# Interaction of *Pseudomonas aeruginosa* biofilm with inflammatory cytokines and immune cells

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## Abstract

Colonisation and persistent lung infection by pathogens, mainly *Pseudomonas aeruginosa* (PA), is an important cause of morbidity and mortality in Cystic Fibrosis patients. PA persists inside the lungs by forming drug-resistant biofilms. Investigation into the behaviour of bacterial cells within these structures and their response to components of the innate immune response will give us an insight into novel approaches to combat infection.

The main aim of this thesis was investigate the effect of inflammatory mediators such as cytokines and immune cells on the development of PA biofilms. For this the BioFlux system and PA expressing green fluorescent protein were used to generate biofilms. After incubation with serum, cytokines such as GM-CSF and IFN-γ, changes in the characteristic of the biofilms were analysed. No significant changes on PA biofilms were observed in the presence of the cytokines but human serum was shown to have an inhibitory effect.

Incubation of PA biofilms with labelled purified human monocytes and macrophages indicate that macrophages, unlike monocytes attached to PA biofilms. Cell surface receptors expressed by monocytes and macrophages were compared and, as previously described, high mannose receptor (MR) expression was found in macrophages. MR is a carbohydrate binding receptor that plays a crucial role in innate immunity. MR binds mannose-rich carbohydrates through C-type lectin-like domains (CTLD) 4 to 7. Using a recombinant protein containing the mannose binding region of MR (CTLD 4-7-Fc) it was found that MR can directly interact with PA biofilms and that this binding is increased when Psl expression is increased. Furthermore CTLD 4-7-

Fc was shown to bind crude extracts of carbohydrates from PA biofilm enriched for the extracellular polysaccharide Psl. We hypothesize that MR expressed by macrophages and dendritic cells might contribute to PA recognition through Psl. Future work will explore the capability of immune mammalian C-type lectins to recognise PA biofilms and purified Psl and elucidate the role of Psl in modulating the recognition of PA by immune cells using PA strains expressing different combinations of extracellular polysaccharides. Our results will shed light on the impact of Psl on the immune recognition of PA, and might open the avenues for the discovery of novel therapeutic targets.

Dispersion is the final stage of the biofilm cycle and contributes to dispersal and transmission of pathogens to colonize new sites. Till now not many studies have focused on the role of dispersion in pathogenesis. Thus the present study also focused on the contribution of a dispersal molecule produced during PA biofilm formation termed *cis*-2-decenoic acid (CDA) and analyse its role in immunomodulation. Previous observations showed that addition of exogenous CDA is able to disperse mature biofilm and inhibit biofilm formation of different gram negative and gram positive bacteria such as PA, *E. coli* and *S. aureus*. The present study showed that exogenous addition of CDA is able to modify some aspects of the inflammatory response by modulating production of the pro-inflammatory cytokine TNF-α by human monocytes and macrophages. Thus the present study has identified the potential impact of CDA as an immune modulator which will offer many opportunities for development of drug with potential anti-inflammatory action.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| 3-oxo-C12 | N-(3-oxo-dodecanoyl)-homoserine lactone |
| AAMs | alternatively activated macrophages |
| AECs | airway epithelial cells |
| AHL | acyl-homoserine lactone |
| ANOVA | analysis of variance |
| AQs | 2-alkyl-4-quinolones |
| Apo-Tf | Apo-Transferrin |
| C4-HSL | butyl-homoeserine lactone |
| CAMs | classically activated macrophages |
| CDA | *cis*-2-decenoic acid |
| CF | cystic fibrosis |
| CFTR | cystic fibrosis transmembrane conductance regulator |
| CLRs | C-type Lectins receptors |
| CTLD | C- type Lectin Like Domain |
| DAPI | 4'6-diamidino-2-phenylindole hydrochloride |
| DCs | dendritic cells |
| DSF | Diffusible signal factors |
| ECM | extracellular matrix |
| EPS | extracellular polymeric substances |
| eDNA | extracellular DNA |
| GM-CSF | granulocyte macrophage colony-stimulating factor |
| (HEK)293 cells | human embryonic kidney 293 cells |
| HHQ | 2-heptyl-4(1*H*)-quinolone |
| HS | Human serum |
| IRAK | IL-1 receptor-associated kinase |
| IFN-γ | interferon-γ |
| LPS | lipopolysaccharide |
| MAPKs | Mitogen-activated protein kinases |
| MBL | Mannose Binding Lectin |
| MDSCs | Myeloid- Derived Suppressor Cells |
| M-CSF | macrophage colony-stimulating factor |
| MR | Mannose Receptor |
| NF- κB | Nuclear Factor kappa-light-chain-enhancer of activated B cells |
| NLRs | NOD (Nucleotide-binding Oligomerization Domain receptors)-like receptors |
| PA-GFP | P.aeruginosa (PAO1) Lausanne strain expressing green fluorescent protein |
| PAMPs | Pathogen-Associated Molecular Patterns |
| PMSs | polymorphonuclear neutrophils |
| PRRs | pattern recognition receptor |
| Psl | Polysaccharide synthesis locus |
| QS | Quorum sensing |
| RSCVs | Rugose small colony variants |
| SCVs | small colony variants |
| TDA | Trans 2 Decenoic acid |
| T3SS | type III secretion system |
| TLRs | Toll-Like receptors |

# Chapter 1

# 1. General introduction

The purpose of the present study was to investigate the mechanisms involved in innate immune recognition of *Pseudomonas aeruginosa* (PA) biofilms and subsequent response specifically in the context of respiratory tract infections (RTIs).

PA is the most important pathogen in Cystic Fibrosis (CF) lung disease and PA infection is highly associated with poor lung function, morbidity and mortality in CF patients (Hoiby et al., 2010, Banerjee and Stableforth, 2000, Brugha and Davies, 2011, Kimberg and Brown, 2008). Persistence of PA in CF lungs involves an advanced process of adaptation, formation of biofilms, development of new phenotypic variants resistant to antibiotics, hyper variability and tailored pathogenicity with virulence determinants being expressed according the infection stages (Sousa and Pereira, 2014, Sriramulu et al., 2005). After initial PA colonization CF patients suffer from subsequent episodes of re-colonization until chronic infection is established; chronic infection can remain years to decades or even never eradicate (Hogardt and Heesemann, 2010, Schelstraete et al., 2013). PA biofilm is often considered a source of high diverse phenotypic variants, and hence one of the most crucial adaptive mechanisms by which PA persist inside CF airways (Hoiby et al., 2010, Hassett et al., 2009).

## 1.1. *Pseudomonas aeruginosa* (PA) as a pathogen

PA is a versatile pathogen ubiquitously distributed in various environments including humans, terrestrial, aquatic, animals, and plants (Favero et al., 1971,

Driscoll et al., 2007). PA is a Gram negative opportunistic microbe that causes infection mostly to the immune-compromised patients for example patients suffering from neutropenia, CF, thermal injury and patients undergoing organ transplants or in intensive care units (Coutinho et al., 2008, Silby et al., 2011). PA can cause both acute and chronic infection. Acute PA infections such as ventilator-associated pneumonia and PA bacteraemia in severe burn patients are often invasive, cytotoxic and result in systemic infection, septic shock, and mortality (Lyczak et al., 2000, Yahr and Greenberg, 2004, Turner et al., 2014). In CF patients PA mostly colonise the airway and cause chronic infection which are minimally invasive, non-cytotoxic, and normally do not progress to systemic infection, rather these are mostly biofilm associated infections(Yahr and Greenberg, 2004). Chronic PA infection is associated with inflammation and lung deterioration which is the primary cause of mortality in CF patients (Yahr and Greenberg, 2004). PA versatility relates to its large genome of about 6.3 MB that includes genes associated with complex metabolic pathways and virulence factors which allow PA to grow well in hostile environments that normally do not support many organisms (Diggle et al., 2002, Stover et al., 2000). Thus this large genome provides PA with a great adaptive ability (Rau et al., 2010, Hogardt and Heesemann, 2010). The remarkable capacity of PA to cause infection depends on several factors including its ability to behave as a population and therefore exist in communities known as biofilms.

### 1.1.1. PA virulence determinants

The way by which PA establishes infection and its subsequent clearance has long been a debated issue. PA expresses a variety of virulence factors that enables it to establish infection in a susceptible host (Lyczak et al., 2000). PA

obtained from acute infections express a distinct subset of virulence factors and differs considerably from the PA isolated from chronic infections (Smith et al., 2006, Gellatly and Hancock, 2013, Hogardt and Heesemann, 2010). In addition PA isolated from chronic infections are largely associated with formation of biofilms and also overexpresses the exopolysaccharide alginate, which causes PA to become mucoid (Sadikot et al., 2005, Kipnis et al., 2006).

#### 1.1.1.1. Flagella and pili

PA cells possess a single polar flagellum that provides swimming motility through a rotating corkscrew motion within an aqueous environment and plays an important role in bacterial chemotaxis (Gellatly and Hancock, 2013). PA involved in acute infection including environmental strains and clinical isolates are flagellated and motile (Soutourina and Bertin, 2003, Feldman et al., 1998), whereas PA isolated from chronically infected CF patients lack flagellin expression and are non-motile (Mahenthiralingam et al., 1994, Mahenthiralingam and Speert, 1995). Also it has been shown that PA strains lacking flagella are phagocytosed less readily by alveolar macrophages and polymorphonuclear leukocytes (Mahenthiralingam et al., 1994, Mahenthiralingam and Speert, 1995). PA flagellin is recognised by TLR5 and activates different transcription factors such as Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Mitogen-activated protein kinases (MAPKs) and trigger cytokine secretion (Feuillet et al., 2006). It has been demonstrated that PA-infected CF patients have increased numbers of Myeloid-Derived Suppressor Cells (MDSCs) which are myeloid derived heterogenous population of cells expanded during infection, inflammation that

supress T cell responses. It has been observed that PA flagellin induces MDSCs *in vitro* which eventually supress T cell proliferation and modulate Th17 responses (Rieber et al., 2013). PA type 4 pili act as adhesins that provide surface specific twitching motility and facilitate biofilm formation (Burrows, 2012, Comolli et al., 1999, Deziel et al., 2001). Similar to flagella, pili are involved in initial colonization by binding with asialoGM1 at the epithelial cell membrane (Gupta et al., 1994, Hahn, 1997). It has been shown that in a mouse model of lung infection piliated strains of PA promote more severe and diffuse pneumonia compared to non-piliated mutants (Tang et al., 1995). Thus similar to flagella, pili could be a target for developing anti-pseudomonas immunotherapy (Hahn et al., 1997, Sheth et al., 1995, Cachia et al., 2004). By using both human CF and control airway epithelial cells it has been shown that the interaction between TLR5 and flagellin is crucial and regulate inflammation following exposure to PA, furthermore translational research has demonstrated that genetic variation in TLR5 results in decrease flagellin responsiveness and was associated to improved health indicators in CF adults (Blohmke et al., 2010).

#### 1.1.1.2. Lectins

PA produce two lectins termed LecA and LecB (formerly PA-IL and PAIIL)(Winzer et al., 2000, Gilboa-Garber, 1982). PA lectins have been shown to play a prominent role in human infections; LecA induces cytotoxic effects on respiratory epithelium by altering their growth rate and thus causing respiratory epithelial injury (Bajolet-Laudinat et al., 1994). Furthermore in a mouse model LecA has been shown to induce a permeability defect in the intestinal epithelial cells resulting in exotoxin A, an important virulence factor secreted by PA, to cross the epithelial barrier (Laughlin et al., 2000). In a

separate study Lec A and Lec B were found involved in PA biofilm formation (Diggle et al., 2006b, Tielker et al., 2005). Diggle et al., 2006b showed that a PA *lecA* mutant was unable to form biofilms and it was proposed that by being part of the extracellular matrix LecA mediates cell-to-cell interactions presumably by cross linking galactosides on the surface of bacterial cells and thus connects the bacteria together and facilitates biofilm development. No such effect of LecB on PA biofilm formation was proposed in this study (Diggle et al., 2006b). In contrast in another study under static conditions PA *lecB* mutant was found unable to develop biofilms on glass slides (Tielker et al., 2005). Differences between these studies are proposed to be due to the use of different growth conditions (Diggle et al., 2006b). Both lectins have been shown to help PA adhere to the glycocalyx of mammalian cells and inhibit ciliary beat in a time-dependent manner (Mewe et al., 2005). Initial studies showed that these lectins are mainly located in the cytoplasm while a small fraction persists on the outer membrane and in the periplasmic space (Glick and Garber, 1983). However more recently (Tielker et al., 2005) showed that a large portion of Lec B persists in the outer membrane of PA.

#### 1.1.1.3. Type III secretory system

Similar to other Gram negative pathogens PA manipulates host cells by utilizing a type III secretion system (T3SS). PA T3SS assemblies into a complex needle-like structure on the PA surface, thus it exerts its function in a highly organised manner and transports T3SS effectors, also known as exoenzymes, and various regulatory and chaperone proteins into host cells (Hauser, 2009). Both cellular and animal infection models have shown that production of T3SS is associated with increased pathogenicity and poor disease

prognosis (Hauser et al., 2002, Roy-Burman et al., 2001). Four known virulence factors/exoenzymes, ExoT, ExoY, ExoS, and ExoU, are secreted by PA via T3SS into host cells (Engel and Balachandran, 2009). ExoT was shown to have both an N-terminal domain with GTPase activity and a C-terminal domain with adenosine diphosphate (ADP) ribosyltransferase activity and ExoS is an ADP ribosyltransferase of ExoT, ExoY acts as an adenylate cyclase and ExoU functions as a powerful cytotoxin (Epelman et al., 2004, Finck-Barbancon et al., 1997, Sawa et al., 1999). Therefore these exoenzymes cause host cell damage summarised as follows: ExoS causes alteration of cell function; it inhibits phagocytosis, endocytosis and host cell DNA synthesis (Barbieri et al., 2001, Fraylick et al., 2001, Pederson et al., 1999). ExoT inhibits the process of epithelial cell wound repair and impairs host cell division (Geiser et al., 2001, Shafikhani and Engel, 2006). Exo Y disrupts epithelial actin cytoskeleton and thus impairs bacterial uptake (Cowell et al., 2005). Exo U has phospholipase activity and in the context of PA infection Exo U targets and impairs host neutrophils function which lead to immunesupression and render the host susceptible to secondary infections (Diaz et al., 2008). PA T3SS has been involved predominantly in acute infections although evidence suggest that T3SS may also be involved in chronic infections including CF patients as many CF patients develop antibodies against T3SS proteins (Moss et al., 2001, Jain et al., 2004). These effectors are expressed at some point during infection but eventually PA lose the ability to produce T3SS over time in CF airways (Lee et al., 2005, Roy-Burman et al., 2001, Jain et al., 2004). PA isolated from chronically-infected CF patients is often found to have a mucoid phenotype which is associated

with overexpression of the exopolysaccharide alginate. Overproduction of alginate is often caused by mutations within the *mucA* gene and it was shown that *mucA* co-ordinates alginate and type III secretion (Govan and Deretic, 1996, Wu et al., 2004). The prevailing assumption is that PA is subject to selection towards altered virulence and reduced cytotoxicity during chronic CF airways infection although this may not be applicable to all clonal variants. For example PA small colony variants (SCVs) which are associated with increased antibiotic resistance, and an enhanced ability to form biofilms showed an upregulation of expression of the T3SS and production of the respective effector proteins (von Gotz et al., 2004).

### 1.1.2. Quorum sensing (QS)

The QS system consists of self-generated signalling molecules known as autoinducers that regulate gene expression in response to changes in population density (Williams and Camara, 2009, Miller and Bassler, 2001). In PA, biofilm development and the expression of different virulence factors are regulated via the action of two different classes of signal molecules: the *N*-acylhomoserine lactones (AHL) and the alkyl-quinolones (AQ) (Fuqua et al., 1994, Cao et al., 2001, Pesci et al., 1999, Heeb et al., 2011, Miller and Bassler, 2001, Williams and Camara, 2009). Both AHL-based QS systems named *las* and *rhl* in PA have been widely studied (Diggle et al., 2006a, Heeb et al., 2011, Lee et al., 2013). The *las* system is composed of a synthase LasI which mediate synthesis of the AHL molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and a transcriptional regulator termed LasR. This system regulates genes involved in the production of virulent factors such as elastase (lasB), the LasA protease (lasA), alkaline protease (aprA) and exotoxin A (toxA)(Diggle et al.,

2003, Gambello et al., 1993, Passador et al., 1993, Pearson et al., 1997, Gambello and Iglewski, 1991, Toder et al., 1991). The *rhl* system is comprised of a transcriptional activator, RhlR, and the auto-inducer synthase RhlI. RhlI mediates the production of the signal molecule *N*-butyryl-L-homoserine lactone (C4-HSL) (Latifi et al., 1995). Virulent factors and metabolites controlled by *rhl* system include the rhamnosyltransferase, encoded by *rhlAB*, which produces the biosurfactant/haemolysin rhamnolipid, elastase, pyocyanin, cyanide, chitinase and LecA and LecB (Pearson et al., 1997, Latifi et al., 1995, Ochsner and Reiser, 1995, Winzer et al., 2000).

The AQ QS network consists of more than 50 compounds including the most active signal molecule 2-heptyl-3-hydroxy-4-quinolone known as pseudomonas quinolone signal (PQS) (Pesci et al., 1999, Deziel et al., 2004).

QS regulates up to 10% of the PA genome (Williams and Camara, 2009, Whiteley et al., 1999, Wagner et al., 2003, Schuster et al., 2003), but the role of QS in PA biofilm development still remains unclear. Some studies have shown that QS is important for biofilm development whereas others indicated that the QS system plays little or no role. Initial reports showed that the *las* QS system was crucial for development of mature, differentiated PA biofilms. In a flow-chamber system a *lasI* mutant was shown to form flat and undifferentiated biofilms compared to wild type (WT) PAO1 and in response to the detergent sodium dodecyl sulfate (SDS), the biofilm formed by the *lasI* mutant quickly dispersed and detached from the substratum (Davies et al., 1998). In a similar study it was also reported that under high shear conditions, biofilms formed by a *lasI* mutant strain showed clear structural dissimilarity when compared with the WT PAO1 strain (Purevdorj et al., 2002). There are also reports suggesting

that the QS system may not be important for biofilm formation. Heydorn et al., 2002 reported that both WT and QS mutant strains formed flat, uniform biofilms which are very similar to each another (Heydorn et al., 2002). Different culture conditions and biofilm developing methods have been suggested behind these observed discrepancies. Difference in PA biofilm formation was observed in presence of different nutrients including glucose, glutamate and succinate, similarly biofilm formed by QS mutants were shown to be different when grown in presence of succinate but no such difference was found when grown in presence of glutamate or glucose. (Shrout et al., 2006). In addition the *pel* biosynthetic operon which is involved in production of PA biofilm extracellular polymeric substances (EPS) was found to be regulated by the QS system; a reduction in transcription of the *pel* operon occurs in *lasI* and *rhlI* mutants (Sakuragi and Kolter, 2007). PA produces extracellular DNA (eDNA) which is a component of the PA biofilm matrix. Experiments with WT PAO1 and QS mutants (*lasIrhlI*, *pqsA* and *pqsL*) have suggested that generation of eDNA is dependent on AHL and AQ signalling systems (Allesen-Holm et al., 2006). Furthermore it has been shown that PA forms robust biofilms under anaerobic conditions, an environment believed to occur in the CF lung. In anaerobic biofilms, *rhl* deficient mutants were observed to be readily killed in response to toxic NO build-up, thus the Rhl QS system was considered essential for anaerobic biofilm survival (Yoon et al., 2002).

Thus the actual QS-controlled factors and their role in developing PA biofilm have not been fully identified. But in general all these studies supported that PA biofilms developed in the absence of functional QS system (caused by either inhibition or mutation) are vulnerable to shear forces, SDS treatment,

antimicrobial treatment, $H_2O_2$ treatment and polymorphonuclear neutrophils (PMNs) phagocytosis (Hassett et al., 1999, Hentzer et al., 2002, Shih and Huang, 2002, Bjarnsholt et al., 2005). Therefore it could be suggested that before considering an *in vitro* study: for example identifying potential antimicrobial agent limiting PA biofilm development or to determine the role of immune response on PA biofilm formation an optimization of PA biofilm development using an appropriate culture media need to be decided. Clinical relevance of QS *in vivo* is supported by the fact that CF sputum contains the two principal PA QS signals associated with biofilm formation (Singh et al., 2000). Details of the role of the QS system in immune modulation has been described in Chapter 5.

### 1.1.3. PA biofilms

Biofilm is a complex community formed by single or multiple species of bacterial cells embedded in self-produced polymeric matrix. It is considered an important strategy used by bacterial cells to survive in a hostile environment (Chen and Wen, 2011). In many pathogens, biofilm formation appears as a major determinant of chronic infection (Mikkelsen et al., 2011). Specialised cells often arise within a biofilm due to changes in gene expression rather than in gene composition (Spormann, 2008, An and Parsek, 2007, Stewart and Franklin, 2008). The developmental process of PA biofilms is considered to consist of five-stages: 1. initial attachment of free planktonic bacteria to a surface; 2. production of EPS which results in irreversible attachment of PA to a surface; 3. development of biofilm architecture and 4. seeding dispersal in which planktonic cells from the biofilm exit to occupy a new surface (Stoodley et al., 2002) (Figure 1.2).

PA can form biofilms on different surfaces such as contaminated catheters and contact lenses despite antibiotic therapy (Ma et al., 2009). It has been proposed that PA biofilms in the sinuses of CF patients may act as reservoirs and promote re-infection of the lungs (Johansen et al., 2012). PA biofilm development in CF lungs is associated with slow growing bacteria, an increased frequency of mutations, low metabolic activity and increased doubling time of PA cells, consistent with adaptation of the bacteria to the conditions in the lungs and antibiotic treatment (Hoiby et al., 2010) (Figure-1.1). Understanding the development and structure of the PA biofilm matrix may guide researchers towards the development of novel therapeutics which could disrupt these structures.

