are typical opportunistic respiratory tract pathogens and their pathogenic interactions have been previously reported (Delisle *et al.*, 2008, Mear *et al.*, 2013). However, the nature of the relationship between these two pathogens is still not entirely clear. We hypothesised that the production of ETA might provide an advantage to *P. aeruginosa* in competition with *C. albicans* as ETA production is up-regulated in the presence of *C. albicans* and, as mentioned above, ETA is the most damaging virulence factor produced by *P. aeruginosa* (Purschke *et al.*, 2012, Webb *et al.*, 2008).

Data from our study demonstrated that *P. aeruginosa* supernatants obtained from PAO1-L and different mutant strains in the Lausanne background altered *C. albicans* growth in a dose dependent manner (Chapter 5). Furthermore,

image analysis showed that these changes in *C. albicans* growth correlate with the induction of hyphal growth (Fox *et al.*, 2013). These observations suggest that *P. aeruginosa* secreted factors are able to control and promote morphological switch in *C. albicans* from yeast to hyphae. Changing *C. albicans* growth to hyphae is critical for *P. aeruginosa* domination within the microenvironment as it selectively binds to and kills the hyphal form (Fox *et al.*, 2013, Hogan and Kolter, 2002).

These results are in agreement with several studies that suggest *P. aeruginosa* has the ability to down regulate *C. albicans* growth *in vitro* and in thermal injury patients (Gupta *et al.*, 2005, Kerr *et al.*, 1999). In addition, it has been shown that there is increased growth of *C. albicans* in CF patients after using anti-bacterial agents to suppress *P. aeruginosa* infections (Burns *et al.*, 1999). However, a striking result obtained in the present study was that the yeast form dominates *C. albicans* growth in the presence of supernatants from the PAAMP1 mutant strain which contains a 58 Kb deletion in its genome. The genome sequence of *P. aeruginosa* strain (PAO1) has been determined that a number of genes which encode functional proteins are located within this region (Llamas *et al.*, 2009). The *hxc* system (*hxcU, P, X, Y, Z, Q)* termed Type II secretion system (T2SS) which secretes one substrate, the low molecular weight alkaline phosphatase LapA was identified within the 58 Kb missing nucleotides (Llamas *et al.*, 2009, Bleves *et al.*, 2010, Ball *et al.*, 2002). This might suggest that the *hxc* gene machinery is a potential candidate for the morphological transition in *C. albicans* from yeast to hyphae form and their absence in PAAMP1 strain might drive the domination of the yeast growth in *C. albicans* culture.

Work is currently underway in our group to confirm these observations and to identify the active element within *P. aeruginosa* exo-products which is responsible for the induction of the hyphal formation in *C. albicans* and determine the advantages of the filamentation triggered by *P. aeruginosa* during infection.

Thus, it could be concluded that enhanced hyphal formation in *C. albicans*, promoted by *P. aeruginosa* secreted products does not rely on the production of already studied virulence factors such as pyocyanins, ETA, rhamnolipids, or the activity of the regulatory genes *toxR* and *Bif*. However, the potential gene that encodes or regulates the active agent which is associated with hyphal induction in *C. albicans* might be located within this 58 Kb region. To determine the active agents in PAO1-L supernatant which are involved in the promotion of hyphal growth in *C. albicans,* experiments are underway in our laboratory .PAO1-L mutants lacking different regions with 58 kb deletion are being generated to study the effect of its supernatants on the morphological transition and compare this impact between different strains of *C. albicans*.

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