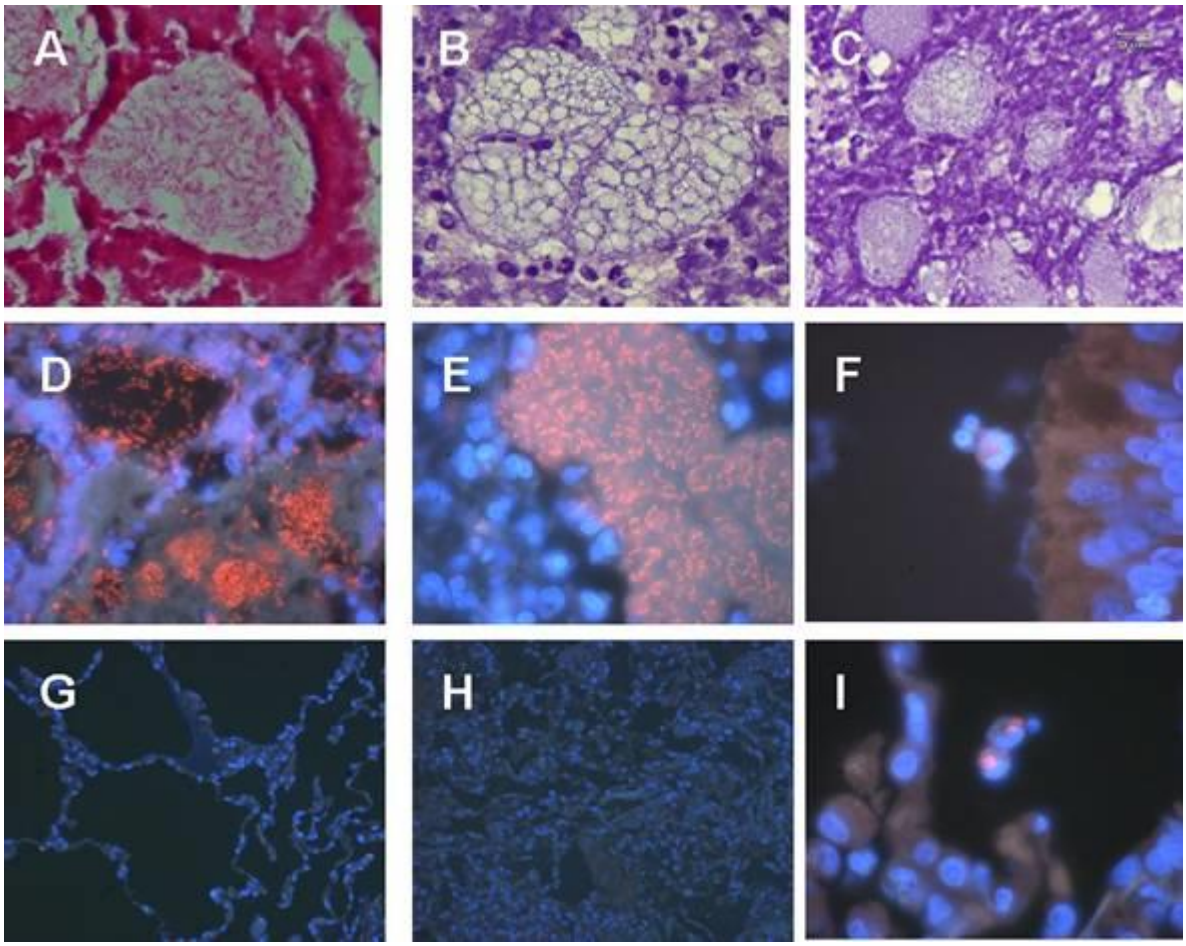



Fig: 1. 1 Microscopic images of PA biofilm obtained from CF patients lung

PA biofilms in the conductive zone, **A.** Gram staining of biofilm **B** and **C.** bacteria inside bronchiole stained with HE **D** and **E**. PA biofilms surrounded by PMNs, **F.** Intact bronchi wall. **G** and **H**. consolidation of alveoli. **I.** Single phagocytosed PA in the respiratory zone. (Bjarnsholt et al., 2009)

### 1.1.4. Role of extracellular polymeric substance (EPS) components in PA biofilm development

EPS is considered to play an important role in maintaining the PA biofilm architecture and functions as a matrix, or glue, holding bacteria cells together and providing protection from shear forces in fluid environments (Stoodley et al., 2002). EPS performs an important role in cell attachment to surfaces and cell to cell interactions (Whitchurch et al., 2002, Ma et al., 2006, Yang et al., 2009, Molin and Tolker-Nielsen, 2003, Jackson et al., 2004). There is not enough information about how the EPS is formed at different stages of PA biofilm development and which EPS components hold biofilm cells on surfaces. Detailed knowledge on biofilm EPS may provide a tool for rational design of inhibitors that could minimise the clinical and environmental complications related to biofilms-associated infection. PA produces three different types of exopolysaccharides that help in EPS formation : alginate, Pel, and Polysaccharide synthesis locus (Psl) and the involvement of each exopolysaccharide to the biofilm matrix varies among strains (Ryder et al., 2007). By providing extracellular matrix alginate facilitates the persistence of biofilm in CF patients and it is commonly associated with mucoid phenotype in PA (Wozniak et al., 2003). Certain strains such as PAO1 and PA14 can produce biofilms without alginate which suggest that one or more polysaccharides might support biofilm formation (Ryder et al., 2007). For example, the PA polysaccharide synthesis locus (*Psl*) is responsible for the synthesis of Psl EPS and supports the initiation and maintenance of biofilm in PA strains such as PAO1 and ZK2870 (Ryder et al., 2007, Ma et al., 2007, Zegans et al., 2012). Similarly the *Pel* locus generates EPS and contributes to

the development of biofilm in the laboratory strain PA14 (Ryder et al., 2007). As mentioned above PA produces extracellular DNA (eDNA) which is an important matrix component. Polymerase chain reaction and southern analysis show that eDNA is similar to bacterial chromosomal DNA. It is suggested that eDNA in biofilms and cultures is generated following lysis of a subpopulation of PA. Furthermore it was suggested that generation of eDNA follows a process which depends on AHL as well as on flagella and type IV pili (Allesen-Holm et al., 2006).

#### 1.1.4.1. Alginate

One of the most important structural changes during PA biofilm development is production of the extracellular polysaccharide alginate consists of linear polymer made up of guluronic acid and mannuronic acid modified with O-acetyl groups. PA can modify or breakdown the biofilm extracellular polysaccharide backbone utilizing the enzyme alginate lyase which cleaves the 4-O-linked glycosidic bonds between uronate residues (Boyd and Chakrabarty, 1994). Alginate provides protection from host defence mechanisms and antimicrobial agents which makes alginate an important target in medical research (Alkawash et al., 2006, Boyd and Chakrabarty, 1994). As mentioned above PA isolated from CF patients lungs often undergo a switch to a mucoid phenotype which results from overproduction of alginate, which provides protection against harsh environment within CF lungs (Govan and Deretic, 1996). Studies comparing isogenic mucoid and non-mucoid strains of PA showed that overproduction and modification of alginate had an important effect on biofilm structure and development of resistance to antibiotics and

phagocytes (Nivens et al., 2001, Pier et al., 2001, Cabral et al., 1987, Stiver et al., 1988).

Exogenous addition of PA alginate limited uptake of both viable and non-viable non-mucoid PA by mouse peritoneal macrophages (Simpson et al., 1988). Alginate also reduced the bactericidal effect of antimicrobial agent such as aminoglycosides *in vitro* and decreases PMN-mediated non-opsonic phagocytosis of mucoid strains (Bayer et al., 1991). Purified PA alginate scavenges free radicals released by macrophages and thus favour survival of mucoid forms of PA in the CF lung (Simpson et al., 1989). This protective property of PA alginate appeared to correlate with its size, viscosity, and biological properties such as changes of O-acetyl groups (Mai et al., 1993).

Transcriptional analysis of PA gene expression showed the mechanism for the overproduction of alginate is complex and involves different regulatory proteins that act in a hierarchical regulatory cascade (Wozniak and Ohman, 1994). Within the CF lung several local predisposing conditions have been suggested to promote mucoid conversion. *In vitro* studies shown that after attachment onto CF airways surfaces PA penetrate actively and/or passively (due to mucus turbulence) into hypoxic mucus zones which subsequently favour increased alginate production and the formation of macrocolonies (Worlitzsch et al., 2002). Thus in chronic lung disease production of alginate enhances biofilm structure and facilitates biofilm resistance to antimicrobial agents such as tobramycin (Nivens et al., 2001, Hentzer et al., 2001). Within the CF lung initial PA colonisation is initiated by non-mucoid isolates, later on these isolates are replaced by mucoid isolates. PA colonisation in CF patients is often associated with production of alginate-specific antibodies (Speert et al.,

1984). However, these antibodies were not usually found to be protective (Meluleni et al., 1995); it was hypothesized that in CF lungs massive neutrophil infiltration may release excessive elastase that may cleave the antibodies and / or their receptors (Govan and Deretic, 1996). Infection with mucoid strains is associated with increased humoral response and poorer lung function; thus it was proposed that alginate is an important virulent determinant in chronic lung infection in CF patients (Hoiby, 1974, Burns and May, 1968, Doggett and Harrison, 1972, Hoiby et al., 1977, Pedersen et al., 1990).

#### 1.1.4.2. Psl

Non-mucoid PA rely on the exopolysaccharides Psl and/or pel for the formation of biofilm matrix (Branda et al., 2005, Ryder et al., 2007, Wozniak et al., 2003). Fifteen co-transcribed genes have been identified to encode the necessary enzymes involved in Psl synthesis (Friedman and Kolter, 2004a, Jackson et al., 2004, Matsukawa and Greenberg, 2004). The resulting Psl polysaccharide consists of D-mannose, D-glucose, and L-rhamnose, and possibly galactose (Friedman and Kolter, 2004b, Jackson et al., 2004, Matsukawa and Greenberg, 2004, Rocchetta et al., 1998). The sugar nucleotide pool of precursors including GDP-D-mannose, UDP-D-glucose, and dTDP-L-rhamnose is involved in Psl biosynthesis (Byrd et al., 2009). Further details on the role of Psl in PA biofilm formation and immune responses have been described in Chapter 4.

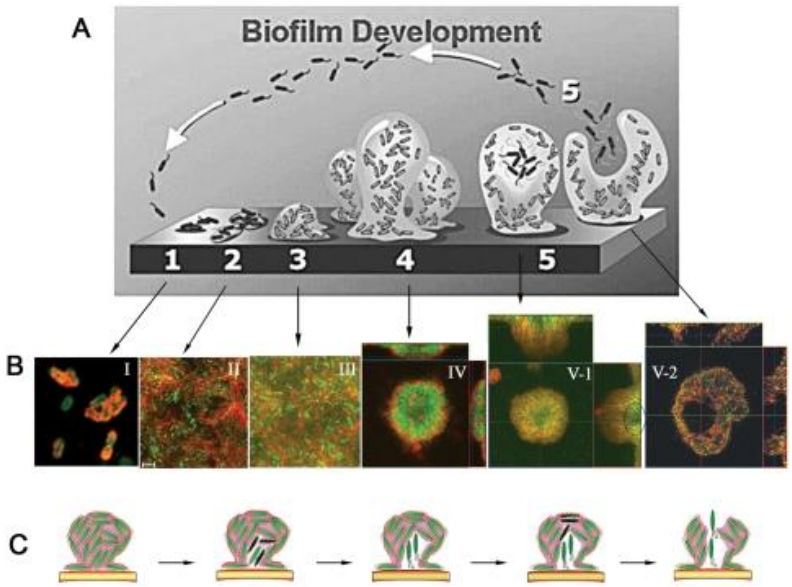



Fig:1.2 Different stages of the PA development and contribution of Psl in PA biofilm formation

(A) Schematic diagram showing the five stages of PA biofilm development (B) Showing Psl (red) during each stage of PA biofilm formation. Green fluorescence signal representing GFP-labeled PA. (C) A suggested event showing how a PA micro-colony sacrifices a portion of cells in the center and damage the existing matrix to free PA for dispersion (Ma et al., 2009).

### 1.1.4.3. Pel

The polysaccharide-producing locus Pel is required for matrix formation and biofilm development in the laboratory model strain PA14. The Pel locus is composed of seven genes and is critical for the self-aggregating properties of the PA14 (Friedman and Kolter, 2004a, Friedman and Kolter, 2004b). Unlike alginate and Psl, composition and nature of the Pel polysaccharide remains largely unknown. Protein products of the Pel genes share sequence similarity with proteins that participate in carbohydrate processing and Pel mutants lack a glucose rich component of their extracellular matrix (Friedman and Kolter, 2004b). The Pel gene cluster has been involved in the formation of pellicles in PA14 strain, a surface-associated biofilm on the air-liquid interface in standing

cultures (Friedman and Kolter, 2004a, Vasseur et al., 2005). Normally in a PA strain in the presence of both intact Psl and Pel loci Psl is dominant with Pel having limited impact on biofilm formation. Under certain circumstances when the Psl operon is absent such as in PA14 strain or when c-di-GMP is maximally elevated Pel was shown to have a clear effect on biofilm formation. Previous studies suggest that PAO1 primary utilize Psl to form biofilm but in an extended biofilm cultivation PAO1 with deletion of Psl upregulate Pel polysaccharide production (Colvin et al., 2012). In addition to maintaining cell-to-cell interactions in PA14 biofilm, Pel increases resistance to aminoglycoside antibiotics (Colvin et al., 2011). Thus it was proposed that Pel is able to play both structural and protective roles in PA biofilms.

### 1.2. Role of pattern recognition receptors (PRRs) in recognition of pathogens including PA

Within the innate immune system a wide range of non-clonally expressed receptors, named PRRs bind highly conserved molecular complexes called Microbe-Associated Molecular Patterns (MAMPS) which include lipids, carbohydrates, peptides and nucleic acids expressed by micro-organisms (Berube et al., 2010). PRRs are also capable of recognizing endogenous molecules which are released after damage to the host cells known as Damaged Associated Molecular Patterns (DAMPs) (Takeuchi and Akira, 2010). Receptor-ligand binding stimulates intracellular signalling leading to activation of NF-κB, activator protein 1, IFN regulatory factors (IRFs) and MAPKs, which induces recruitment and activation of neutrophils and other innate immune cells to tissues (Parker and Prince, 2011). Four major types of PRRs have been described these include Toll-Like receptors (TLRs), C-type lectins

receptors, the retinoic acid-inducible gene (RIG)-I-like receptors and NOD (Nucleotide-binding Oligomerization Domain receptors)-like receptors (NLRs) (Takeuchi and Akira, 2010).

### 1.2.1. Toll like Receptors (TLRs)

TLRs are non-phagocytic, signal transducing PRRs that recognise a diverse array of MAMPs (Janeway and Medzhitov, 2002). Members of the TLR family consist of two domains a cytoplasmic Toll/ Interleukin (IL-1) receptor homology (TIR) domain involved in downstream signalling (Janssens and Beyaert, 2003) and an extracellular domain containing leucine-rich repeats (LRR) which mediates ligand binding (Opitz et al., 2010). Within the respiratory system, different host cells express TLRs such as macrophages, dendritic cells (DCs), lung epithelial cells (Opitz et al., 2010). Among different TLRs TLR2, TLR4 and TLR-5 were shown to mediate host defence against PA infection (Ramphal et al., 2008, Raoust et al., 2009, Balloy et al., 2007, Descamps et al., 2012). PA has a variety of MAMPs, among them Lipopolysaccharides(LPS) and flagellin play an important role in signalling bacterial presence by interacting with TLR-4 and TLR-5, respectively (Williams et al., 2010). PA infection induces the expression of TLR2 on airway epithelial cells and the binding of TLR2 regulates the function of gap junction through Ca2+ signalling (Martin and Prince, 2008). An enhanced recruitment of TLR5 to the apical surface of airway epithelial cells (AECs) as well as an increased expression of the TLR5 was observed following incubation with PA flagella (Adamo et al., 2004). Binding of TLR5 to flagella has been shown to activate the MAPKs p38 and (in concert with asialoGM1) ERK1/2 (McNamara et al., 2006, Zhang et al., 2007) and both p38 and ERK1/2 MAPKs are crucial

for IL-8 production by AECs in response to PA (Delgado et al., 2006). Also flagellin facilitates activation of NF-κB which eventually upregulates the expression of matrilysin a matrix metalloproteinase involved in tissue repair and immunity, and the antimicrobial peptide β-defensin-2 in AECs (Cobb et al., 2004). As mentioned above in CF patients a prominent feature of PA strains is the conversion to a mucoid, exopolysaccharide alginate-overproducing phenotype; mannuronic acid polymers which is one of the main components of alginate have been shown to exert immune modulatory response using the TLR2 and TLR4 pathways (Flo et al., 2002). Also among all effector proteins secreted by the PA T3SS ExoS has been shown to activate TLR2 and TLR4 and thus contribute to immune responses (Epelman et al., 2004). After recognizing bacterial ligands TLR signalling relies on the MyD88 adaptor protein that eventually activates NF-kB and produce pro-inflammatory cytokines. Thus in absence of MyD88 mice infected intranasally with PA exhibit increased bacterial counts in lung (Skerrett et al., 2004). Besides PA also rely on Toll-IL-1 receptor domain containing adaptor molecule inducing IFN-β (TRIF) mediates a distinct TLR signaling pathway and induce expression of certain cytokines such as RANTES (also known as CCL5), tumour necrosis factor (TNF) and keratinocyte-derived chemokine ( also known as CXCL1) (Power et al., 2007). All TLRs except TLR3 signal via MyD88 pathway, while TLR3 and TLR4 signal through TRIF pathway (Yamamoto et al., 2003, Feng et al., 2011). During infection Pathogens are able to modify their structures to avoid or regulate immune recognition which has been termed as an “immune evasion strategies”(Cigana et al., 2011). Studies suggest that PA has evolved the capacity to exploit PAMPs modification as a

tool to evade immune response and favour survival in CF patients (Amiel et al., 2010, Cigana et al., 2009).

It has been observed that LPS isolated from *E.coli* is a more potent stimulant than the LPS isolated from PA (Williams et al., 2010). During conversion from acute to chronic infection evolutionary changes in the LPS structure make it less immunogenic and poorer agonist for TLR-4 (Cigana et al., 2009).

### 1.2.2. NOD (Nucleotide-binding Oligomerization Domain receptors)-like receptors (NLRs)

Besides TLR, the NLR family can recognize MAMPs. The NLR family comprises 22 members. All of them have a central nucleotide-binding oligomerization (NOD) domain with different N-terminal effector binding domains including caspase recruitment domains (CARD) and pyrin domains (PYD) (Opitz et al., 2010). A subgroup of NLRs, NLRP1–3, 4 display a pyrin domains and form multi-protein complexes known as inflammasomes. Inflammasomes particularly NLRP3 respond to a multitude of infectious agents including influenza virus, *Staphylococcus aureus*, and *Candida albicans* (Craven et al., 2009, Gross et al., 2009, Allen et al., 2009). PA pilli has shown to activate inflammasome NLRP3 in bone-marrow-derived murine macrophage (Arlehamn and Evans, 2011).

### 1.2.3. C-type Lectins (CLRs)

C-type lectins are considered as a superfamily containing a large group of extracellular metazoan proteins including soluble and membrane-attached proteins with immune and non-immune functions identified by the presence of one or more C-type lectin-like domains (CLTDs) with diverse functions (Zelensky and Gready, 2005, Weis et al., 1998). The CTLDs have a

characteristic double-loop ('loop-in-a-loop') structure which is connected by two highly conserved disulfide bridges situated at the bases of the loops as well as a set of conserved hydrophobic and polar interactions. The second loop is known as the long loop region; it is structurally flexible and is involved in Ca2+ dependent carbohydrate binding and interaction with other ligands (Zelensky and Gready, 2005, Weis et al., 1998). The region involved in Ca2+-dependent carbohydrate binding was identified first as a globular structure in rat mannose-binding lectin (MBL) and was named as carbohydrate recognition domain (CRD) (Weis et al., 1991a).The nomenclature of the CRD was modified based upon crystallographic studies suggesting that the distinct folding structure of the domain is different to any known protein folds hence CRD was named by C-type CRDs (Weis et al., 1991b). Furthermore these domains were then referred as C-type lectin-like domains (CTLD) due to the discovery of non-standard CRDs with no Ca2+ binding ability and the fact that most of them are able to bind non-carbohydrate ligands (Vales-Gomez et al., 2000). CTLDs bind a wide variety of ligands, but as the name suggests, carbohydrates (in various contexts) are the main ligands for CTLDs (Weis and Drickamer, 1996). Based on features including phylogeny and structure CLRs have been classified into 17 groups (Zelensky and Gready, 2005). The section below will be focusing on the CLRs members of Group I (mannose receptor), Group II (i.e. DC-SIGN, dectin-2,) and V (i.e. dectin-1) which have been considered in the present study.

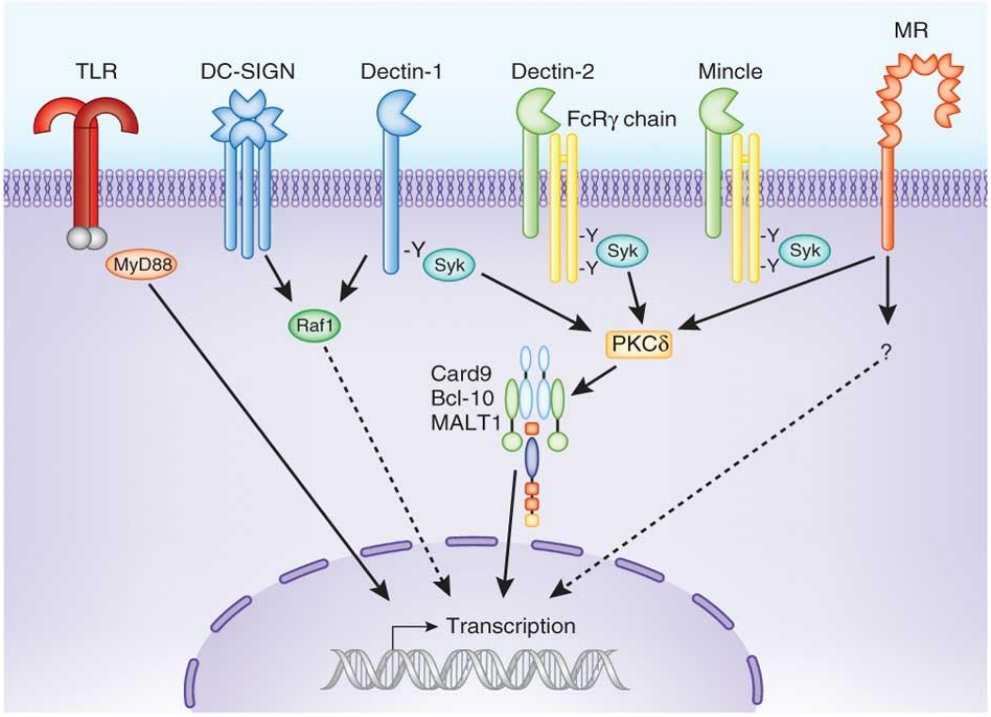



Fig: 1.3 Transmembrane CLRs involved in innate immunity and intracellular signalling pathway

Dectin-1, Dectin-2 and Mincle induce intracellular signalling using tyrosine (Y)-based activation motifs, which recruit and activate Syk kinase either directly or indirectly through the FcRγ adaptor chain. DC-SIGN and Dectin-1 can signal utilizing the Raf-1 kinase pathway, which subsequently regulate (dotted line) other signalling pathways, including those regulated via TLR and Syk pathways. The MR can also mediate intracellular signalling but the mechanism is still unknown. CLR signalling collaborate with that of the TLR induce signalling pathway which synergistically induce or repress the induction of differnt cytokines and chemokines.(Hardison and Brown, 2012)

### 1.2.3.1. DC-SIGN

The 'SIGN' (specific ICAM-3-grabbing non-integrin) family of Group II CLRs include two human DC-SIGN orthologues (i.e. DC-SIGN, and DC-SIGNR) and the eight murine (SIGNR1 to SIGNR8) (Powlesland et al., 2006). The members of the SIGN family contain one CRD region that defines their ligand specificity. DC-SIGN is specifically expressed on phagocytic cells such as dendritic cells (DCs) and on macrophages (Krutzik et al., 2005, Koppel et al., 2005, Tailleux et al., 2005, Lai et al., 2006) Expression of DC-SIGN during the monocyte to DC differentiation pathway depends on IL-4. Furthermore GM-CSF co-operates with IL-4 to produce a high level of DC-SIGN mRNA, whereas IFN-α, IFN-γ, and TGF-β downregulate DC-SIGN expression (Relloso et al., 2002). DC-SIGNR showed 77% amino acid identity with DC-SIGN, both DC-SIGN and DC-SIGNR display differences in their ligand specificities for example DC-SIGN can detect both mannose- and fucose-containing glycans but DC-SIGNR can only detect high mannose oligosaccharides (Powlesland et al., 2006). DC-SIGN binds to high-mannose-content N-glycans and also blood group Lewis antigens (Feinberg et al., 2001, Guo et al., 2004, Mitchell et al., 2001, Geijtenbeek et al., 2002). Carbohydrate profiling studies showed that DC-SIGN can also bind to a wider range of glycan ligands including fucosylated glycans found in many pathogens (Appelmelk et al., 2003, Guo et al., 2004, Naarding et al., 2005, van Liempt et al., 2006).

DC-SIGN was initially identified as a receptor involved in binding intercellular adhesion molecule-3 (ICAM-3) during DC-mediated T-cell proliferation. Now it is well known that DC-SIGN is involved in detection of

multiple pathogens including fungi in a Ca2+-dependent manner (Khoo et al., 2008, Serrano-Gomez et al., 2004). DC-SIGN also detects viruses including Dengue virus, HIV-1, HCV, HCMV and Ebola, and bacteria such as *Mycobacterium tuberculosis* by detecting high-mannose glycans(Geijtenbeek et al., 2000, Alvarez et al., 2002, Halary et al., 2002, Lozach et al., 2004) and *Helicobacter pylori* by detecting fucosylated ligands (Appelmelk et al., 2003). After detection of antigen DC-SIGN mediates internalisation through endocytosis, while its role in phagocytic ability is indirect and has not been characterised conclusively (Koppel et al., 2005, Cambi et al., 2009). It has been shown that pathogens engage DC-SIGN on human DCs and activate the serine and threonine kinase Raf-1 and thus modulate activation of the NF-kB. Activation of Raf-1 is regulated by its phosphorylation at Ser338, Tyr340, and Tyr341 by p21-activated kinases (PAKs) and Src kinases, after its association with GTPase Ras proteins (Gringhuis et al., 2007, Hodges et al., 2007). DC-SIGN modulates TLR-mediated responses at transcriptional level; this cross-talk relies on prior activation of NF-κB by TLR signalling (Gringhuis et al., 2007, Geijtenbeek et al., 2003, Chen et al., 2002).

#### 1.2.3.2. Dectin-2

Dectin-2, also known as CLEC6A, is primarily expressed by tissue macrophages, langerhans cells and DCs and is up-regulated during immune responses (Ariizumi et al., 2000, Taylor et al., 2005b). Dectin-2 performs an crucial role in the activation of various genes, such as genes encoding pro-inflammatory cytokines, by detecting ligands bearing high mannose carbohydrates expressed on various fungal cells (Sun et al., 2013). Dectin-2 has

shown to use the FcRγ chain for syk-mediated signalling pathway to regulate respiratory burst and produce cytokines that eventually promote the *in vitro* differentiation of Th17 cells (Drummond et al., 2011, Strasser et al., 2012). Dectin-2-deficient mice displayed reduce survival rate during infection with *Candida albicans* (Saijo et al., 2010, Saijo and Iwakura, 2011, Vautier et al., 2010). Similar to DC-SIGN dectin-2 consists of a single extracellular CTLD domain followed by a stalk region, a transmembrane domain and a short cytoplasmic domain (Graham and Brown, 2009). Studies demonstrated that both Dectin-1 and Dectin-2 function as the PPRs for *C. albicans* (Saijo et al., 2010, Sato et al., 2006, Willment and Brown, 2008, Drummond et al., 2011, Kerrigan and Brown, 2011, Rogers et al., 2005, Brown, 2011). In these infection Dectin-1 (see below) detects β-glucan in the cell wall of the yeast form of *C. albicans* (Gantner et al., 2005, Brown et al., 2002) and Dectin-2 has been shown to recognize the mannoprotein coat in the cell wall of the hyphal form of *C. albicans* (Saijo et al., 2010, Sato et al., 2006). The signalling pathways triggered by Dectin-2 following *C. albicans* infection are not fully understood. Studies suggest that activation of both Dectin-2 and Dectin-1 by the cell wall components of *C. albicans* can induce Syk (Rogers et al., 2005, Robinson et al., 2009, Underhill et al., 2005) which subsequently activate multiple signalling cascades (Kerrigan and Brown, 2011, Brown, 2011) and activate NF-κB through a CARD9-dependent pathway (Robinson et al., 2009, Gross et al., 2006). Phospholipase Cγ2 (PLCγ2) which is an important signalling component of the B cell receptor signalling pathway, functions downstream of Dectin-2 triggered by the hyphal form of *C. albicans* and deficiency of PLCγ2 results in the ineffective activation of NF-κB and MAPK

and thus limits the production of reactive oxygen species against fungal infection (Gorjestani et al., 2011).

#### 1.2.3.3. Dectin-1

Dectin-1 (βGR) is a major β -glucan receptor expressed by different cell types including DCs, macrophages, monocytes, neutrophils and a subset of T cells (Taylor et al., 2002, Brown et al., 2002). Dectin-1 consists of a single CRD with a short stalk and a cytoplasmic tail that contains an immunoreceptor tyrosine-based activation motif and recognises particles including zymosan, *Saccharomyces cerevisiae*, and *C. albicans* in a β-glucan dependent manner (Brown et al., 2002). After binding of the particulate β-glucans ligands dectin-1 can facilitate the production of many cytokines and chemokines such as TNF-α, CXC-chemokine ligand 2 (CXCL2, also known as MIP2), IL-2, IL-10 and IL-12 (Gantner et al., 2003, Rogers et al., 2005, Steele et al., 2003). Dectin-1 can also promote the respiratory burst and perform ligand uptake through phagocytosis (Herre et al., 2004, Gantner et al., 2003, Underhill et al., 2005). Further biochemical characterization of the specificity of Dectin-1 showed that Dectin-1 recognizes (1,3)-linked β-glucans and that the differential high affinity interactions of Dectin- 1 and β-glucans is depend on the degree of branching and polymer length (Adams et al., 2008, Kimberg and Brown, 2008). Dectin-1 has been shown to perform a protective role *in vivo* during *C. albicans* infection which is strain dependent with many of the responses being mediated in collaboration with TLRs(Gow et al., 2007) . In a mouse model deletion of dectin-1 function during intratracheal challenge with *A.fumigatus*, using a soluble inhibitor significantly decreased lung inflammatory responses and increased fungal burdens (Steele et al., 2005). Work with Dectin 1

knockout mice support the role for this receptor in antifungal immunity in vivo, but there was doubt about the relevant role of this receptor. For example Dectin-1-deficient leukocytes resulted in defects in inflammatory responses including fungal killing with an increased susceptibility to systemic infection with *C. albicans* (Taylor et al., 2007). In a similar study increased susceptibility to this fungal pathogen was observed in mice deficient in CARD9 which is a downstream component of the Dectin-1 signalling pathway (Gross et al., 2006, Kimberg and Brown, 2008). In contrast, other authors showed that dectin-1 is required for inducing immune responses to some fungal infections and provide protective immune response to Pneumocystis but not to *Candida* (Saijo et al., 2007). Similarly no significant differences both in clinical course and cytokine production was observed between dectin-1-deficient and control mice against *C. neoformans* infection (Nakamura et al., 2007).

#### 1.2.3.4. Mannose Receptor (MR)

The MR was initially discovered as a 175kDa endocytic receptor on rabbit AM engaged in the clearance of endogenous glycoproteins in the late 1970s (Wileman et al., 1986). MR was the first member to be discovered as a part of the MR family of C-type lectins, other MR family members include DEC-205, M-type phospholipase A2 receptor (mPLA2R), and Endo-180 (East and Isacke, 2002, Zelensky and Gready, 2005).

Among the C-type lectin superfamily MR family members are unique as they have multiple CTLDs within a single polypeptide backbone. They also possess an N-terminal cysteine-rich (CR) domain, a second lectin domain in MR, followed by a single fibronectin type II domain (FNII) that binds collagen. The

cytoplasmic tails contains motifs that promote transport into the cellular endocytic machinery (Zelensky and Gready, 2005, East and Isacke, 2002). Although MR family receptors share structural similarities they differ in the number of CTLDs within a single polypeptide backbone, eight CTLDs in the case of MR, PLA2R and Endo 180 and ten CTLDs in the case of DEC205. They also have different ligand binding properties that relate with their different roles *in vivo* (East and Isacke, 2002, Zelensky and Gready, 2005).

1.2.3.4.1. MR expression and specificity

Several studies on MR expression in murine tissue have been performed in great detail and compared to its expression on human tissue (Taylor et al., 2005a). MR receptor is expressed by selected populations of DCs and macrophages, hepatic, splenic, lymphatic, and dermal microvascular endothelium (Groger et al., 2000, Martinez-Pomares, 2012) as well as mesangial cells kidney cells, retinal pigment epithelium and trachea smooth muscle cells (Taylor et al., 2005b). An upregulation of MR expression on CD14+ human monocytes was demonstrated in response to *C. albicans* (Smeekens et al., 2011). As an endocytic receptor MR can potentially internalise ligands bound by the three distinct binding regions situated in its extracellular region. MR has been shown to perform a role in homeostatic processes including involved in clearance of endogenous products and also mediate pathogen recognition and antigen presentation (Taylor et al., 2005a). The majority of MR is expressed intracellularly and MR at the cell surface corresponds to only 10-30% of total MR in the cell (Taylor et al., 2005a, East and Isacke, 2002). Regulation of MR expression by different cytokines has been demonstrated in several studies. It has been shown that in recruited

inflammatory peritoneal macrophages MR levels has been elevated in response to cytokines such as IL-4, IL- 10, IL-13 (Stein et al., 1992, Doyle et al., 1994, Martinez-Pomares et al., 2003). In contrast IFN- γ which is involved in classic macrophage activation has been shown to downregulate MR expression (Harris et al., 1992, Cowan et al., 1992). These findings suggest an important role of MR in limiting self-damage and regulating resolution of inflammation. Also MR has been shown to facilitate removal of lysosomal enzymes, neutrophil-derived myeloperoxidase and tissue plasminogen activator and deficiency of MR was associated to higher levels of lysosomal hydrolases in serum (Shepherd and Hoidal, 1990, Noorman et al., 1995, Lee et al., 2002).

1.2.3.4.2. Structural features of MR

Among all members within MR family only MR contains a functional CR domain (Leteux et al., 2000, Martinez-Pomares, 2012). The CR domain binds sulphated carbohydrates such as galactose or GalNAc sulphated in position 3 or 4 in a calcium-independent manner (East and Isacke, 2002, Taylor et al., 2005a, Leteux et al., 2000, Liu et al., 2000). Binding requires Tryptophan 117, a residue that interacts with the galactose ring of the ligand (Taylor et al., 2004). Although FN II domain is the most conserved domain among all members of the MR family, each family member is able to recognize different types of collagen (East and Isacke, 2002). The FN II domain of MR binds collagen type I, II, III, and IV and weakly collagen V, PLA2R detects type I and type IV collagen and Endo-180 recognize collagen types I, IV, and V (East and Isacke, 2002, Martinez-Pomares et al., 2006, Napper et al., 2006, East et al., 2003, Wienke et al., 2003).A recent study showed that the FNII domain of DEC205 does not bind collagen (Jurgensen et al., 2014).

MR mediates collagen internalisation by both human and mouse macrophages (Martinez-Pomares et al., 2006, Madsen et al., 2011). MR CTLDs are responsible for the binding to sugars terminated in mannose, fucose or N-acetyl glucosamine (Taylor et al., 2005a, Taylor et al., 1992, Taylor and Drickamer, 1993). It has been shown that multiple CTLDs are necessary to enhance avidity of binding, thus the CTLD4-8 construct was found able to interact with multivalent ligands with affinity similar to that of the whole receptor (Taylor et al., 1992, Taylor et al., 2005a). Characteristically, MR is able to recycle between the plasma membrane and early endosomes. Recycling is mediated by an endocitosis motif present in the cytoplasmic tail, similar to that of the low density lipoprotein (LDL)-receptor (Schweizer et al., 2000, Taylor et al., 2005a, East and Isacke, 2002). Thus rapid internalisation and recycling from the endosomal system back to the cell surface is regulated by a di-aromatic sequence consisting of the tyrosine present in the endocytosis motif with an adjacent phenylalanine (Schweizer et al., 2000).

1.2.3.4.3. Role of MR in immunity

MR functions as a PRR through its CTLDs and is able to interact with numerous pathogens including *C. albicans* (Martinez-Pomares et al., 1998, Marodi et al., 1991), *P. carinii* (O'Riordan et al., 1995), HIV (gp120) (Nguyen and Hildreth, 2003), mycobacterial lipoarabinomannan (Tailleux et al., 2003), Leishmania (Chakraborty et al., 2001, Chakraborty et al., 1998), *Streptococcus pneumoniae* (capsular polysaccharide, selected strains) (Zamze et al., 2002) and *Klebsiella pneumonia* (endotoxin, selected strains) (Zamze et al., 2002).

1.2.3.4.4. Phagocytosis

There is limited information about the role of MR in phagocytosis. MR was involved in the phagocytosis of pathogens including *P. carinii* (Ezekowitz et al., 1991), yeast (Ezekowitz et al., 1990), Leishmania mexicana amastigotes (Peters et al., 1995), *M. tuberculosis* (Kang et al., 2005), *Francisella tularensis* (Schulert and Allen, 2006) and *C. albicans* (Marodi et al., 1991).Chinese hamster ovary (CHO) cells expressing human MR were found to have a functional MR-mediated endocytic capacity but failed to phagocytose particulate ligands such as zymosan, *Mycobacterium kansasii*, or mannosylated latex beads (Le Cabec et al., 2005). Also normal host immune response was observed in MR deficient mice in response to *C. albicans* (Lee et al., 2003). Similarly although MR is able to recognize mannose residues on the surface of Leishmania parasites, no differences in clinical course was observed between MR-deficient and WT mice (Akilov et al., 2007).

Furthermore engagement of MR was associated with suppression of phagosome-lysosome fusion when macrophages phagocytosed glycopeptidolipid (GPL) (isolated from *Mycobacterium avium*) coated heat-killed *Staphylococcus aureus* (SA) (Shimada et al., 2006). Also engagement of the MR by mannose-capped lipoarabinomannan (ManLAM) during the phagocytic process is a key step which limits phagosome-lysosome fusion (Kang et al., 2005). In contrast another study showed that MR mediates uptake of pathogenic and nonpathogenic mycobacteria, but this MR-dependent phagocytosis is not associated with activation of NADPH oxidase or with the maturation of phagosomes (Astarie-Dequeker et al., 1999).

In support for a role for MR in phagocytosis regulation of MR in monocytes-derived macrophages by exposure to IL-4 is associated with increased *Francisella tularensis* Ft phagocytosis, as did expression of MR in J774 (murine macrophage cell line) cells. MR involvement was confirmed by the use of an MR-specific antibody (Schulert and Allen, 2006). Similarly transfection of the MR complementary DNA into Cos-I cells enabled phagocytosis of *C. albicans* and *P. carinii* (Ezekowitz et al., 1990, Ezekowitz et al., 1991). Thus all these studies suggested that the effect of MR on phagocytosis and phagosome maturation might depend on the cell-type used.

Studies performed on MR phagocytosis showed that the cytoplasmic tail is crucial in phagocytosis; both MR transmembrane and cytoplasmic domains are required (Kruskal et al., 1992).

1.2.3.4.5. MR role in antigen processing and presentation

Expression of MR is a characteristic of immature human monocyte-derived DCs and MR was identified as an important system that contributes to antigen (Ag)-internalization in these cells and facilitates Ag presentation through the MHC II and CD1b pathways. Thus targeting MR has been suggested as a way to increase Ag immunogenicity (Taylor et al., 2005a, Gazi and Martinez-Pomares, 2009). Co-localisation of MR with CD1b and lipoarabinoman (LAM) in MIIC (compartment where antigen is loaded on MHC class II molecules) was observed (Prigozy et al., 1997). A distinct localisation of MR and MHC class II molecules was reported by (Engering et al., 1997). These differences could be explained by the fact that different types of MR ligand were used for these studies; mannosylated-BSA vs. LAM. Also there might have been

engagement of additional PRR including DC-SIGN and dectin-2 which share similar ligands with MR.

1.2.3.4.6. Intracellular signalling

As described earlier MR is able to recognize both endogenous molecules and pathogens, thus participate in maintaining physiological balance between homeostasis and immunity.

MR has also been suggested to play an important role in intracellular signalling and has been involved in regulation of gene expression in several studies (Zhang et al., 2004, Tachado et al., 2007, Yamamoto et al., 1997, Fernandez et al., 2005, Lopez-Herrera et al., 2005, Zhang et al., 2005, Chieppa et al., 2003). Human alveolar macrophages (AM) are able to recognize unopsonised *Pneumocystis carinii* organisms mainly through MR leading to phagocytosis, release of reactive oxygen species, activation of NFκB and production of IL-1β, IL-6, and TNF-α. MR requires assistance of other PRRs in order to activate any signalling cascade (Shibata et al., 1997, Zhang et al., 2005, Tachado et al., 2007). One potential PRR that MR is suggested to cross-talk with is TLR2. Incubation with *Pneumocystis carinii* does not induce IL-8 production by non-phagocytic human embryonic kidney (HEK)293 cells transfected with human MR cDNA whereas HEK293 cells released IL-8 when co-transfected with both human TLR2 and human MR cDNA. It was suggested that after binding with the pathogen MR might form a functional complex with TLR2 on the cell surface and facilitate signal transduction (Tachado et al., 2007). Similarly MR has been shown to perform a crucial role as a phagocytic receptor for unopsonized PA and collaborates with TLR2 to produce pro-inflammatory cytokine including TNF-α and IL-1 β by human monocytes in response to PA

infection, also activates NF-κB and MAPK pathways and facilitates TLR2-mediated signalling in MR transfected HEK293 cells (Xaplanteri et al., 2009). In addition MR was found to directly participate in induction of a regulatory phenotype in human DC; DC incubated with specific anti-MR mAb were found unable to release Th-1 recruiting chemokines and rather produce Th2 and T regulatory cell recruiting chemokines. But not all ligands were shown to exert the same effect on these cells. Ligands such as MUC III (a partially purified mucin), biglycan (a purified complex proteoglycan), and Man-LAM from *M. tuberculosis* induce production of IL-10, IL-1R antagonist, IL-1R type II, and decrease production of IL-12 in LPS-maturing DC, whereas ligands such as mannan, dextran, and thyroglobulin had no significant effect on cytokine production (Chieppa et al., 2003). It was suggested that the effect of Man-LAM is likely directly related to inhibition of NF-κB activation and dampening of TLR signalling. Indeed Man-LAM limits IL-1 receptor-associated kinase (IRAK)-TRAF6 interaction and IκB-α phosphorylation and directly attenuates nuclear translocation and DNA binding of c-Rel and p50. It was proposed that Man-LAM exerts of these effects by stimulating the expression of IRAK-M, a negative regulator of TLR signalling (Pathak et al., 2005). However there are multiple manuscripts showing that MR-deficiency in mice does not lead to increased susceptibility to infection. For example MR deficient mice showed no difference in phagocytosis and recruitment of inflammatory cells and humoral response towards *C. albicans* (Lee et al., 2003). Similarly MR deficient mice didn't showed increased susceptibility against *P.carinii* (Swain et al., 2003)

## 1.3. Role of innate immune cells during PA infection

Progressive and chronic lung disease is the major cause of morbidity and mortality in CF patients and the majority of chronic pulmonary infections in these patients are caused by PA (Chmiel et al., 2002, Gibson et al., 2003, Hauser et al., 2011). In CF patients, high systemic and pulmonary expression of the T helper cell 1 (Th1) cytokine Interferon-gamma (IFN-γ) correlates with better pulmonary function whereas expression of the T helper cell 2 (Th2) IL-4 correlates with chronic PA infection and poor pulmonary function (Moser et al., 2000, Moser et al., 2005, Hartl et al., 2006). Although the adaptive immune response is very important in shaping up the outcome of PA infection the present study will focus on innate immune response which is the first line defence against pathogens. There are three important innate immune cells mainly involved in mediating host defence against PA infection: these are Airway Epithelial Cells (AECs), neutrophils and macrophages. This chapter will only focus on the role of neutrophils and macrophages in PA infection especially in context of chronic infection in CF patients. Within this condition the role of neutrophils has been the focus of attention due to the presence of numerous neutrophils and their failure to eradicate PA biofilm form CF airways (Bjarnsholt et al., 2009). The role of macrophages regarding CF pathophysiology has been largely overlooked. Macrophages might have an important role in the initial stages of CF disease and dysfunction of macrophages may have a role in initiation of cascade of events which leads to develop chronic infection/ inflammation in these patients (Simonin-Le Jeune et al., 2013). Alveolar macrophage is the lung resident macrophages plays a critical role providing defence against infection and it has been reported an

elevation of alveolar macrophage numbers in the lung of the CF patients (Brennan et al., 2009, Aberdein et al., 2013).

### 1.3.1. Role of neutrophils in PA infections

Neutrophils act as potent phagocytic cells against extracellular bacteria and fungi (Segal, 2005) and originate from the bone marrow from myeloid precursor cells. Neutrophils constitute about 50–70% of the circulating white blood cells (WBC) in humans and only 10–25% in mice (Doeing et al., 2003, Mestas and Hughes, 2004). The proliferation and differentiation of neutrophils from the bone marrow is regulated by the granulocyte colony stimulating factor (G-CSF) which is synthesized in response to IL-17 A (IL-17A). IL-17A can be produced from a variety of T subsets including Th17 cells, γδ T cells, natural killer (NK) T cells and a subset of CD8+ cytotoxic T cells (Kondo et al., 2009). In response to infection neutrophils get mobilised into the blood stream and are recruited to site of infection, and migrate across the endothelium by adhering to vascular endothelium. This process involves a number of adhesion molecules expressed on endothelial cell surfaces in response to various cytokines and chemokines (Balamayooran et al., 2010). Initial recruitment, activation and subsequent survival of neutrophils can be induced by AECs and macrophages through the release of cytokines including G-CSF, GM-CSF, IL-8, and TNF-α, neutrophils themselves can promote further neutrophils recruitment on at site of infection by releasing IL-17A and IL-8 (Linden et al., 2005, Mantovani et al., 2011).

Different mechanisms are used by neutrophils to kill pathogens including the respiratory burst, enzymes that can be released through degranulation of neutrophils granules such as elastase, lysozyme, cathepsin G, azurocidin, and

antimicrobial peptides such as defensins (Smith, 1994, Papayannopoulos and Zychlinsky, 2009).

Similar to other infectious agents neutrophils are quickly recruited to the infection site in response to PA to aid subsequent clearance. Several studies have shown the unequivocal role of neutrophils in control of PA infection. For example neutrophil-depleted mice are more susceptible to PA infection even in presence of low numbers of bacteria (Koh et al., 2009). Similarly neutrophil depletion was associated with higher mortality in PA-infected mice (Tsai et al., 2000). Fujimoto et al have demonstrated the important role of neutrophils in the defence towards PA infection using murine models of PA induced sepsis (Fujimoto et al., 2002).

Neutrophil elastase is a major contributor to PA infection-associated host tissue inflammation and damage (Hirche et al., 2008, Meyer and Zimmerman, 1993, Lee and Downey, 2001). A mouse model showed that elastase is required for maximal intracellular killing of PA by neutrophils. It was suggested that neutrophil elastase degrades PA major outer membrane protein F which acts as a PAMP and maintains PA structural integrity (Hirche et al., 2008).

In order to provide effective protection against PA airway infection early recruitment of sufficient numbers of neutrophils as well as efficient bacterial killing by these cells at the site of infection is important (Lavoie et al., 2011). Among CF patients a Th17-biased immune response has been suggested to contribute to the deterioration of lung function (Singh et al., 2015). CF airways contain abundant neutrophils which are believed to be the primary mediators for pulmonary injury in this disease and IL-17A is considered involved in co-ordinating neutrophil accumulation in this disorder (Khan et al., 1995, Hayes et

al., 2011, Linden et al., 2005). In CF patients dysfunction of CF transmembrane conductance regulator (CFTR) function was found associated with decreased phagocytic capacity through reduced intraphagolysosomal HOCl production and thus results in defective killing of PA (Painter et al., 2006, Painter et al., 2010, Bonvillain et al., 2010, Young et al., 2011). An extracellular structure named neutrophil extracellular traps (NETs) has been found as an alternative mechanism of bacteria killing (Young et al., 2011). Among CF patients, NET-mediated killing was found effective against PA (Young et al., 2011).

### 1.3.2. Role of Macrophages in PA infection

Macrophages perform a crucial role in immunity by initiating and regulating innate immune responses. They act as scavengers of foreign particles and as sentinels able to detect infectious agents and orchestrate the inflammatory response. In any lung pathology, alveolar macrophage provides the first line of defence.

Depending upon the stimulation from neighbouring cells and components of extracellular matrix, resident macrophages can show high degree of heterogeneity (in morphology, phenotype and life span)(Gordon and Taylor, 2005). Macrophages can follow different activation pathways; these include the classical activation pathway that takes place in response to the combined effect of the Th1 cytokine IFN-γ and a microbe or microbial product such as LPS (Mosser, 2003). Macrophages activated by this pathway have high microbicidal activity and are associated with pro-inflammatory cytokine release and cellular immunity; whereas macrophages activated through the alternative pathway in response to Th2 cytokines such as IL-4 and IL-13 are

less microbicidal and inflammatory (Mosser, 2003, Gordon, 2003, Nathan, 1991). Several reports suggested that macrophages could potentially control the growth of PA via phagocytosis, production of reactive oxygen species, release of hydrolases and iron sequestration (Speert et al., 1988, Speert and Gordon, 1992, Heale et al., 2001, Kannan et al., 2008). In contrast recent study by our laboratory showed that macrophages activated with IFN-γ in the presence and absence of GM-CSF were unable to control PA growth or protect macrophages from PA-induced cytotoxicity (Singh et al., 2015). Various receptors that have been proposed to be involved in PA phagocytosis including complement receptor 3 (CR3), the LPS co-receptor CD14, MR, TLR5 and protease-activated receptor 2 (PAR2) (Heale et al., 2001, Descamps et al., 2012, Speert et al., 1988, Moraes et al., 2008). The pulmonary surfactant proteins A and D (SP-A and SP-D) and antimicrobial peptide Trappin-2 has shown to induce receptor-mediated uptake of PA by macrophages (Kuroki et al., 2007, Wilkinson et al., 2009). Also there appears to be a requirement for glucose in mediating non-opsonic phagocytosis of PA by both murine and alveolar macrophages (Speert and Gordon, 1992, Wong et al., 1999). An report showed that non-opsonic phagocytosis of PA and subsequent superoxide release by murine alveolar macrophages rely upon activation of Lyn (an Src family tyrosine kinase) and the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, although the receptors responsible for Lyn phosphorylation remained unidentified (Kannan et al., 2008).

Murine alveolar macrophages secrete chemokines and cytokines (TNF-α and IL-6) upon activation of TLR-4 and TLR-5 by PA LPS and flagellin, respectively (Raoust et al., 2009). Alveolar macrophages respond to PA T3SS

by activating caspase-1 and inducing IL-1β processing via the NLRC4 (IPAF) inflammasome (Mijares et al., 2011).

Besides the intrinsic phagocytic function alveolar macrophages recruit other phagocytic cells during PA infection (Cheung et al., 2000). AM were found phagocytosis-competent *in vitro* but not effective against unopsonized PA (Cheung et al., 2000). CF patients infected with PA showed a higher percentage of M2 macrophages in their lungs and this inversely correlates with pulmonary function (Murphy et al., 2010) It was suggested that in CF patients, the phagolysosomes within macrophages were found defective in acidification, thus allowing the bacteria to survive (Di et al., 2006).

## 1.4. Hypotheses and aims

We hypothesise that (i) recognition of PA biofilms by innate immune cells will be influenced by inflammatory conditions; (ii) different receptors will contribute to the recognition of PA biofilms and planktonic PA and (iii) the dispersal molecule produced during PA biofilm formation termed *cis*-2-decenoic acid (CDA) will influence the activation of immune cells.

To test these hypotheses this project will study biofilm formation in the presence of inflammatory cytokines, analyse the interaction of PA biofilms with human monocytes and macrophages and determine the effect of CDA on the activation of human monocytes and macrophages.

# Chapter 2

## Materials and Methods

### 2.1. Bacterial strains, plasmids and culture conditions

The strains used in this study are listed in Table 2.1. All PA strains were routinely grown in X-Vivo 15 (LONZ04-744Q, LONZA, UK) medium. *P.aeruginosa* (PAO1) Lausanne strain (PA-GFP) expressing green fluorescent protein (GFP) grown in X-Vivo 15 was used for biofilm assays. PAO1-N wild-type (WT) and the following reporter strains: *lasB::lux, lasI::lux, rhlA::lux, rhlI::lux, pqsA::lux* and *lecA::lux* were used to determine expression of QS molecules and Virulence factors in LB broth vs X-Vivo 15 medium as reporter strains required were not available in the PAO1-L background. These reporter strains had been generated previously by members of the Quorum Sensing Group, University of Nottingham by chromosomal fusion of the *luxCDABE* genes to the promoters of the genes under study.

Table 2.1 List of bacterial strains and plasmid used in this present study

| **Strains** | Relevant characteristics | Source/Reference |
|---|---|---|
| PAO1-L | Wild-type *P. aeruginosa* from Université de Lausanne, Switzerland | (Holloway et al., 1979) |
| Wt Parsek | Wild-type *P. aeruginosa*<br>Normal cellular level of c-di GMP Expresses psl and pel | (Holloway et al., 1979) |
| PA105 | Δ*wspF* Δ*Pel* Parsek strain mutant with high cellular levels of c-di GMP<br>Overexpresses psl<br>Lacks pel | (Kirisits et al., 2005) |
| Plasmids | | |
| miniTn7(Gm)$P_{A1/04/03}$–<br>–*ecfp* | $P_{A1/04/03}$ –*ecfp* cloned into *Not*I site of pBK-miniTn7-ΩGm; Gm; Cm | Lambertsen; Unpublished |

### 2.2. Assessment of quorum sensing molecules and virulence factors

Overnight cultures of PAO1-N WT (5ml) and the reporter strains *lasB::lux, lasI::lux, rhlA::lux, rhlI::lux, pqsA::lux, lecA::lux*, and *pKan::lux* were prepared in LB broth or X-Vivo 15 and adjusted to $OD_{600nm}$ = 1.0 and diluted 1:100 in either LB broth or X-Vivo 15. 200 µl of each diluted culture was added to the wells of a 96-well microtitre plate (Greiner 115 Bio-One, Germany). For each variable triplicate channels were used. The plate was then incubated at 37$^{o}$C for 24 h in a Tecan Infinite® M1000 PRO (Tecan Group Ltd., UK) plate reader which measured absorbance ($OD_{600nm}$) and luminescence (relative light units, RLU) of the cultures every 30 min. To correct for any differences in the growth of different cultures, gene expression was calculated as luminescence divided by absorbance.

### 2.3. Biofilm assays

The bacteria were streaked from glycerol stocks onto an LB agar plate and incubated at 37$^{o}$C overnight. The following day a single colony was inoculated into 5 ml of X-Vivo 15 medium and incubated overnight at 37$^{o}$C with shaking at 200 rpm. The overnight culture was then adjusted to $OD_{600nm}$ = 1, diluted 1:100 in 25 ml of fresh X-Vivo 15 in a 250 ml flask, and incubated at 37$^{0}$C, 200 rpm for 3 hours to get mid-log phase culture. The mid-log phase culture was then diluted in X-Vivo 15 medium to obtain $OD_{600nm}$ 0.04 and use to establish biofilms.

#### 2.3.1. Biofilm assay using the BioFlux system

PA biofilms were grown in a BioFlux™ device (Fluxion Biosciences, South San Francisco, CA) which has a microfluidic platform designed to run automated shear flow (Fig- 2.1). 48-well BioFlux Plates containing 24 shear

controlled microfluidic channels were used for this study. To grow biofilms, inlet wells were primed with 100µl of X-Vivo 15 medium and the microfluidic channels (depth, 70 µm; width, 370 µm) were inoculated with diluted PA cultures ($OD_{600nm}$ 0.04). Presence of cells and movement into the channel was confirmed using light microscope. After 30 min of incubation at 37°C to allow cell attachment, inlet wells were loaded with 1 ml of fresh X-Vivo 15 media and the plate was incubated for 15 hours with a previously optimized shear pressure of 0.05 dyn/cm$^2$ which is equivalent to flow rate 67 µl/hr. Prior analysis PA-biofilms were stained with propidium iodide (8µM in X-Vivo 15, Sigma,) for 30 min at a flow rate of 0.05 dyn/cm$^2$ and were visualised using 20 x magnifications using a Zeiss LSM 510uv META Kombi confocal with a Zeiss Axiovert 100 microscope (Carl Zeiss, Germany).

### 2.3.2. Psl staining within biofilms

To visualize Psl within PA biofilm, PAO1-Parsek and Δ *wspf* Δ *pel* PA105 (Parsek) biofilms were grown in BioFlux channels as above. Biofilms were stained with SYTO9 (FilmTracer Live/Dead Biofilm Viability Kit; LifeTechnologies, USA). Non- attached cells were washed using X-vivo 15 using shear pressure of 1 dyn/cm2. To stain Psl, HHA- Lectin conjugated to Texas Red (EY Laboratories) at a concentration of 20 µg/ml in X-Vivo 15 was added to the inlet and incubated for 1 hour with a shear pressure of 0.5 dyn/cm$^2$. Excess dye was removed using X-Vivo 15 media using a shear pressure of 1 dyn/cm$^2$. Staining was visualised using a Zeiss LSM 510uv META Kombi confocal with a Zeiss Axiovert 100 microscope. Green channel images were acquired using 488 nm excitation and 515 nm emission. Red channel images were acquired using 596nm excitation and 615 nm emission.

### 2.3.3. Incubation of PA-biofilms with cytokines and immune cells

Cytokines either rhGM-CSF (50ng/ml, MiltenyiBiotec, UK) or IFN-ɣ (20ng/ml, R&D Systems, UK) were added to PA-biofilms within BioFlux channels 2 hours post-inoculation. For each variable triplicate channels were analysed to minimize experimental error. Biofilms were analysed after 15 hour incubation. On separate experiments monocytes or macrophages generated from peripheral blood mononuclear cells (PBMCs) as described in Chapter 4 were added to 15 hours grown PA- biofilms within the BioFlux system (n=1); for each variable duplicate channels were analysed. Co-cultures were incubated for 1 or 3 hours.

### 2.3.4. Preparation of supernatants from biofilms generated using the BioFlux system

After 15 hours of incubation supernatants were collected from the outlet wells and centrifuged to pellet cells. Supernatants were stored at – $80^0$C until required.

### 2.3.5. Analysis of QS molecules in supernatants from biofilms generated using the BioFlux system

The extraction procedure to quantify QS molecules was carried out as described previously (Ortori et al., 2011). Briefly, PA biofilm supernatants (1.0 ml) were centrifuged at 8,500×g for 10 min. Therefore A volume of 5 µl of d9 C5-HSL (10 µM stock) was used as an internal standard and was added alongside 1.0 ml of acidified (0.01% acetic acid) ethyl acetate to the biofilm supernatant. This mixture was vortex mixed for 1 min and thus the organic phase was removed. This extraction step was repeated twice and finally the pooled thyl acetate extracts was evaporated to dryness. The dried samples were

stored at −80 °C until use and reconstituted in 50 µl methanol immediately prior to LC-MS/MS analysis.

QSSMs analysis was conducted using a LC-MS/MS at a flow rate of 0.45 ml min−1 (Shimadzu series 10 AD VP, Columbia, MD, USA) and a Phenomenex Gemini Column C18, (150×2 mm) with a 5 µm particle size was maintained at 50 °C. The HPLC system was used as the mobile phase A developed using 0.1% formic acid (Sigma-Aldrich, Gillingham, UK) and 200 µM EDTA (Sigma-Aldrich, Gillingham, UK) in water and a mobile phase B developed using 0.1% formic acid (Fisher Scientific, Loughborough, UK) in acetonitrile (Fisher Scientific, Loughborough, UK). Mass spectrometry (MS) was carried out on a 4000 QTRAP hybrid triple-quadrupole linear ion trap mass spectrometer (Applied Biosystem, Foster City, CA, USA). Data acquisition and processing were processed using Analyst software (version 1.4.1) (QSSMS analysis was performed by Nigel Halliday).

A B C

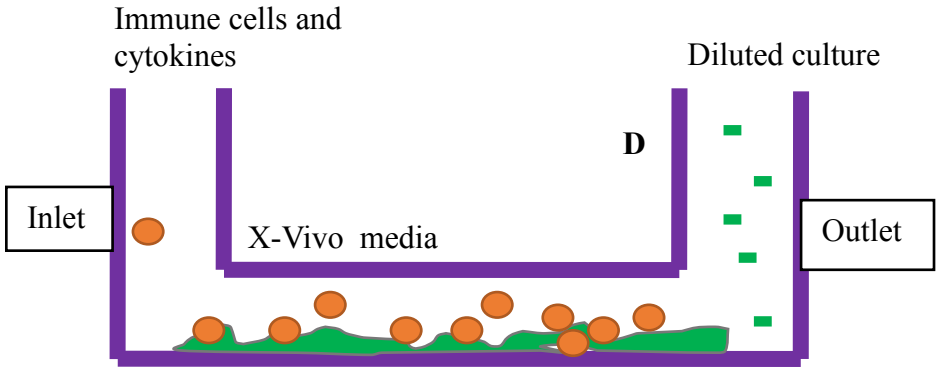



Fig: 2.1 BioFlux systems for biofilm generation under flow conditions

(**A**) Photograph of the BioFlux system (**B**) A diagram showing the BioFlux plate containing 48 wells and 24 independent channels connecting pairs of wells. (**C**) Close-up view of two microfluidic channels (black lines). Each channel has a serpentine part (one serpentine part is enclosed within a box) to supply enough back pressure and a chamber for microscope viewing (**D**) Diagram showing the inlet and outlet wells respectively. Pneumatic pressure was delivered to the top of the inlet well to pushes fresh medium through the microfluidic channel containing PA biofilm and into the outlet well. The PA biofilm can be viewed with a microscope.

### 2.3.6. Confocal Analysis

Confocal microscopy was performed using a Zeiss LSM 510uv META Kombi confocal with a Zeiss Axiovert 100 microscope (Carl Zeiss, Germany) under 20x magnification. GFP-tagged biofilms were excited at 488 nm and collected with a Long Pass LP 505nm filter.

Using the visual mode, the biofilm were first visualised by phase contrast microscopy at the inlet of the channel. The microscope was then adjusted to LSM mode. After that fine focus was adjusted to detect the area of maximum brightness using fast scanning. The detector gain and amplifier offset were then adjusted at this location in order to reduce over (red pixels) and under saturation (blue pixels) of all images which was found comparable within each experiments.

At the beginning of the inlet of the channel when the biofilm first appeared a Z series of the first image was collected. The focus was then moved to the top of biofilm to set the last image of the Z series. Within the Z series interval between slices was between 1-2 µm. In total 5 Z stacks were captured within each channel started from the inlet towards the outlet. Images were processed using Zen 2009 (Zeiss, Germany).

### 2.3.7. COMSTAT image analysis

Confocal images of biofilms were analysed using the COMSTAT software package (Heydorn et al., 2000). The program was written as a script in MATLAB 5.1 (The Maths Works Inc., Natick, Massachusetts), which is equipped with the Image processing Toolbox.

Before starting COMSTAT, the CONVERT000 program was used to name the image stacks in the right format. The CHECKALL program makes sure that

the whole image stacks were unaltered. Threshold values were set with the support of the LOOK and LOOKTIF program. Adjusting threshold makes an image stack into a 3D matrix with a value of one in position where the pixel values in the original image were above or equal to the threshold value and ZERO where the pixel values were below the threshold value. The value ONE is considered biomass and ZERO exhibit background. The threshold was adjusted using a random selection of some images. The most representative threshold value was chosen subjectively after examining LSM acquired images. This value was then applied to all images.

A total of 11 different statistical parameters defining biofilm 3D structure can be generated using the software. For this study, three parameters, biomass, average thickness and surface coverage were used to assess potential differences among biofilms.

### 2.3.8. Static biofilm assay

Mid-log phase PA culture was adjusted to $OD_{600nm}$=0.04 in X-Vivo 15 medium,100µl of diluted culture was added to 96-well polystyrene microtitre plates (Costar) and was incubated at 37°C for 4 and 24 hours in presence 5% $CO_2$. To quantify PA biofilm formation, microtitre plates were washed 3 times with HPLC water. The washing procedure is as follows: HPLC water was dripped with care down the side wall of each well using a Pasteur pipette. The washing solution was then removed from the very edge of the well by tilting the plate and by inserting the prong (attached to the suction pump on its lowest power). This aimed to minimize damage to the PA biofilm. Afterthat PA biofilm was stained with 125µl of crystal violet solution (1% in HPLC water) and was incubated for one hour at room temperature. The wells were then

washed with HPLC water three times to remove excess dye. 70% ethanol (200µl) was used to solubilise the crystal violet. Finally 125µl of this solution was transferred to a new polystyrene microtitre plate and the absorbance was measured at 595 nm on a plate reader. Novel antimicrobial agents are continuously being investigated for potential anti biofilm activity and for this besides microscopic analysis a colourimetric microtitre plate method has been widely used by numerous research groups. Thus this procedure was optimized based on previous studies (O'Toole, 2011, Guo et al., 2014, Parks et al., 2009).

### 2.3.9. Biofilm Binding Assay (Plate assay)

The following binding assay was adapted from a protocol established in (Zamze et al., 2002). At first biofilms grown for 24 hours in 96 well microtitre plates (Costar) were fixed using 2% formalin at 4°C for 10 minutes. Afterthat the microtitre plates were washed with Tris-buffered saline (TBS) three times. After washing with TBS, 50ul of CTLD4-7-Fc/ CR-FNII-CTLD1-3-Fc proteins (10 and 2ug/ml in TBS) was added and the plate was incubated for two hours at room temperature. Furthermore the washing step was performed with TBS; therefore anti-human IgG Fc-specific conjugated to alkaline phosphatase (Sigma, dilution 1:1000) was added and incubated for one hour at room temperature. The washing step was repeated again using TBS and Alkaline Phosphatase (AP) buffer. The plate was then developed using 50uL of p-nitrophenyl phosphate substrate (Sigma) solution (one tablet per 15 mL (1 mg/mL) prepared in AP-buffer. Absorption was then measured at 405nm on a plate reader.

### 2.3.10. Biofilm Binding Assay without fixation (BioFlux channel)

Biofilms grown within BioFlux channels using previously discussed method were stained with SYTO9 (FilmTracer Live/Dead Biofilm Viability Kit; Life Technologies, USA) for 30 minutes using a shear pressure of 0.05 dyn/cm$^2$. Non- attached cells were washed using X-Vivo 15 media using a shear pressure of 1 dyn/cm$^2$. CTLD4-7-Fc protein (10 µg/ml in X-Vivo 15) or X-Vivo 15 without CTLD4-7-Fc protein as a negative control was added within the inlet and incubated for 1.5 hour with a sheared pressure of 0.05 dyn/cm$^2$. Washing with X-Vivo 15 was repeated before anti-human IgG Fc-specific conjugated to Alexa fluor 647 (Molecular probes) (10ug/ml in X-Vivo 15) was added. Excess dye was removed using X-Vivo 15 media using shear pressure of 1 dyn/cm$^2$ and biofilms were visualised using 20 x magnifications by a Zeiss LSM 510uv META Kombi confocal with a Zeiss Axiovert 100 microscope (Carl Zeiss, Germany). Alexa fluor 647 images were acquired using 650nm excitation and 668 nm emission.

### 2.3.11. Biofilm Binding Assay with fixation (coverslip-based assay)

PA biofilms were generated on coverslips on 6 well plates. For this, cover slips with a thickness of 0.13-0.17 mm were utilized. PA culture in X-Vivo-15 (1 ml of culture diluted to $OD_{600nm}$ 0.04) was added to the plates. Plates were incubated at 37$^o$C at 60 rpm for 72 hours. Biofilms were then fixed using 4% paraformaldehyde in PBS, for 10 minutes at 4°C. The plates were then washed with TBS three times gently using a Pasteur pipette. The non-attached cells were removed by tilting the plate and inserting the prong (attached to the suction pump on its lowest power) and sucking the solution from one point at the very edge of the well. Biofilm were incubated with CTLD4-7-Fc or CR-

FNII-CTLD1-3--Fc protein (10ug/ml in TBS) or TBS only as a negative control for 1.5 hour. The washing with TBS was repeated before anti-human IgG Fc-specific conjugated to Alexa fluor 647at three different concentrations (10, 5, 2.5 µg/ml) in the presence of 3% Donkey serum (Sigma-Aldrich) in TBS was added and the plate was incubated for a further one hour. Excess dye was removed using TBS. Finally to detect PA, biofilms were stained with Hoechst 33342 (Cell Signalling Technology, UK) (1 ug/mL in PBS) for 15 minutes and were visualised using 60x magnification using a Zeiss LSM 510uv META Kombi confocal with a Zeiss Axiovert 100 microscope (Carl Zeiss, Germany).

### 2.3.12. Extraction of crude EPS from Δ *wspf* Δ *pel* PA105 (Parsek) culture

Extraction of EPS from Δ *wspf* Δ *pel* PA105 (Parsek) cultures was based on (Byrd et al., 2009). PA105 (Parsek) was grown in 140-mm diameter Petri dishes in humidified chambers at 37°C for 24 h in M63 minimal medium ($(NH4)_2SO_4$, 2g/L; $KH_2PO_4$, 13.6 g/L; $FeCl_3$, 0.5 mg/L ) pH 7; supplemented with 0.5% Casaminoacids (Difco), 1 mM MgCl2 and 0.2% glucose (Byrd et al., 2009). The following day cells, growth medium and the transparent gelatinous materials were collected from bottom of the petri dishes. Cells and cells associated matrix were separated from the viscous growth media by centrifugation (6000g, $4^0$C, 30 min) and pellet was re suspended in 0.9% Nacl. Cell associated polymers were separated by mild sonication (3 × 30 s, 50% cycle, and intensity 0.5). The same procedure was repeated twice in order to extract more cell associated matrix. Trichloroacetic acid (TCA) to a final concentration of 5% w/v was added to precipitate extracellular DNA and proteins. Further centrifugation was performed (10,000g, 10 minutes) to collect

the clear supernatant which was dialyzed using 10 kDa MWCO membrane (Vivaflow) and lyophilized. Finally the crude extract was re-suspended in HPLC water in a concentration of 270 mg/ml.

### 2.3.13. EPS Binding Assay

The binding assay was performed using the protocol previously described (section 2.6.4.) (Zamze et al., 2002). MAXISORP 96 well plates (Nunc) were coated with 50 µl of different concentration of EPS in PBS, sealed with parafilm and incubated at 4°C overnight. The following day the plates were incubated with the chimeric proteins as above.

## 2.4. Generation of Primary Human Monocytes

Fresh blood (20ml) was pooled from EDTA vacutainer (Fisher Scientific) (Ethical approval was available and informed consent was obtained from each donor before performing the study) in a 50 ml falcon tube and diluted with 20 ml of Hank's buffer (Sigma). This mixture was layered on 10 ml of Histopaque-1077 (Sigma-Aldrich, UK) in a 50ml falcon tube. Tubes were then centrifuged at 800×g for 30 minutes, acceleration-1, deceleration-0 at room temperature and the PBMC layer was collected. Monocyte purification was performed by washing PBMCs with Hank's buffer three times and incubating in 80 µl of cold Macs buffer (PBS containing 0.5% bovine serum albumin and 2mM EDTA in PBS, pH 7.2, sterile filtered) and 20 µl of CD14 Micro Beads (human) per $10^7$ cells at $4^0$C in the dark for 15 minutes. Monocytes (CD14+ cells) were then selected using MS MACS columns (Miltenyi Biotec, UK) following the manufacturer's instructions. Purified monocytes were resuspended at a density of 1 x $10^6$ monocytes/ml in RPMI-1640-complete

medium [RPMI (Sigma-Aldrich, UK) containing 15 % human AB serum (Sigma-Aldrich, UK or PAA Laboratories Ltd, UK), 2 mM L-glutamine (Sigma-Aldrich, UK), and 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, UK)]. Trypan blue exclusion was performed to assess cell viability.

### 2.5. Generation of primary human macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from leucocyte cones (Blood Transfusion Service, Sheffield, UK) as follows. Cells were collected into a sterile T-75 flask (10 ml) and diluted with 40 ml of Hank's solution (Sigma-Aldrich, UK). This mixture (25 ml) was layered onto Histopaque-1077 (15 ml, Sigma-Aldrich, UK) in 50 ml falcon tubes. Tubes were centrifuged for 30 minutes, acceleration-1, deceleration-0 at room temperature and the PBMC layer was collected. Monocyte purification was performed by washing cells three times with Hank's solution (Sigma). Monocytes (CD14+ cells) were then selected using CD14 MicroBeads (human) and LS MACS columns (Miltenyi Biotec, UK) following the manufacturer's instructions. Purified monocytes were resuspended at a density of 2 x $10^6$ monocytes/ml in RPMI-1640-complete medium containing recombinant human M-CSF (rhM-CSF, 50 ng/ml, R&D Systems, UK), 5 ml of this cell suspension was seeded into T-25 ultra-low attachment flasks (Corning Life Sciences, the Netherlands) and incubated at 37$^o$C, 5% $CO_2$ for 6-7 days. Besides ultra-low attachment flasks, Teflon bottle (Nalgene, UK) (total capacity of each Teflon bottle= 125 ml) were also used for culturing the monocytes following the same culture conditions. In both instances cultures

were fed at day 3 with 1.5 ml per flask of fresh RPMI complete medium containing M-CSF 50 ng/ml.

On Day 7, the culture medium containing suspended cells was first collected and placed on ice. Finally following centrifugation macrophages were re-suspended in RPMI-1640 containing 15% human AB serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES and 50 ng/ml M-CSF (R&D Systems). Before performing further experiments these cells were counted and viability was asccessed by Trphan blue staining when no clumping among these cells was observed.

### 2.5.1. Staining of monocytes and macrophages with PKH 26 red fluorescent dye

Labelling was performed following manufactures recommendations. Monocytes/macrophages were suspended in RPMI with 10 % human AB serum and centrifuged at 350 g for 5 min at $37^0$C. 500 µl of diluents C was added to the cell pellet and gentle pipetting was done to ensure complete dispersion. Cells were incubated for 5 minutes at $37^o$C. A dye solution was prepared by mixing 4µm or 2µm of PKH 26 dye solution (Sigma-Aldrich, catalogue number- PKH26PCL) into 500 µl of diluent C in a polypropylene centrifuge tube. 500 µl of the cell suspension in Diluent C was added to 500 µl of dye solution and mixed immediately by pipetting and incubated for 5 minutes at $37^0$C. Staining was stopped by adding an equal volume of Human AB serum (1ml) and incubating for 1 min at $37^0$ C to allow binding of excess dye.

Cells were centrifuged at 400 ×g for 10 minutes at $25^0$C and re-suspended in RPMI medium. For further experimentation cells were washed with respective

media, as for example for ROS assay labelled cells were washed with PBS containing $Ca^{2+}$ and $Mg^{2+}$ (Invitrogen, UK). To observe the nuclei counterstaining was performed with Hoechst (Cell Signalling Technology) at 1µg/ml in PBS. Finally before adding those on bioflux channel cells were counted and viability was assessed by Tryphan blue staining.

### 2.5.2. Assessment of cell surface marker expression by flow cytometry

Cell surface markers expressed on freshly isolated monocytes and PKH 26-stained monocytes were analysed by flow cytometry. 50,000 cells were washed with 2 ml cold PBA [PBS containing 0.5% bovine serum albumin and 0.1% sodium azide (Sigma-Aldrich, UK)] and then blocked with 5% mouse serum (Sigma-Aldrich, UK) in PBA at $4^{o}$C for 45-60 min. Cells were then washed once with cold PBA and incubated with one of the mouse anti-human monoclonal antibodies or their corresponding isotype controls (Table- 2.2) at the same concentration at $4^{o}$C for 30 min in the dark. Cells were then washed once again with cold PBA and fixed overnight in 0.5% paraformaldehyde in PBS at $4^{o}$C. Samples were analyzed using a Beckman Coulter Epics Altra flow cytometer within one week of staining. 30,000-50,000 events were collected depending upon the initial cell number. Data was processed using Weasel v3.0.1.

Table 2.2 List of mouse anti- human monoclonal antibodies

| Specificity | Conjugate | Source | Catalogue number | Dilution used |
|---|---|---|---|---|
| CD14 | FITC | BC, UK | IM0645U | 1:10 |
| CD11b | FITC | BC, UK | IM0530 | 1:10 |
| CD16 | PE | BC, UK | A07766 | 1:10 |
| CD206 (MR) | PE | BC, UK | IM2741 | 1:10 |

### 2.5.3. ROS production assay

Production of ROS by monocytes was assessed by the luminol chemiluminescence assay. Briefly, 100 µl of luminol solution [67 µg/ml luminol (Sigma-Aldrich, UK) in Buffer A, 20 mM HEPES (Invitrogen, UK) and 0.1% BSA (Sigma-Aldrich, UK) in Hank's buffered saline solution containing $Ca^{2+}$ and $Mg^{2+}$ without phenol red (Invitrogen, UK)] was added to the wells of a 96-well chemiluminescence microtitre plate (BD Biosciences, UK). Next, either zymosan at a concentration of 50 particles/cell or PA at a concentration of 10 live PA/cell were added as stimuli. Mid log phase PA culture in X-Vivo 15 was prepared as above. Counting of the bacteria was done after fixing them with 4 % formaldehyde in PBS using the Thoma bacterial counting chamber (Hawksley, UK). Controls were treated only with Buffer A. Both unlabelled and PKH 26-labelled monocytes were washed three times in PBS containing $Ca^{2+}$ and $Mg^{2+}$ (Invitrogen, UK), resuspended in Buffer A and added to the microtitre plate at a density of 50,000 cells/well immediately prior to measurement. Chemiluminescence was measured at 3-5 min intervals for 2

hr at 37$^{o}$C using a Microlum at LB96P luminometer. All conditions were carried out in duplicate or triplicate.

### 2.5.4. Analysis of pro-inflammatory cytokines and chemokines expression by both unstimulated and LPS stimulated Monocytes and Macrophages in response to CDA

CDA pure compound from (U-Chemo/China) was added at a concentration of 10 µM. Diluent Methanol (HPLC-grade Methanol, Sigma-Aldrich, UK) was used as negative control. Freshly purified monocytes or day 7 macrophages isolated from leucocytes cones were re-suspended in RPMI complete medium, for monocytes $5\times10^{5}$ cell per well in 500 µl and macrophages 250,000 macrophages per well in 500 µl was used. Cells were stimulated with LPS 100 ng/ml (Enzo Life-science) in presence or absence of CDA 10 µM, TDA 10µM or Methanol. For monocytes 1, 3 hours and for macrophages 3, 6 hours incubation was performed at 37$^{o}$C, 5% $CO_2$.

### 2.5.5. Relative quantification of gene expression

#### 2.5.5.1. RNA isolation

RNA was extracted from monocytes and macrophages using the RNA Miniprep Kit (Sigma-Aldrich, UK) according to manufacturer's instruction. Briefly the cells were lysed in reconstituted Lysis Buffer containing β-mercaptoethanol and homogenised by pipetting. Homogenates were then transferred to a Pre-filter Spin column and centrifuged. Filtrates were collected and mixed with an equal volume of 70% ethanol and vortexed. Afterwards the mixture was transferred to the RNA Binding Spin Cup and centrifuged. Following a salt-gradient extraction from low salt to high salt washes, RNA

was eluted and resuspended in elution solution (Sigma-Aldrich, UK). The RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, UK). A260/A280 ratio ranging between 1.9 and 2.1 was considered acceptable.

#### 2.5.5.2. Reverse transcription to cDNA

The quantity of RNA from all samples was normalized to 0.5 µg before reverse transcription. Using the manufacturer's instruction cDNA strand was synthesized using random hexamers and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Life Technologies, UK). In brief RNAs were mixed with random hexamers (Life Technologies, UK) and incubated at 70$^{o}$C for 5 minutes to eliminate any secondary structures that may have formed. The product was then incubated in ice for 5 min to limit reformation of secondary structures. Bulk mix was then added (composed of 5x first strand buffer (250 mM Tris-HCl, [pH 8.3]. 375 mM KCl, 15 mM $MgCl_2$ and 0.1 M of dithiothreitol) to the template and primer mixture. Afterwards the reverse transcriptase was added to the whole mixture right before starting the reaction. Mixtures were incubated at 37$^{o}$C for 1 hour. Finally the cDNA was incubated at 95$^{o}$C for 10 minutes to inactivate the reverse transcriptase. Reverse transcript negative controls were also performed. cDNA were stored at -20$^{o}$C until quantitative real-time PCR analysis was performed.

#### 2.5.5.3. Quantitative real-time PCR

For quantitative real-time PCR (qPCR) amplification assay mixtures (25 µl) of 2x SYBR green I qPCR master mix with 2.5 mM $MgCl_2$ (Stratagene, UK) along with template cDNA 5µl were used. A reference dye ROX was used in order to correct the pipetting error. An Agilent Mx3005P qPCR system was used to run the reactions. The cycling conditions were 2 min at 95°C and 40

cycles including 95°C for 10 sec, and 59$^{o}$C for 33 sec. Triplicates were performed for each sample. Primer efficiencies were determined by a standard curve with templates in 4-log serial dilutions and efficiencies between 99-110% are considered appropriate. HPRT expression was used as house keeping control.

Table 2.3 Primers used in this study

| Primer(Accession number) | Primer Sequences |
|---|---|
| **NM_000194.2** | |
| HPRT_f | GTAATGATCAGTCAACGGGGGAC |
| HPRT_r | CCAGCAAGCTTGCAACCTTAACCA |
| **NM_000758.3** | |
| GM-CSF_f | TCTVAGAAATGTTTGACCTCC |
| GM-CSF_r | GCCCTTGAGCTTGGTGAG |
| **NM_000600.3** | |
| IL-6_f | GATGAGTACAAAAGTCCTGATCCA |
| IL-6_r | CTGCAGCCACTGGTTCTGT |
| **NM_000594.3** | |
| TNFaH_f | GACAAGCCTGTAGCCCATGT |
| TNFaH_r | TCTCAGCTCCACGCCATT |
| **NM_000576.2** | |
| IL-1β_f | TACCTGTCCTGCGTGTTGAA |
| IL-1β_r | TCTTTGGGTAATTTTTGGGATCT |
| **NM_000584.3** | |
| IL-8_h_f | AGACAGCAGAGCACACAAGC |
| IL-8_h_r | ATGGTTCCTTCCGGTGGT |
| **NM_002982.3** | |
| MCP-1_f | AGTCTCTGCCGCCCTTCT |
| MCP-1_r | GTGACTGGGGCATTGATTG |
| **NM_174088** | |
| IL-10_ f | ACT GCA CCC ACT TCC CAG T |
| IL-10_r | TGT CCA GCT GGT CCT TTG TT |

### 2.5.5.4. Cytokine protein quantification

**Enzyme-linked immunosorbent assay (ELISA)**

The levels of TNF- α, IL-10 and IL-8 in the supernatants of Macrophages cultures were quantified following manufacturer's instructions (R&D, UK). Culture supernatants were centrifuged to remove cell debris. Between each step, solutions were aspirated and the wells were washed three times with 400 µl wash buffer (0.05% Tween-20 (Sigma-Aldrich, UK) in PBS, pH 7.2-7.4). Wells of 96-well MAXISORP surface microplates (Thermo Scientific, UK) were coated with 100 µl of mouse anti-human capture antibody and incubated at room temperature overnight. The solution was aspirated and wells were washed three times with 400 µl of wash buffer. Each well was blocked with 300 µl of Reagent Diluent (1% bovine serum albumin of protease free/ fraction V grade (Millipore, US) in PBS, pH 7.2-7.4, 0.2 µl filtered) for 1 h at room temperature. 100 µl of samples and standards was then added and incubated for 2 h at room temperature. Wells were aspirated, washed and 100 µl of the Detection Antibody (biotinylated goat anti-human TNF-α/ IL-10, IL-8) was added and incubated for 2 h. After washes, 100 µl of horseradish peroxidase conjugated (HRP)-streptavidin at working concentration (1:200) was added to each well and incubated for 20 min. 100 µl of substrate solution containing 1:1 mixture of tetramethylbenzidine (TMB) and $H_2O_2$ was then added and the plate was incubated in dark for 20 min. 50 µl of 2N $H_2SO_4$ was added to each well to stop the reaction. OD at 450 nm was measured. The concentration of Cytokines in samples was calculated according to the equation obtained from the standard curve readings.

### 2.6. Preparation of CTLD4-7-Fc

The CTLD4-7-Fc strain of *E. coli* was taken from glycerol stocks (Martinez-Pomares et al., 2006) and streaked onto an LB agar plate containing antibiotic (ampicillin 50 µg/ml) and incubated at 37°C overnight. From the overnight plate, a colony was picked and added to a sterile tube containing LB broth (10ml) and 100µl of ampicillin (50 µg/ml ), then incubated at 37°C overnight in a shaker (200rpm). The overnight culture (1.3ml)was transferred to a flask containing LB broth (125ml) and 125µl of ampicillin (50 µg/ml) and incubated overnight at 37°C in a shaker (200rpm).This overnight culture was used to purify the plasmid using the Nucleobond® EF PC/AX 500 kit. The plasmid stock was stored at -80°C freezer.

#### 2.6.1. Protein production

HEK293T cells were grown in DMEM media containing L-glutamine (2mM), Penicillin (100 units/ml), streptomycin (100µg/ml) and 10% Foetal bovine serum. The cells were passaged into five T125 flasks and incubated at 37°C until they were around 50-60% confluent (determined by light microscopy). The media was then removed from each flask and 18ml of fresh complete DMEM media was added per flask. A mixture of Opti-MEM, L-glutamine (2mM), penicillin and streptomycin (100U/ml) (no serum) was prepared and 270µl of GeneJuice (Novagen) was added. After being gently mixed, the solution was left for 10 minutes at room temperature. Following this, 90µg of plasmid DNA (CTLD4-7-Fc) was added and left for 15 minutes at room temperature. The mixture solution was divived and added to the five flasks of HEK293T cells. The flasks were then incubated at 37°C overnight. The following day, the media was changed to 30ml (per flask) of optimem, L-

glutamine, penicillin and streptomycin. Therefore the flasks were incubated for five days at 37°C tempareture. Supernatants were then collected and centrifuged at 3000 rpm for 15 minutes at 25°C. The supernatant were poured into fresh 250ml Corning bottle and the centrifugation was repeated. Finally the supernatant was stored at 4°C until use.

### 2.6.2. Protein purification

For purification of protein a protein A sepharose column was utilized. Briefly, PBS (75 µl) was added to the column and the position the PBS reached was marked. The PBS was then removed and protein A-Sepharose gel (GE Healthcare, Amersham, UK) was added up to the mark. Therefore the column was washed with 25ml of PBS and the supernatant was added. The column was eluted with 0.1M glycine (pH 2.8) after performing washing step again with 50ml of PBS. The effluent was collected in clean eppendorf tubes containing 50µl of Tris-HCl (pH 8, 1M, SIGMA), for this ten drops of effluent were collected in each tube. The protein content in each fraction was evaluated using a nanodrop.

Fractions containing proteins were pooled and dialysed against PBS and BCA (Bicinchoninic Acid- Pierce, UK) assay was performed to assess the protein concentration.

# Chapter 3

## Effect of inflammatory mediators on *Pseudomonas. aeruginosa* biofilm formation

### 3.1. Introduction

Pseudomonas biofilms play a significant role in persistent infection in cystic fibrosis (CF) patients where immune responses are marginally or severely compromised (Brazova et al., 2005, Moser et al., 2005). One key defence mechanism within the innate immune system involves phagocytes, including neutrophils and macrophages. This defence mechanism is effective against planktonic bacteria however it is less effective when phagocytes encounter bacteria in biofilms. Neutrophil-mediated inflammation is essential for bacterial clearance but extensive and on-going neutrophil-dominated inflammation during chronic infection can lead to persistent damage of the lung and poor bacterial clearance (Bjarnsholt et al., 2009, Jensen et al., 2007). In CF there seems to be an association between presence of the Th2 cytokine IL-4 and chronic PA infection and poor pulmonary function, while high expression of the Th1 cytokine interferon-gamma (IFN-γ) appears to confer protection against infection and improved pulmonary function (Moser et al., 2005, Brazova et al., 2005, Singh et al., 2015). In addition to neutrophils, macrophages are important components of the innate immune system act as phagocytic effector cells and also modulate the action of other immune cells by secreting a variety of cytokines, chemokines and growth factors (Gordon, 2003, Martinez et al., 2009). Human and murine macrophages phagocytose PA

(Speert et al., 1988, Speert and Gordon, 1992, Kannan et al., 2008). Macrophages can adopt different activation states in response to changes in their environment. The Th1 cytokine IFN-γ promotes the induction of classically activated macrophages (CAMs) which are more microbicidal, and pro-inflammatory, and promote Th1 responses, while the Th2 cytokines IL-4 and IL-13 induce alternatively activated macrophages (AAMs), which are less microbicidal and inflammatory, and involved in wound healing and promotion of Th2 responses (Gordon, 2003, Martinez et al., 2009, Moser et al., 2005, Singh et al., 2015, Hartl et al., 2006). Human macrophages treated with IFN-γ in presence or absence of the pro-inflammatory cytokine granulocyte macrophage colony-stimulating factor (GM-CSF) are able to influence the inflammatory response to PA by upregulating monocyte chemoattractant protein-1 (MCP-1) and, tumour necrosis factor-α (TNF-α) and down regulating IL-10 but failed to inhibit bacterial growth or display improved survival upon PA infection. GM-CSF in combination with IFN-γ facilitates macrophages IL-6 production and further reduce IL-10 production (Singh et al., 2015). Thus cytokines affect the response of macrophages to PA infection.

### 3.1.1. Role of cytokines (GM-CSF and IFN- γ) during bacterial infection

The objective of the present study is to investigate the effect of immune cells and mediators involved in immune responses on PA biofilm development, specifically in the context of respiratory infection. In CF patients cytokines such as IL-4 expressed by T helper cells 2(Th2) associate with poor pulmonary function whereas higher expression of the Th1 cytokine IFN-γ promotes better lung function. CF patient also have a Th17-biased immune response which co-exists alongside a Th-2 immune response and contributes to deterioration of

lung function (Chan et al., 2013, Decraene et al., 2010, Tiringer et al., 2013). Th17 cells and in particular their effector cytokine IL-17A promote defence against various pathogens including extracellular bacterial infections (Ouyang et al., 2008). IL-17A induce the expression of a variety of pro-inflammatory cytokines including IL-1, IL-6 TNF-α, CXCL8, granulocyte colony-stimulating factor (G-CSF), and GM-CSF which eventually leads to the recruitment and activation of neutrophils to the site of infection (Ouyang et al., 2008).

Within the present study the effect of the cytokines GM-CSF and IFN-γ on PA biofilm formation have been investigated. GM-CSF and IFN-γ are important activators of inflammatory immune cells. GM-CSF promotes dendritic (DC) cells maturation and facilitates survival and activation of phagocytes such as macrophages, neutrophils and eosinophils (Hamilton, 2008). GM-CSF can be produced by a wide variety of cell types, including activated T cells, macrophages, B-cells, fibroblasts, endothelial cells, and tumour cells (van Nieuwenhuijze et al., 2013, Fleetwood et al., 2005). In addition to the activation and maturation of phagocytes from precursors, GM-CSF is also involved in other functions: for example promote antigen presentation, induce complement and antibody-mediated phagocytosis, and mediate leukocyte adhesion and chemotaxis (Cook et al., 2004, Fleetwood et al., 2005, Gomez-Cambronero et al., 2003). Besides being highly expressed in both stromal and hematopoietic compartments, some recent murine studies showed that GM-CSF can be released by CD4+ T cells (Codarri et al., 2011, Piper et al., 2014). It has been showed that GM-CSF–secretion from helper T cells plays an important role during chronic inflammation (Codarri et al., 2011, Piper et al., 2014). Numerous studies have been performed to elucidate the effect of GM-

CSF on cells of the granulocyte and monocyte lineage and contribution to anti-microbial immunity. Subcutaneous administration of GM-CSF in 7 healthy volunteers showed increase number of neutrophils in blood mainly by accelerating delivery of neutrophils from bone marrow to blood and reducing delivery from blood to tissue while no effect was found on neutrophil production and half-life (Dale et al., 1998). A similar result was found in HIV-infected patients after GM-CSF treatment where a dose-dependent increase in circulating neutrophils, monocytes and eosinophils was observed (Groopman et al., 1987). Also it has been observed that inhalation of GM-CSF steers the alveolar environment from a Th2-dominated response towards a Th1 response which is related to better preservation of pulmonary function and thus improved outcomes in CF patients (Heslet et al., 2012). GM-CSF played a critical role in the response to *P. carinii*; levels of infection and inflammation increased significantly in GM-CSF-deficient mice compared to wild type mice in the absence of CD4+ T cells (Paine et al., 2000). Similarly GM-CSF significantly improved survival in a neonatal rat model of staphylococcal sepsis when it was administrated prophylactically (Frenck et al., 1990). In contrast, it was found that administration of GM-CSF failed to improve survival in rats with peritonitis induced by caecal ligation and puncture (Toda et al., 1994). Similar to bacterial infection different research approaches have been conducted to explore the role of GM-CSF in fungal infection. In a neutropenic mouse model, prophylactic administration of GM-CSF increase the overall survival of mice experimentally infected with *C.albicans*, PA, or *S. aureus* (Mayer et al., 1991). Also during neutropenia collaboration between macrophages and vaccine induced CD4+ T cells and production of GM-CSF

by the CD4+ cells was shown able to protect against PA pneumonia (Kamei et al., 2013).

IFN-γ is produced predominately by Th1 CD4+ and CD8+ T cells and NK cells (Gallin et al., 1995, Liles and Van Voorhis, 1995). Monocytes/ macrophages and neutrophils are major target cells for this cytokine. Besides IFN-γ activates nonprofessional host defence cells, including platelets, endothelial and epithelial cells, fibroblasts, hepatocytes, astrocytes, and microglia cells (Gallin et al., 1995). IFN-γ has been shown to stimulate the ability of macrophages to kill intracellular pathogens: for example *Mycobaterium*, *Leishmania*, *Rickettsia*, *Legionella* and *Chlamydia* species (Gallin et al., 1995, Murray, 1994, Liles, 2001). Mice with targeted disruption of either IFN-γ or the IFN-γ receptor have shown impaired ability to control infections with intracellular pathogens, such as viruses, *Mycobacterium tuberculosis*, *bacillus Calmette-Guérin* and *L. monocytogenes* (Murray, 1994, Gallin et al., 1995, Dalton et al., 1993, Huang et al., 1993). Beneficial effects of IFN-γ treatment in a wide variety of experimental infections in animal models have been reported by different groups. For example, systemic administration of IFN-γ facilitates resistance against acute disseminated *C. albicans* infection and invasive aspergillosis in mice (Kullberg et al., 1993, Nagai et al., 1995). Local administrations of IFN-γ (intra-tracheal) in corticosteroid-treated rats enhance pulmonary clearance against experimental legionellosis (Skerrett and Martin, 1994). An increase in survival and reduction of lung colony forming units was found in a *Cryptococcus neoformans* infection mouse model when mice were treated with IFN-γ, compared with untreated controls (Joly et al., 1994). IFN-γ has been reported to be a beneficial

therapeutic agent in experimental infection models by at least 22 bacterial, fungal, protozoan, and helminthic pathogens (Murray, 1994, Liles, 2001). IFN-γ has been approved by the FDA for the treatment of chronic granulomatous disease (CGD). On the basis of a published report showing that CGD cells treated with IFN-γ displayed enhanced activation with stimulation of the oxidative burst-independent antimicrobial mechanisms. A group of CGD patients (n=128) were treated twice a week with IFN-γ or placebo. Patients treated with IFN-γ developed significantly fewer infections and required fewer days of hospitalization (Gallin, 1991). Also further long-term follow-up studies showed that patients who were treated with IFN-γ experienced no unexpected complications (Gallin et al., 1995, Bemiller et al., 1995). IFN-γ has also been considered as an immunomodulatory therapeutic agent for other specific immunodeficiency syndromes such as hyperimmunoglobulinemia E (hyper-IgE) syndrome (Grimbacher et al., 1999) and including protection against the pathogen considered responsible for infections in these patients (Grimbacher et al., 1999). IFN-γ is considered an effective adjunctive therapeutic agent when used in conjunction with conventional antimicrobial chemotherapy for patients suffering from cutaneous and visceral leishmaniasis, disseminated atypical mycobacterial infection, or lepromatous leprosy (Murray, 1994, Holland et al., 1994, Badaro et al., 1990). Several studies have reported the effect of IFN-γ on AM which are important in protection against *M. tuberculosis* infection. Aerosol administration of IFN-γ (3 times per week for 1 month) to a number of patients with multidrug-resistant tuberculosis (Condos et al., 1997) showed a conversion of sputum acid-fast bacillus positive smears to negative in all 5 patients. Furthermore a reduction in the size of cavitary lesions was observed

in each patient after 2 months of IFN- γ treatment. However, within one month sputum smears reverted to positive in 4 of the patients due to discontinuation of IFN-γ therapy. These preliminary observations imply a potential use of aerosolized IFN-γ as an adjunctive therapy for multidrug-resistant tuberculosis patients. A few patients with atypical (nontuberculous) mycobacteria respiratory tract infections have also been reportedly treated successfully with both systemic and aerosolized IFN-γ (Holland et al., 1994, Chatte et al., 1995, Holland, 2000). In a chronic PA lung infection rat model intraperitoneal treatment with recombinant rat interferon-γ (rIFN-γ) showed increased Th1 responses compared to controls which showed Th2 type responses (Johansen et al., 1996). More recently it was shown that IFN-γ production from the peripheral blood mononuclear cells (PBMCs) from CF patients positively correlates with pulmonary function and thus IFN- γ could be protective among CF patients by modulating immune responses against PA infection (Singh et al., 2015).

Thus in the present study we aimed to analyse whether GM-CSF and IFN-γ have any direct effect on PA biofilm development. Previous studies carried out in our lab showed that PA formed smaller microcolonies when associated with GM-CSF/IFN-γ treated human macrophages compared with M-CSF-treated macrophages (Sonali Singh, PhD thesis). Furthermore, IFN- γ has been shown to bind OprF in PA and induce expression of the QS-dependent virulence determinants PA- lectin and pyocyanin (Wu et al., 2005). These studies suggested that PA biology and, in turn, biofilm formation could be differentially affected by immune cells depending on inflammatory conditions. To investigate this possibility the first step was to study potential direct effects

of the cytokines used to activate the immune cells (IFN-γ and GM-CSF) on PA biofilm formation.

## 3.2. Hypothesis

PA biofilm development will be affected by the presence of human cytokines GM-CSF and IFN-γ.

## 3.3. Aims

1. Compare PA biofilm formation in LB media and X-Vivo 15 media using the BioFlux system. Besides biofilm formation QS molecules and different virulence factors produced by PA biofilms will be investigated.
2. Analyse the effect of human GM-CSF and IFN-γ on PA biofilm formation using the BioFlux system.

## 3.4. Results

### 3.4.1. Optimisation of biofilm formation in X-Vivo 15 media using the BioFlux system

The process of PA biofilm formation consists of several phases including (1) initial attachment of planktonic bacteria to a surface; (2) formation of micro colonies; (3) formation of mature biofilm and biofilm dispersion and release of new phenotypic variants in planktonic form (Ma et al., 2009). Different environmental conditions such as nutrient concentrations, pH and age of the culture can affect PA biofilm development as well as PA phenotype and pathogenesis (Mata-Sandoval et al., 2001, O'May et al., 2006, Abdallah et al., 2015), effects that can be controlled by the QS system (Duan and Surette,

2007, Sakuragi and Kolter, 2007). Early in this study it was considered important to optimize PA biofilm formation using an appropriate culture media compatible with survival of immune cells before including immune cells or cytokines. For this generation of PA biofilm was first optimized using X-Vivo 15 media (Chapter 2). X-Vivo 15 media is a serum-free cell culture media used in the laboratory to culture human macrophages. Biofilms generated in X-Vivo 15 were compared with those produced in diluted LB broth which is traditionally used for PA biofilm studies. LB broth contains animal and yeast products such as tryptone and yeast extract which make it less desirable for growing biofilm that are to be incubated with immune cell such as macrophages. QS molecules produced by PA biofilms generated in X-Vivo 15 and LB were also analysed.

#### 3.4.1.1. X-Vivo 15 supports PA biofilm development

PA biofilms were grown in a BioFlux™ device which has a microfluidic platform designed to run an automated shear flow that permits development of multiple biofilms in parallel with controlled flow rates. Both X-Vivo 15 media and LB media were tested for their ability to support PA biofilm formation. Based on the confocal images and COMSTAT analysis X-Vivo 15 appeared to be appropriated for PA biofilm generation (Fig 3.1) although compared to LB broth a slight reduction in biofilm biomass and average thickness was seen using the X-Vivo 15 media (Fig 3.2). QS molecules were quantified in the supernatants collected from the BioFlux channels by LC-MS (Chapter 2). Culture medium/conditions might affect the expression of QS molecules and related virulence factors in *in vitro* cultures of PA. This analysis showed a reduction in the production of *N*-butanoyl-L-homoserine lactone (C4-HSL), 2-

heptyl-3-hydroxy-4-quinolone (PQS) and 2-heptyl-4-quinolone (HHQ) by PA biofilms grown in X-Vivo15 media compared to LB broth. No differences were found on the production of *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) which was detected at very low levels overall in both X-Vivo 15 and LB media (Fig 3.2). Based on these results X-Vivo15 was considered appropriate to generate PA biofilms suitable for the study of their interaction with eukaryotic cells.

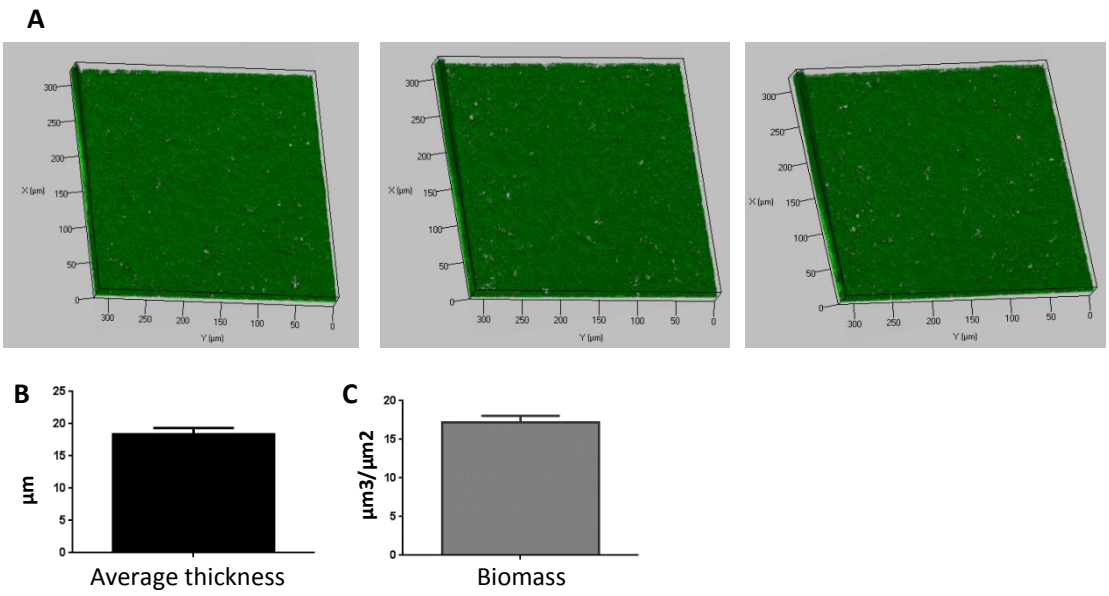



Fig: 3.1 X-Vivo 15 supports PA biofilm formation

**A**. Representative confocal 3D images of 15 h biofilms generated in a BioFlux system. Images correspond to random areas from a single channel. **B and C.** Biomass (B) and average thickness (C) of PA biofilms grown in X-Vivo 15. Bar shows mean ± SEM calculated from 9 experiments. First the mean of five images stacks were calculated from each channel and then the mean from the three channels from each experiment were calculated. Data analysed using COMSTAT software and Grad Prism 6

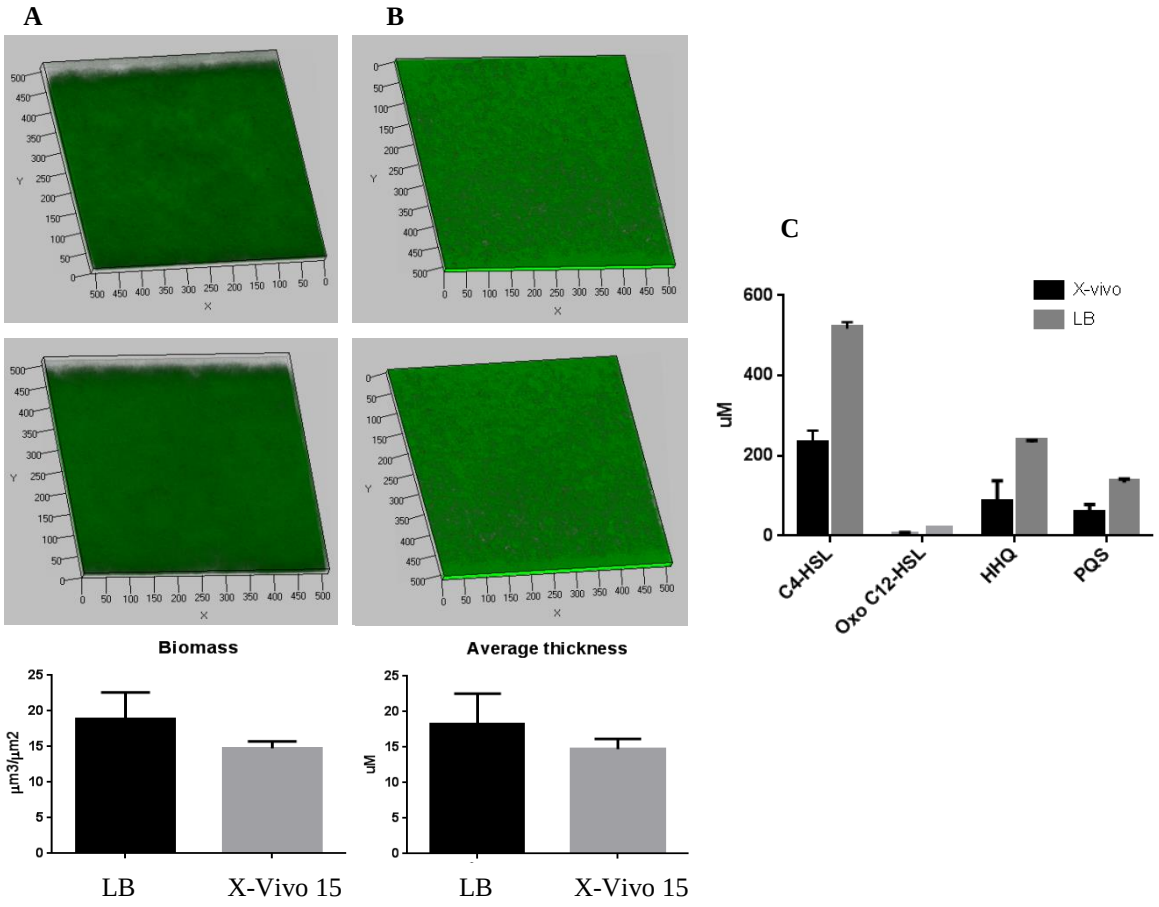



Fig: 3.2 Biofilm formation and production of QS molecules in LB and X-Vivo 15

A, B Confocal images and COMSTAT analysis of PA biofilms grown in LB (A) and X-Vivo 15 (B) using the BioFlux system. C. Quantification of QS molecules in the supernatants from PA biofilms grown in LB or X-Vivo 15 (n=2).

### 3.4.1.2. Effect of buffering X-Vivo 15 with HEPES on PA biofilm development

There were concerns regarding the buffering capacity of X-Vivo 15 and as changes in pH could affect biofilm development the use of HEPES was considered as a mean to minimise potential variations in pH. HEPES is widely employed as an organic buffering agent in cell culture medium. The effect of 10 mM HEPES on PA biofilm formation in X-Vivo 15 was investigated (Fig. 3.3). A small reduction of PA biofilm formation (biomass and average thickness) was observed in the presence of HEPES. Slightly reduced levels of C4-HSL and PQS were observed on the supernatants of PA biofilms developed in the presence of HEPES (Fig 3.3).

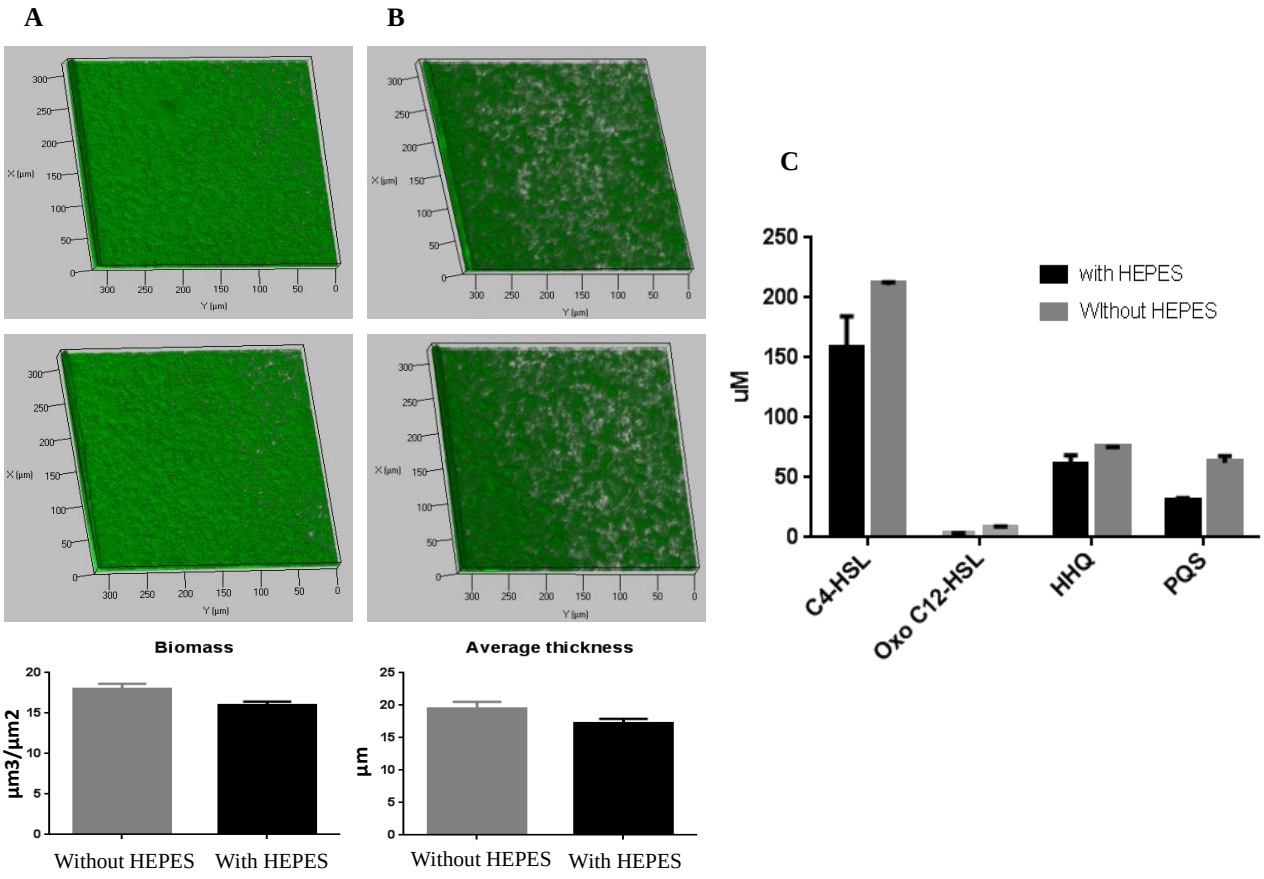



Fig: 3.3 The presence of HEPES does not improve PA biofilm formation in X-Vivo 15

A, B Representative confocal images and COMSTAT analysis of PA biofilms generated in the presence B or absence A of HEPES (10mM) using the BioFlux system. C. Quantification of QS molecules in the supernatants from PA biofilms generated in the presence or absence of HEPES (10mM). N=2

### 3.4.1.3. Expression of PAO1 las, rhl and pqs quorum sensing networks but not lecA is reduced in X-Vivo 15 compared to LB media

To further characterise the behaviour of PA in X-Vivo15 it was considered important to analyse the expression of QS and QS-induced virulence factors during bacterial growth in this medium. For this, the expression of the following genes: *lasI*, *rhlI*, *pqsA*, *rhlA*, *lasB*, and *lecA* in X-Vivo15 and LB media under static conditions was investigated using PAO1-N reporter strains in which the promoters of the genes under study had been fused with lux reporter genes. Expression of all QS networks except *lecA* was found lower in

X-Vivo15 compared to LB broth which demonstrated attenuation of QS virulence factors in X-Vivo 15 compared to LB (Fig 3.4).

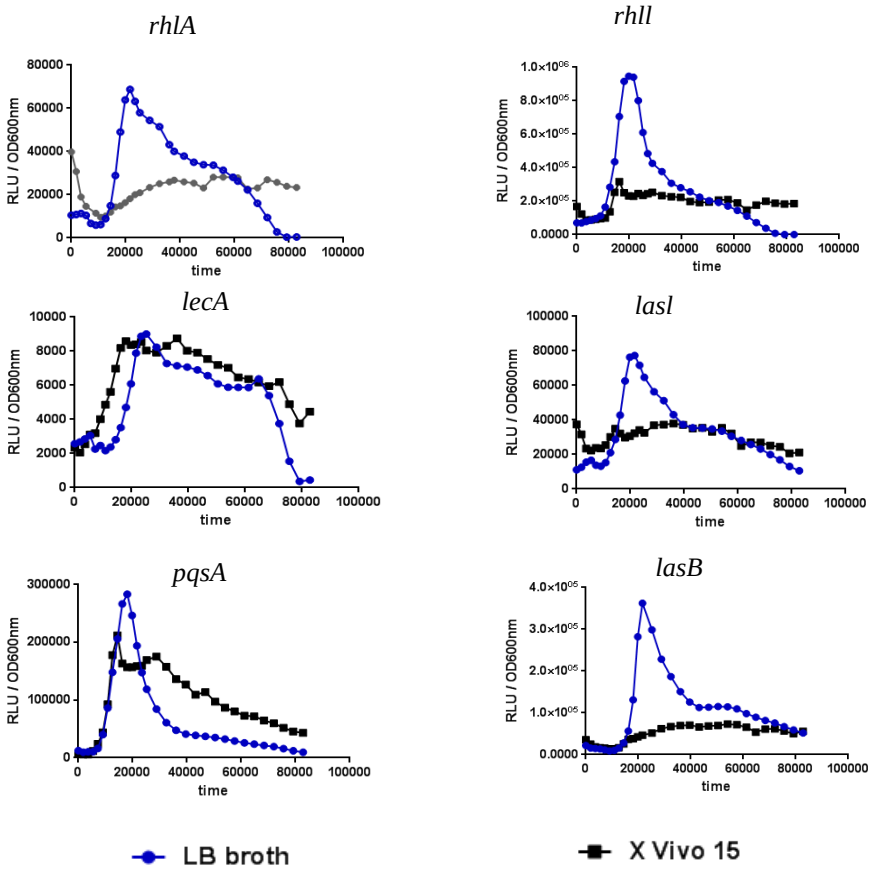



Fig: 3.4 Comparison of QS and virulence factor expression by PAO1-N grown in LB broth and X-Vivo15 under static conditions

The following reporter strains in the PAO1-N background were used: *lasB::lux*, *lasI::lux*, *rhlA::lux*, *rhlI::lux*, *pqsA::lux*, and *lecA::lux*. These strains were generated by chromosomal fusion of *luxCDABE* genes. 1:100 dilutions of OD600nm=1 overnight cultures of each strain in either LB broth or X-Vivo 15 were placed in triplicate in the wells of a 96-well luminescence microtitre plate and incubated at 37$^{o}$C for 24 h in a Tecan Infinite® M1000 PRO plate reader. Luminescence and OD600nm were measured for each cultures every 30 min. To negate any differences in luminescence due to differences in growth of the reporter strains, gene expression was reported as luminescence (relative light units, RLU) divided by OD600nm for that culture. Data presented are mean of triplicate wells from a single experiment.

### 3.4.2. Effect of the inflammatory mediators, GM-CSF and IFN-γ on PA biofilm formation

To study the effect of cytokines on PA biofilm development biofilms were generated in the presence of GM-CSF or IFN-γ using the BioFlux system. Different parameters as biomass, average thickness, surface coverage, QS molecule production and rhamnolipid production were measured.

Initially the cytokine stocks used were prepared in RPMI with 10 % human AB serum (HS) (GM-CSF main stock 10 µg/ml and IFN- γ 100 µg/ml in RPMI with 10 % HS) because they were normally used for the differentiation of human macrophages. After that to rule out the contribution of serum to the results GM-CSF and IFN-γ stocks were prepared directly in X-Vivo 15 (both GM-CSF and IFN-γ main stock were prepared as 100µg/ml in X-Vivo 15 media). For clarity the results are shown in chronological order.

### 3.4.3. Effect of GM-CSF and IFN-γ (main stock in RPMI-1640 with 10 % HS) on PA biofilm parameters

To test the effect of GM-CSF and IFN-γ on PA biofilm formation PA biofilms were grown in a BioFlux™ device in X-Vivo 15 media in the presence and absence of cytokines. In spite of the variability among experiments a significant reduction in surface coverage was observed in response to GM-CSF (50ng/ml) which was added after 2 hours of initial PA attachment in three independent experiments. The reason behind choosing GM-CSF 50ng/ml was that this concentration has been used for all studies of immune cell activation. Besides surface coverage, reduction of biomass and average thickness was also seen in the presence of GM-CSF (Fig 3.5). These differences were similar to the effects observed in the presence of IFN-γ (20ng/ml) which induced a mild

reduction in three independent experiments (Figs 3.6 and 3.7). It appeared that these cytokines had an inhibitory role on the development of PA biofilm rather than promote killing and/or increased levels of eDNA because PI staining was similar among controls and GM-CSF or IFN-γ treated biofilms. After analysing all data it appeared that between these two cytokines the effect of GM-CSF (50ng/ml) was more profound. To facilitate understanding Fig 3.5 and 3.6 show the results from one individual representative experiment for each of these cytokines. Fig 3.7 shows the results from three independent assays performed in triplicate.

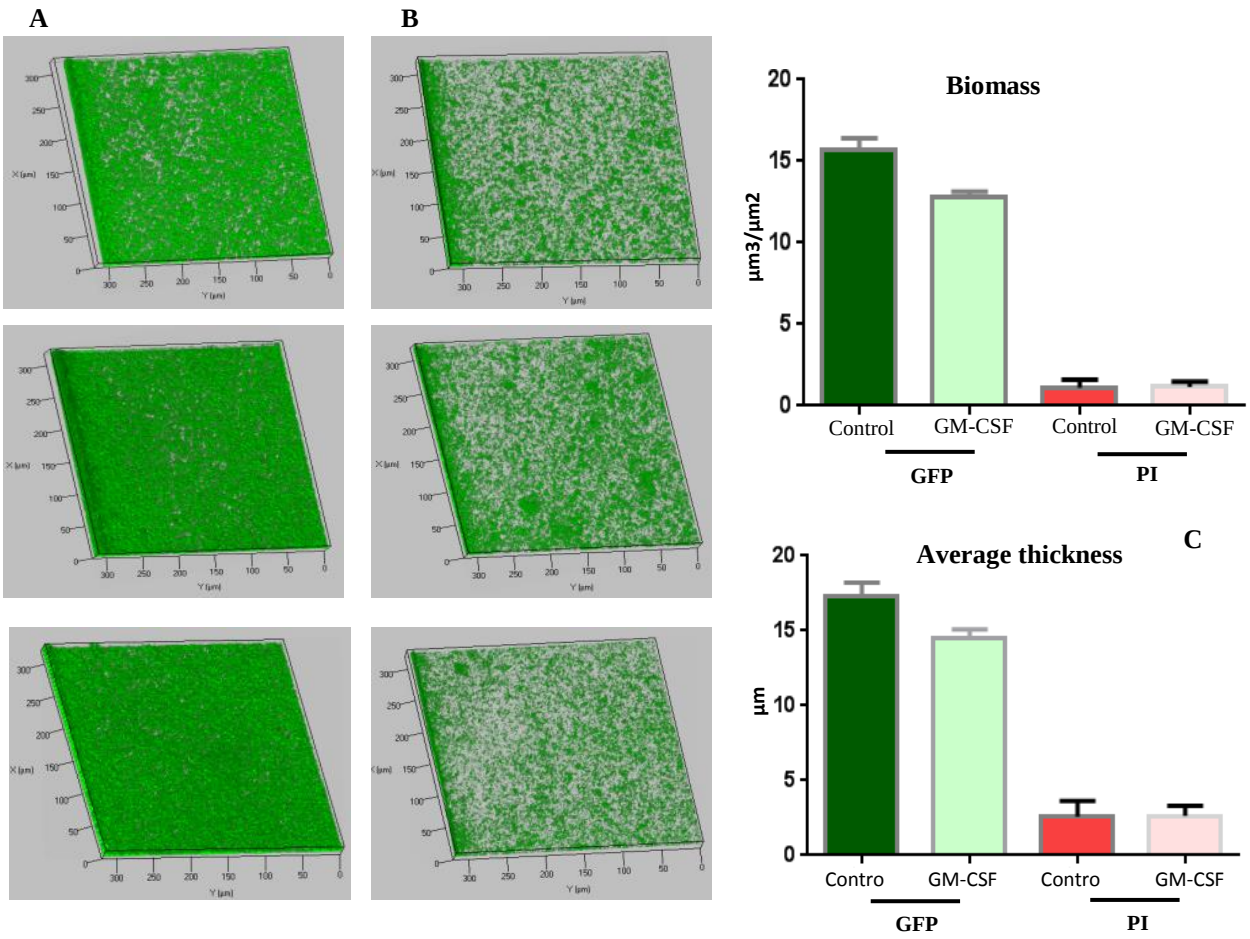



Fig: 3.5 Effect of GM-CSF prepared in RPMI-HS on PA biofilms

Confocal images and COMSTAT analysis from a single representative experiment of PA biofilm in the absence (A) or presence (B) of GM-CSF 50ng/ml using the BioFlux system. (C) Comparison of the biofilm parameters: biomass and average thickness in a single experiment from a 15 hours biofilm grown in presence and absence of GM-CSF .Each bar represents mean± SEM of one experiment done in triplicate channels.

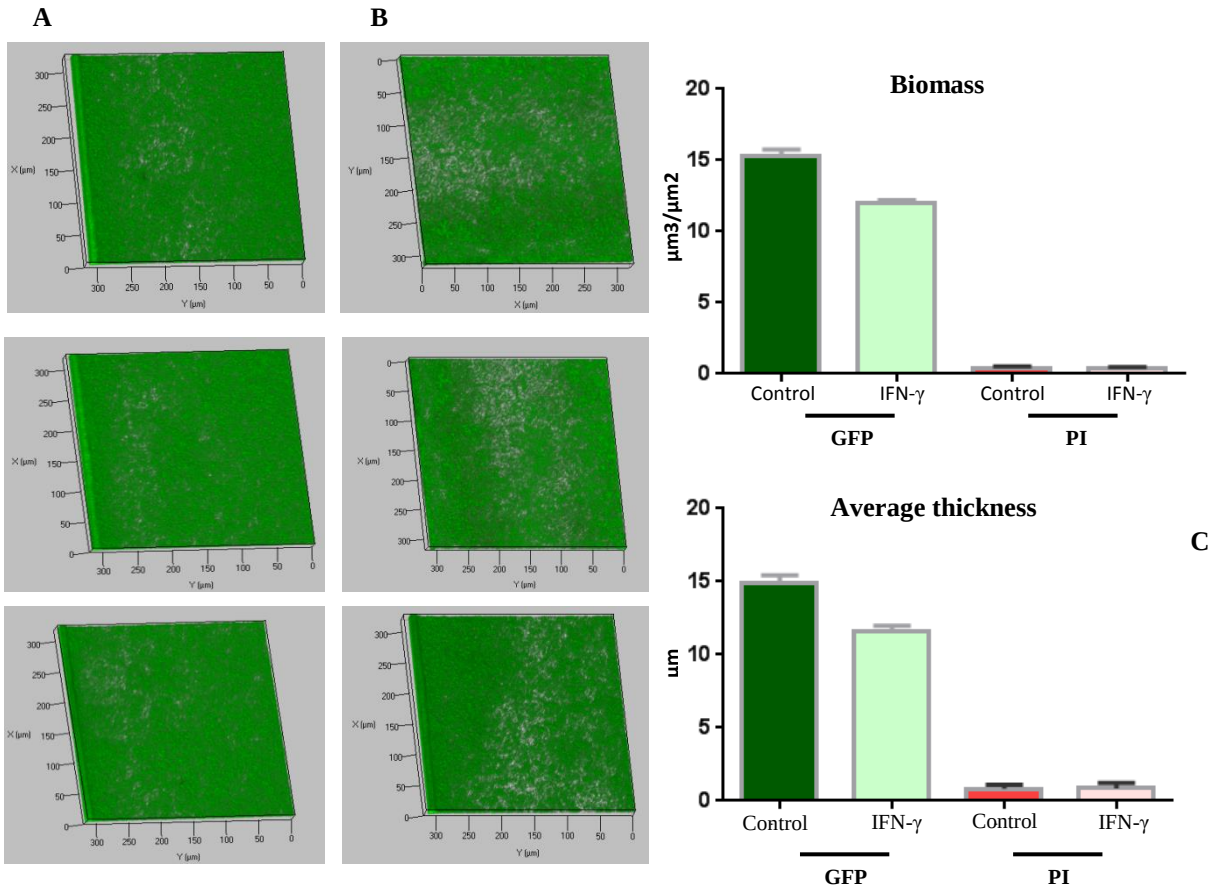



Fig: 3.6 Effect of IFN-γ prepared in RPMI-HS on PA biofilms

Confocal images and COMSTAT analysis of PA biofilm from a single representative experiment in the absence (A) or presence (B) of IFN-γ 20ng/ml using the BioFlux system. (C) Comparison of the biofilm parameters: biomass and average thickness in a single experiment from a 15 hours biofilm grown in presence and absence of IFN-γ. Each bar represents mean ± SEM of one experiment done in triplicate channels.

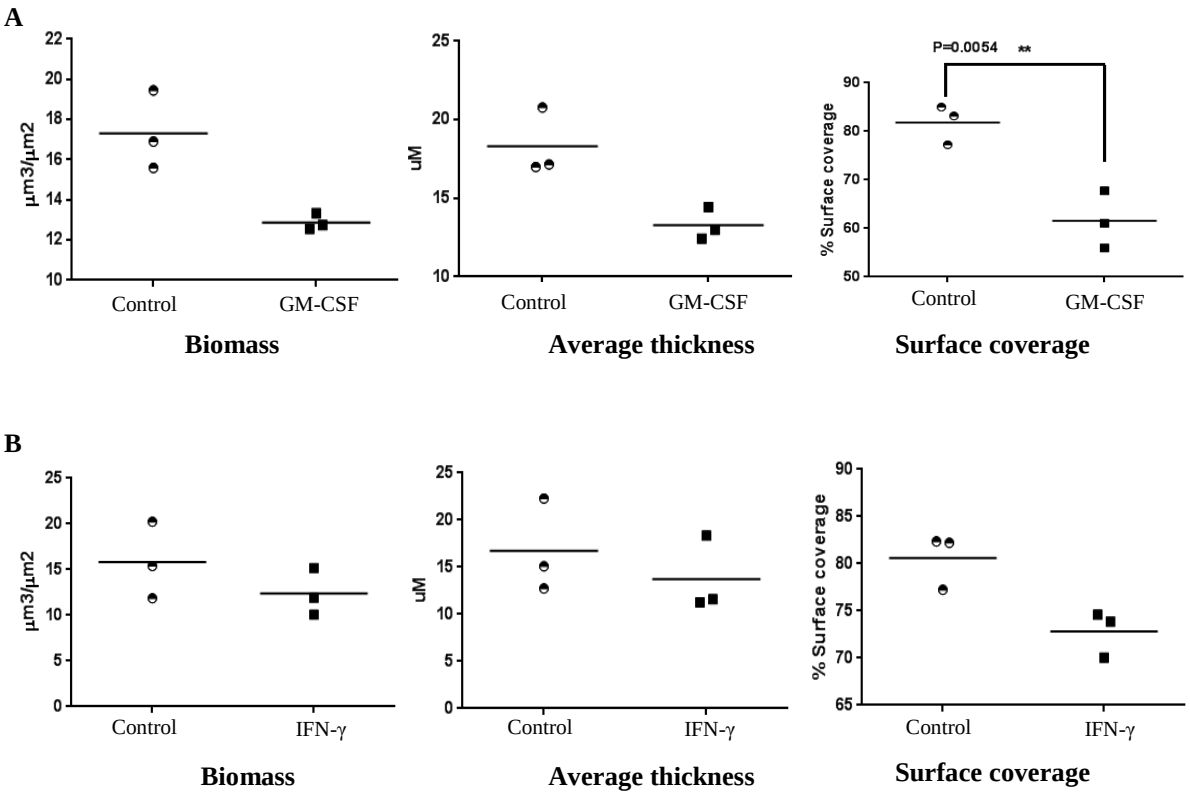



Fig: 3.7 Biomass, average thickness and surface coverage of PA biofilms in presence and absence of GM-CSF (A) or IFN-γ (B) (stocks prepared in RPMI-HS)

Data points represent mean of three channels for each variable. For each channel mean of five images stacks were calculated first and these were used to calculate the mean of three channels. Data analysed was done using COMSTAT software. Statistical significance was calculated using paired Student's t test. ** $p \leq 0.01$ .

### 3.4.4. Effect of GM-CSF and IFN-γ (main stocks 100 µg/ml, in X-Vivo 15) on PA biofilm parameters

According to previous results reduction of PA biofilm were seen in response to both GM-CSF and IFN-γ and GM-CSF appeared to cause a significant reduction on surface coverage. Due to the consistent effect of cytokines on PA biofilms concern arose to whether the serum had any additive effect over the cytokines. To determine whether the serum present in the cytokine stock could influence biofilm formation new stocks of these cytokines were prepared in X-Vivo 15 (main stock 100 µg/ml) and all the experiments were repeated. After

COMSTAT analysis of the confocal images no significant reduction on PA biofilm was observed in response to either cytokine (n=3). To determine whether these cytokines had any effect on PA viability PI staining was performed as above but no changes were observed (Fig 3.8, 3.9, 3.10, 3.11).

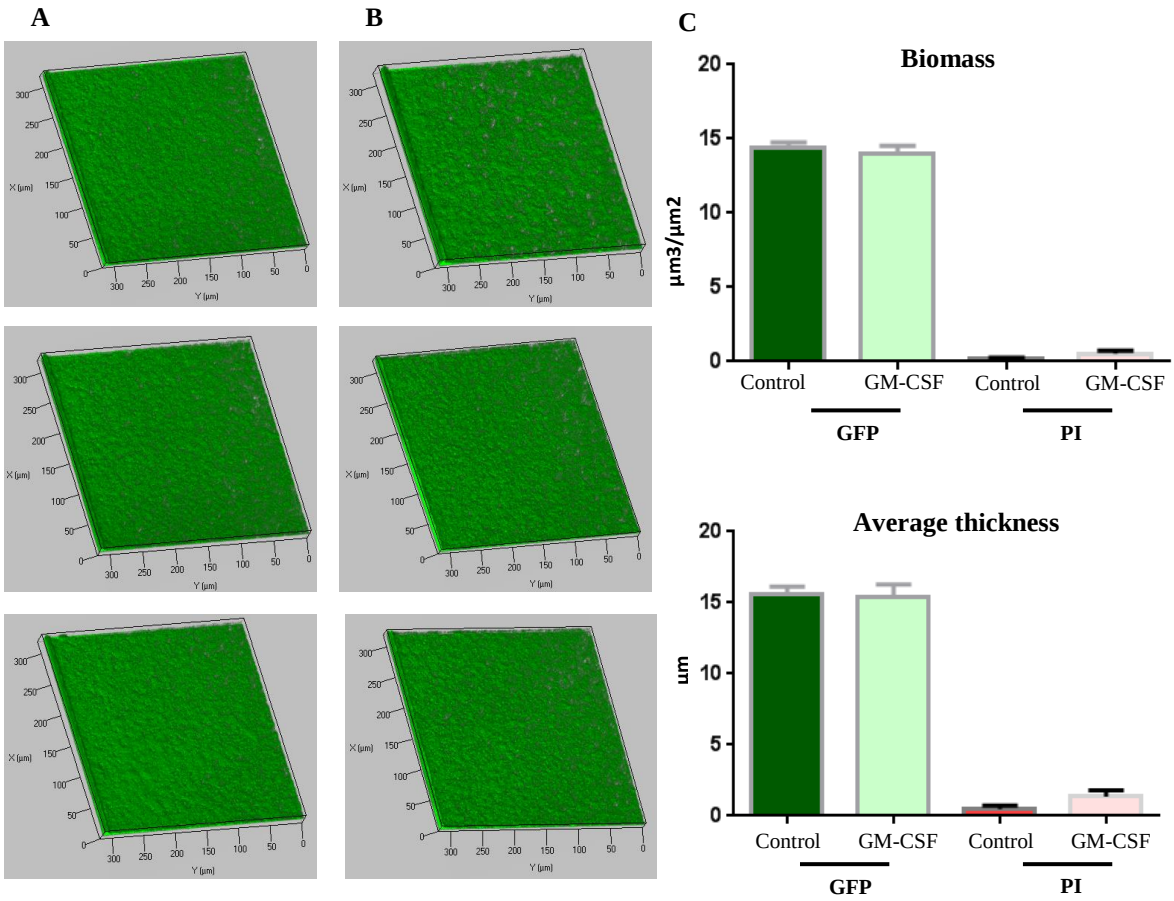



Fig: 3.8 GM-CSF (X-Vivo 15 stock) doesn't affect PA biofilm formation

A and B Confocal images of PA biofilm from a single representative experiment in the absence (A) or presence (B) of GM-CSF 50ng/ml in X-Vivo 15 (from main stock 100µg/ml in X-Vivo 15). C Comparison of the biofilm parameters: biomass and average thickness, determined using COMSTAT, from a 15 hours biofilm grown in presence and absence of GM-CSF. Each bar represents mean ± SEM of one experiment done in triplicate channels.

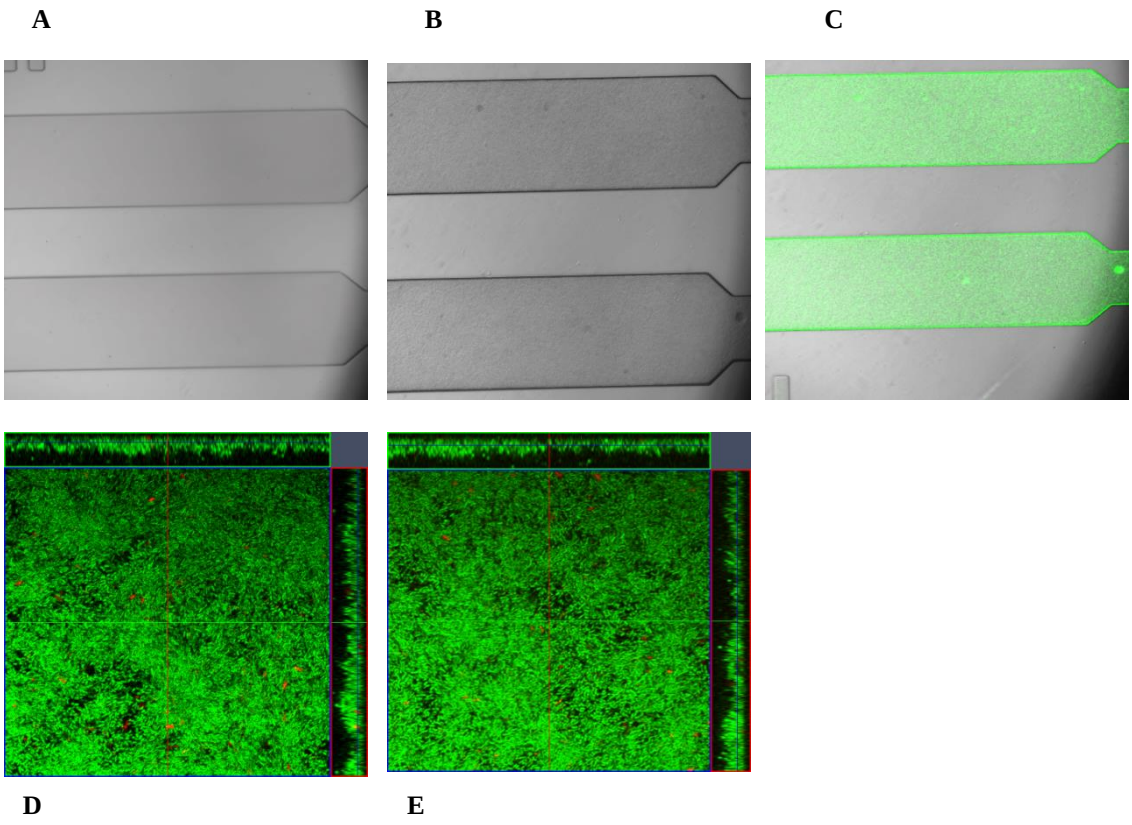



Fig: 3.9 GM-CSF (X-Vivo 15 stock) does not affect PA biofilm formation

A. Images from two adjacent empty BioFlux channels visualised using bright field microscopy (5X magnification). B. Images from two adjacent BioFlux channels containing PA biofilm incubated with or without GM-CSF (50 ng/ml) using bright field microscopy (5X magnification). C. Confocal images of two adjacent BioFlux channels showing GFP expressing PA biofilm incubated with or without GM-CSF(5X magnifications). D and E Confocal images showing GFP expressing PA biofilm incubated without (D) or with (E) GM-CSF (60X magnification).

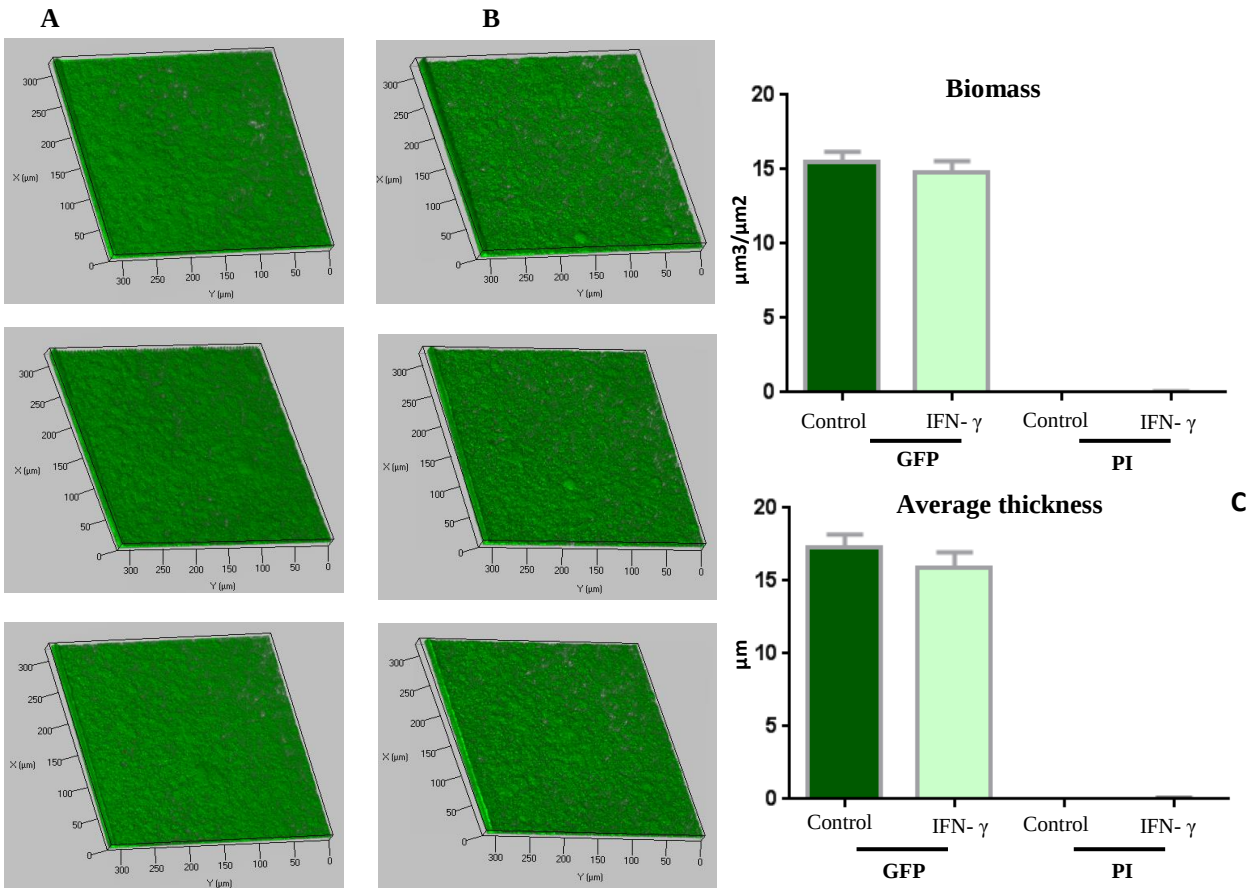



Fig: 3.10 IFN-γ (X-Vivo15 stock) does not affect PA biofilm formation

A and B Confocal images from a single representative experiment of PA biofilm generated in the absence (A ) or presence (B) of IFN-γ 20ng/ml (from main stock 100µg/ml in X-Vivo 15) using the BioFlux system. (C) Comparison of the biofilm parameters biomass and average thickness from a 15 hours biofilm grown in presence and absence of IFN-γ 20ng/ml. Each bar represents the mean ± SEM form one experiment done in triplicate channels.

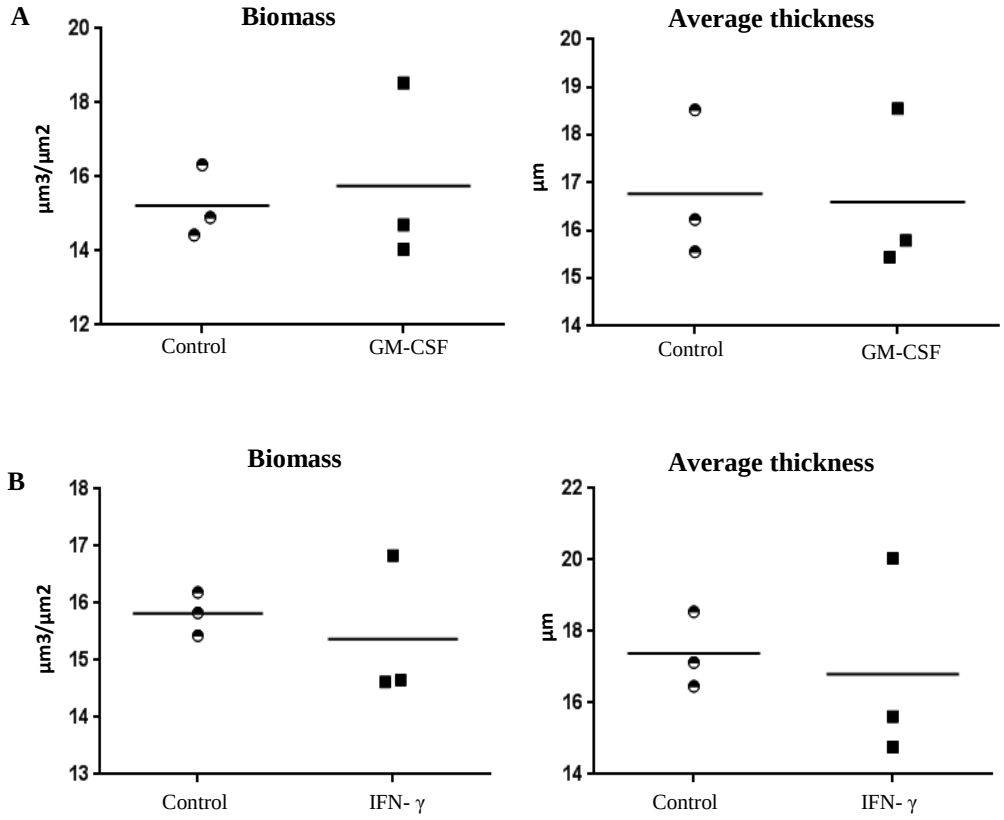



Fig: 3.11 GM-CSF and IFN-γ do not affect PA biofilm formation

Representation of biomass and average thickness of PA biofilms grown in presence and absence of GM-CSF 50 ng /ml (A) or IFN-γ 20ng/ml (B) (main stock of both GM-CSF and IFN-γ was prepared as 100 µg/ml in X-Vivo 15 media). Each data point represents mean of three channels from a single experiment. Mean of five images stacks were calculated from each channel first, after that mean of three channels were calculated for each experiment. Data analysed by COMSTAT software. Statistical significance was calculated by paired Student's t test.

### 3.4.5. Effect of cytokines on the production of QS molecules

To complement the previous findings it was tested whether GM-CSF and IFN-γ had any effect on QS molecules production. For this supernatants produced by the PA biofilms were tested for the presence of QS molecules using LC-MS. All QS molecules C4-HSL, 3-oxo-C12-HSL, PQS and HHQ, which is the precursor of PQS, were detected in the samples but no significant changes were seen among the PA biofilms developed in presence or absence of GM-CSF or IFN- γ (Figure 3.12.).

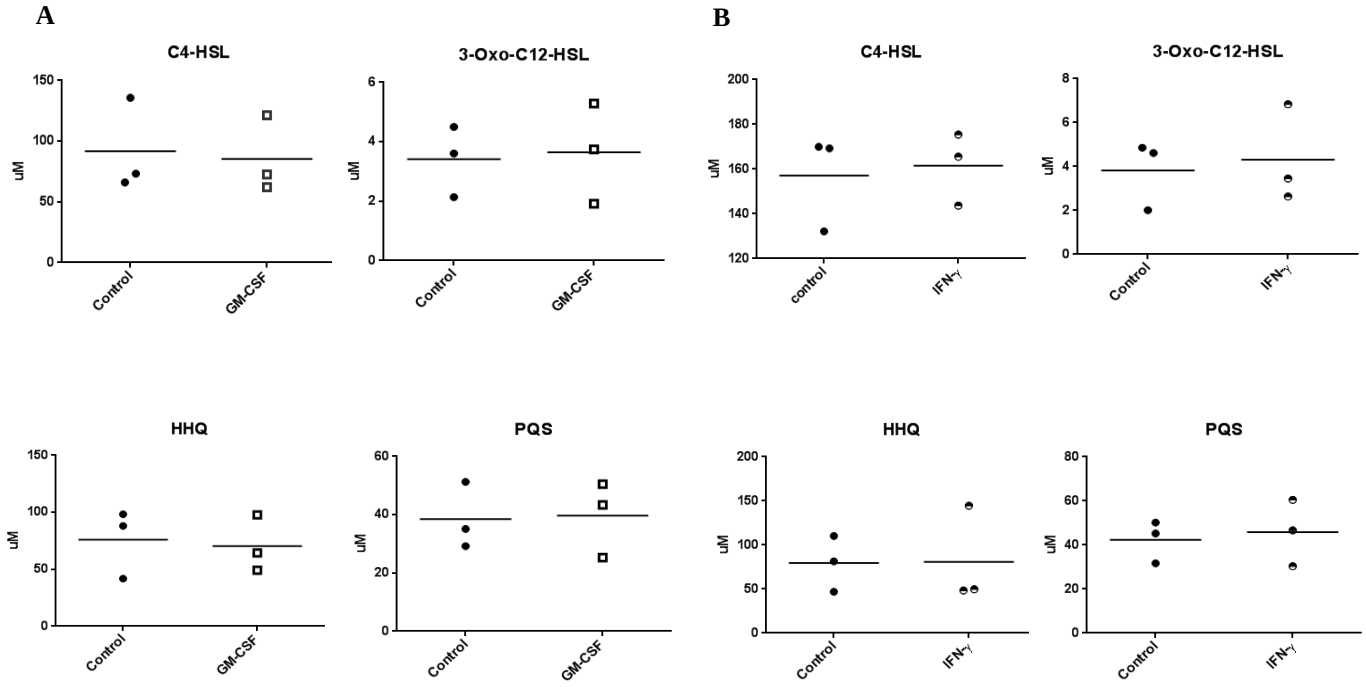



Fig: 3.12 GM-CSF and IFN-γ do not affect production of QS molecules by PA biofilms

Quantification of QS molecules in the supernatants from PA biofilms generated in the presence of GM-CSF 50 ng/ml (A), or IFN-γ 20ng/ml (B). Each point represents mean value of concentration in μM detected by LC-MS.

### 3.4.6. Effect of cytokines on production of rhamnolipid

The results presented above indicated that cytokines do not have any direct effect on PA biofilm development or QS molecule production. Given the important role of rhamnolipids on biofilm dispersion, the production of this biosurfactant was measured to determine whether it could be affected by cytokines. The levels of rhamnolipids were quantified from the supernatants of PA biofilm incubated with or without cytokines. Results showed that biofilm incubation with cytokines (both GM-CSF and IFN- γ) do not significantly affect the productions of rhamnolipids although a minor reduction was observed (Figure 3.13).

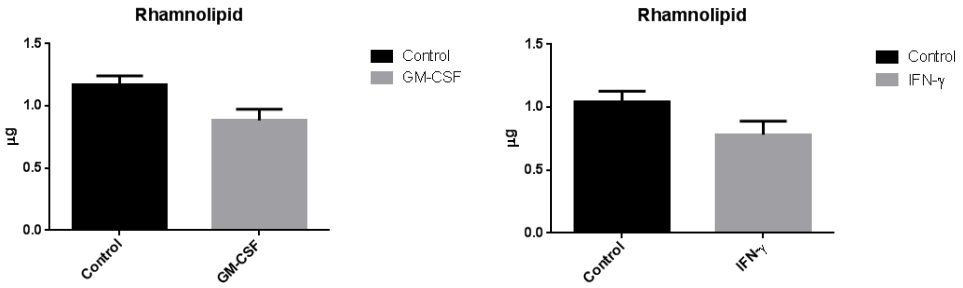



Fig: 3.13 Impact of GM-CSF and IFN- γ on rhamnolipid production by PA biofilms

PA biofilms were grown in presence and absence of GM-CSF 50ng/ml or IFN-γ 20 ng/ml (main stock 100 µg/ml in X-Vivo 15) and supernatants were collected and analysed for rhamnolipid production using the orcinol method. The Y axis indicates the mean rhamnolipid production in µg from three independent experiments ± SEM.

### 3.4.7. Effect of human serum (HS) on PA biofilm development

The results shown above indicate that the human cytokines GM-CSF and IFN-γ at the concentrations tested do not influence PA biofilm development in absence of HS. Thus the original results might have been due to the presence of HS in the cytokine stocks. HS has already been shown to inhibit PA biofilm formation by blocking the direct interaction between PA and substrates and by enhancing twitching motility (Hammond et al., 2010).

To confirm the effect of serum on PA biofilms the effect of HS diluted in the same way cytokines had been diluted was tested. HS was first diluted to 10 % in RPMI media and then it was added to X-Vivo 15 so that HS was present at a final concentration of 0.05% in the final media. This media was then used to generate PA biofilms. After analysing these biofilms using confocal microscopy and COMSTAT analysis a reduction in PA biofilm formation was found. Approximately a 20 % reduction in surface coverage in the presence of 0.05 % HS was detected which is similar to the reduction observed with cytokines dissolved in RPMI-10% HS. Reduction of biomass and average thickness were also seen. PI staining were performed as before to determine whether the serum is inducing killing of bacteria within the biofilm but no such changes were seen (Figure 3.14, 3.15) (n=2).

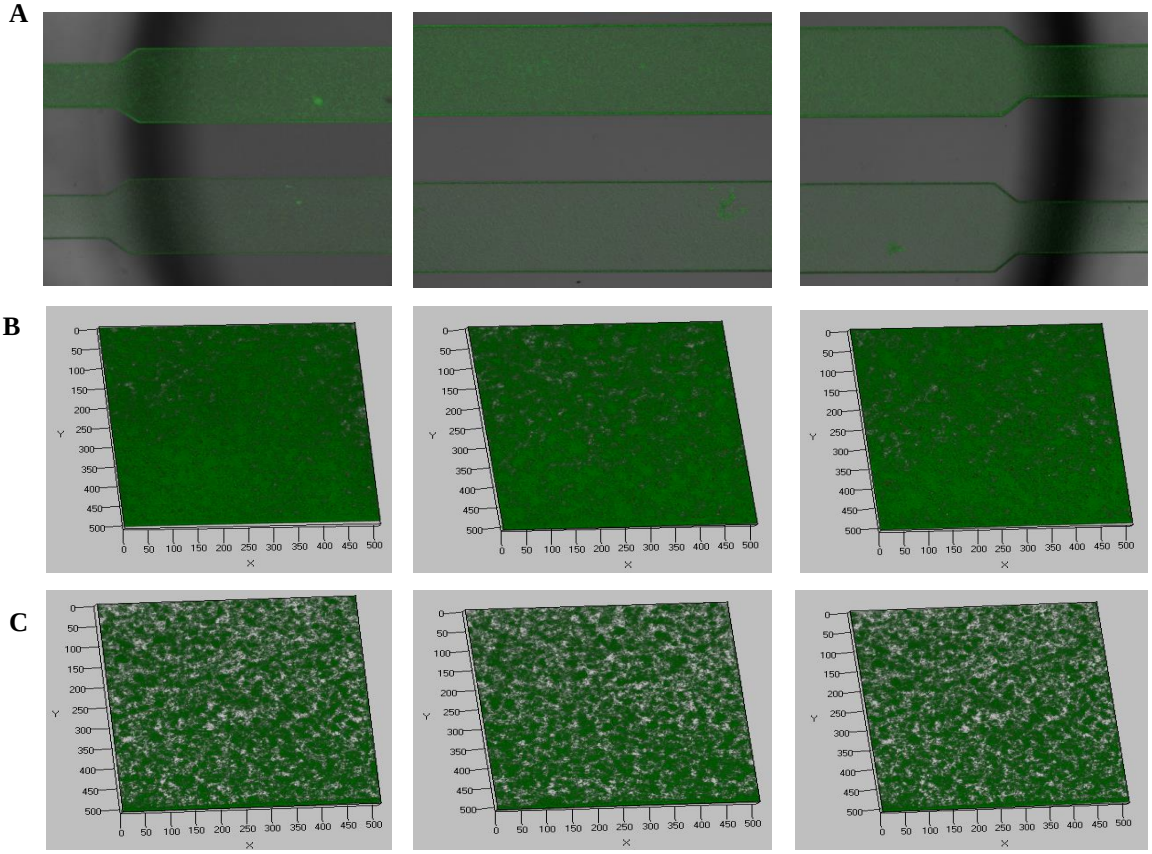



Fig: 3.14 HS reduces PA biofilm formation

Representative confocal images of PA biofilms incubated in presence or absence of 0.05 % HS. **A.**Confocal images (5X magnification) showing three different regions of two adjacent BioFlux channels grown in presence (lower channel) or absence (upper channel) of 0.05% HS in X-Vivo 15. **B and C** 3D Confocal images (20 X magnification) of PA biofilms developed in absence (**B)** or presence **(C)** of HS.

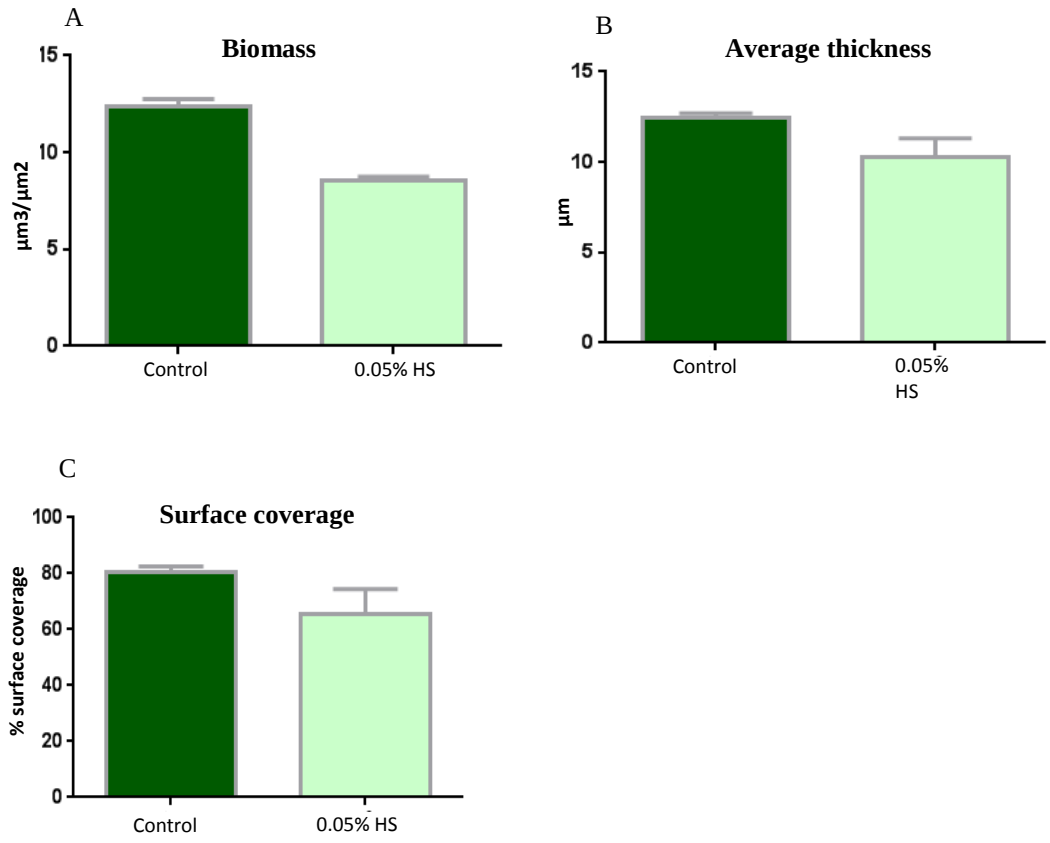



Fig: 3.15 Analysis of PA biofilm formation in the presence and absence of 0.05% HS

COMSTAT and image J software analysis of PA biofilms from a single representative experiment showing the biofilm parameters: biomass (A), average thickness (B) and percentage surface coverage (C). Each bar represents the mean ± SEM of two experiments done in triplicate channels. Statistical significance was calculated by paired Student's t test.

### 3.4.8. HS inhibits early PA attachment

The BioFlux system has some limitations that hamper the study of some aspects of biofilm development such as (i) inability to perform time course experiments because the software was automated to run only one plate at a time and (ii) it was unfeasible to include many variables within the same experiments because once the flow was stopped dispersion of biofilms was seen. To overcome these technical problems an alternative setup was applied where PA biofilm were grown within 96 well plates at $37^{o}$C in presence of $CO_2$ and crystal violet staining was used to measure biomass as an indication of biofilm formation (see Chapter 2)(O'Toole, 2011). To test the effect of HS in this setting, different concentrations of serum were added to PA cultures and cultures were incubated for 4 and 24 hours. Results at 4 hour show that HS was able to inhibit bacterial adhesion and attachment significantly in a dose dependent manner at the concentrations tested (0.2, 0.1, and 0.05, 0.025 %) (n=2) and the effect was lost at (0.0125 %). Therefore a similar dose dependent but mild reduction was also observed after 24 hours which was found insignificant (n=2) (Fig 3.16). Heat inactivation did not affect the ability of HS to restrict initial PA biofilm formation (n=2) (Fig 3.17). In this instance it can not be ruled out a direct effect of serum on PA viability. However, results on the effect of serum on biofilm development in the Bio-flux system suggest that this might not be the case as no increase in cell death was observed. (Fig- 3.5, 3.6).

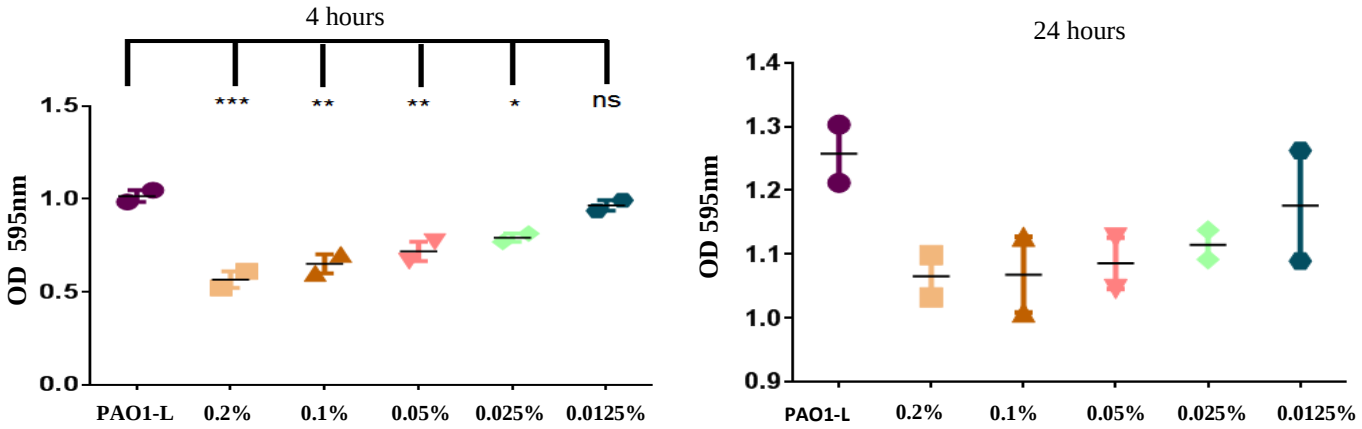



Fig: 3.16 HS inhibits early PA attachment

Crystal violet staining of PA biofilms incubated for 4 hours show significant dose dependent inhibition of earlier PA attachment in the presence of HS **(A)**. Mild but no such significant changes were observed after 24 hours **(B)**. Data points represents mean ± SEM of triplicate or quadruplicate wells from two experiments. Statistical significance was calculated by one way ANOVA (Dunnett's multiple comparisons test) * = p≤0.05.

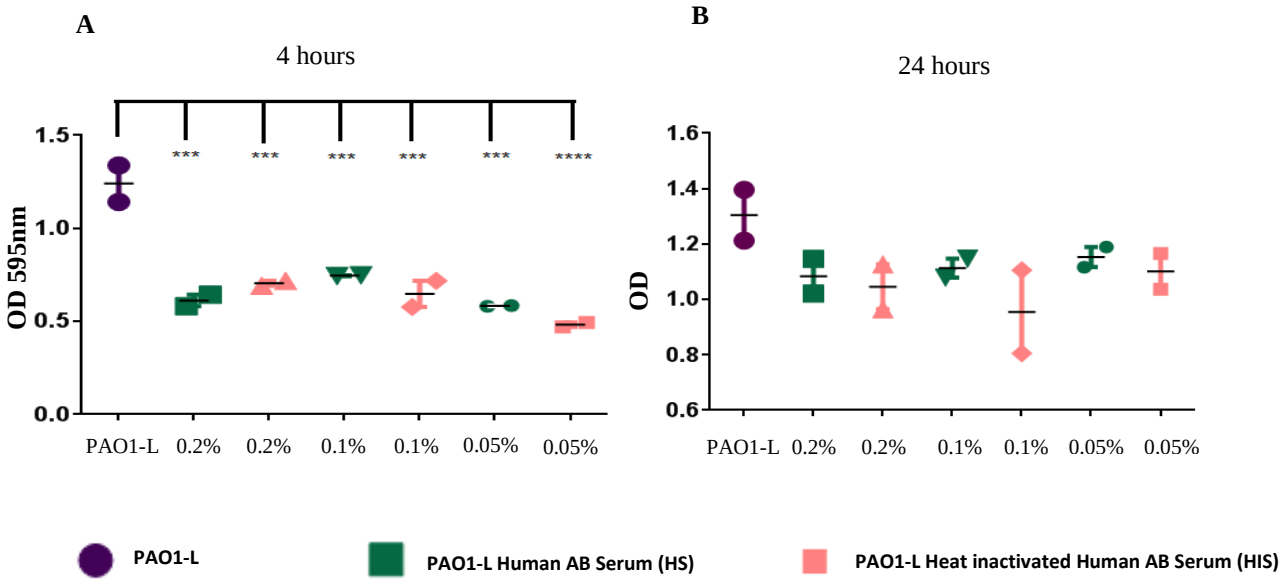



Fig: 3.17 Heat inactivation does not affect the ability of HS to restrict early PA attachment

Crystal violet staining of PA biofilms incubated for 4 and 24 hours from two experiments show inhibition of earlier PA attachment in the presence of HS regardless of heat inactivation (A). No such changes were observed after 24 hours (B). Data points represents mean ± SEM of triplicate wells from two experiments. Statistical significance was calculated by one way ANOVA (Dunnett's multiple comparisons test) * = p≤0.05.

### 3.4.9. Fractionated serum components were unable to prevent earlier PA attachment

Fractionation of HS was performed in order to remove all larger proteins and to analyse whether small peptide or other components of serum such as lipids inhibited earlier PA attachment (see Chapter 2). For this an organic solvent Acetonitrile (ACN) was utilized. ACN is miscible in water and protein, especially small peptides are soluble in ACN while all larger protein including albumin precipitate in the presence of ACN (Chertov et al., 2004). After 4 hours of incubation it was observed that the fractionated HS components was unable to inhibit bacterial attachment at all tested concentrations (0.2, 0.1, 0.05 %) compared to unfractionated HS (0.2, 0.1, and 0.05 %) (n=2) (Fig 3.18).

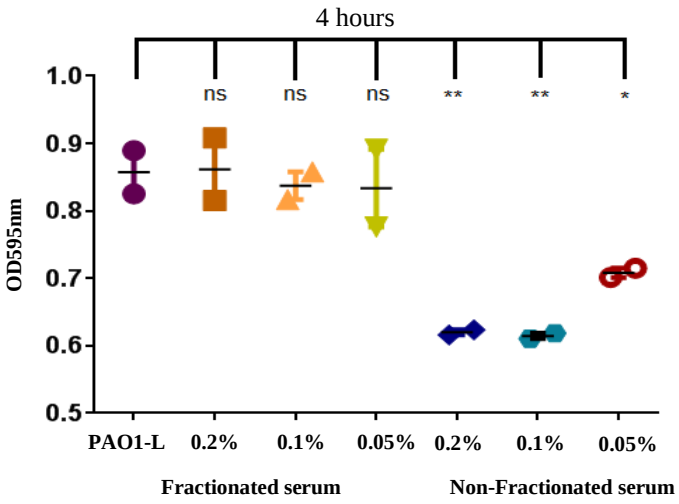



Fig: 3.18 Fractionated serum was unable to restrict earlier PA attachment

Crystal violet staining of PA biofilms incubated for 4 hours from two experiments show fractionated serum do not have any effect on earlier PA attachment .Data points represent mean ± SEM of triplicate or quadruplicate wells from two experiments. Statistical significance was calculated by one way ANOVA (Dunnett's multiple comparisons test) * = $p \leq 0.05$.

## 3.5. Discussion

### 3.5.1. Significance of the study

The innate immune response plays a crucial role in mediating the initial resistance to bacterial infection (Kimbrell and Beutler, 2001). Numerous studies have been undertaken to understand the innate immune response towards planktonic PA. Much less is known about the immune response to PA biofilms. The present study aimed to explore the effect of innate immune components (cytokines and primary immune cells) on the development of PA biofilm under non-opsonic conditions. Towards this aim two cytokines GM-CSF and IFN-γ were investigated to test whether they could alter PA biofilm development. GM-CSF was considered because it is an important inducer of inflammatory macrophages during infection and inflammation (Handman and Burgess, 1979, Hamilton, 2002, Singh et al., 2015). GM-CSF may have a role in inducing IFN-γ and thus promotes a Th1-dominated response through modulation of dendritic cells (Caux et al., 1992, Mariotti et al., 2008). In a recent study treatment of chronically-infected CF patient with inhaled GM-CSF along with several antibacterial and antifungal agents GM-CSF was found to have a positive role in shifting the response from Th2 to Th1, a condition considered to better preserve lung function (Moser et al., 2005, Heslet et al., 2012). Regarding the function of GM-CSF during PA infection it was shown that GM-CSF$^{-/-}$ mice have increased bacterial burden and mortality rate after an intratracheal challenge with PA compared to wild type mice. No defect was observed in early recruitment of PMNs to the site of infection in both GM-

$CSF^{-/-}$ and WT (wild type) mice at 6 hours post infection but the numbers of PMNs in GM-$CSF^{-/-}$ mice were significantly increased compared to WT mice at 24 hours of post infection which likely reflects the fact that the bacterial burden in the lungs of GM-$CSF^{-/-}$ mice is also significantly higher at 24 h. In contrast Alveolar macrophage function including phagocytosis, killing, and $H_2O_2$ production was found defective in GM-$CSF^{-/-}$ mice. It was speculated that defective Alveolar macrophage function in GM-$CSF^{-/-}$ mice may be secondary due to reduced production of TNF-α and IFN-γ (Ballinger et al., 2006). IFN-γ was considered because it can induce classical macrophage activation which makes macrophages more phagocytic and bactericidal (Martinez et al., 2009). Additionally IFN-γ has been found to bind the outer membrane protein OprF resulting in altered expression of the QS-regulated virulence determinant, PA-Lectin (Wu et al., 2005). The present study found (i) PA can form biofilms in X-vivo 15, a defined serum free media, (ii) both GM-CSF and IFN- γ are unable to modulate PA biofilm development and QS molecule productions at the concentrations previously used to activate macrophages and (iii) serum might control PA biofilm development.

### 3.5.2. Optimization of PA biofilm formation using a serum free cell culture media

Since PA biofilm development in itself can be considered to be a virulence factor for chronic infection, it is important to understand the underlying mechanisms which can influence PA biofilm generation, behaviour and structure. It has been reported that hydrodynamics and cell signalling have a role in modulating the structure of PA biofilms (Purevdorj et al., 2002).

For example under conditions of low shear flow biofilms consist of a monolayer of cells with small circular micro colonies but under high-shear, turbulent flow conditions, biofilms formed filamentous structures (Purevdorj et al., 2002). In addition analysis performed using COMSTAT a program used to measure different variables describing biofilm structure showed that different Pseudomonas strains display different modes of biofilm growth (Heydorn et al., 2000). In the present study PA biofilm generation was performed within the BioFlux system using X-Vivo 15 media which is a serum free cell culture media optimised for hematopoietic cells. The reason not to select LB was the presence of yeast extract and animal products such as tryptone that can activate immune cells and could interfere with cytokine action. Within the BioFlux system a microfluidic device is used which is able to controls fluid flow accurately and permits simultaneous growth of multiple biofilms with a large range of precisely controlled flow rates and thus biofilms were grown with a continuous flow of fresh medium. In our assays we used laminar flow (low shear flow 0.05 dyn/cm2) to generate PA biofilms. The results showed that levels of GFP and PI fluorescence signal remained consistent and reproducible among experiments. These results contrast with results obtained using alternative traditional approaches such as growing biofilm within coverslips or metal coupons. The BioFlux viability screening method relied on the use of bacterial strains expressing GFP, while the dead cells were measured using PI staining. For non-GFP-expressing bacteria another method, such as LIVE/DEAD staining was performed (see Chapter 2). The main disadvantage of the BioFlux system was bacterial dispersion which

occurred once the flow was stopped which made it difficult to assess biofilm development at different times; biofilm structures were substantially altered when the flow was stopped to take confocal images. For this besides the BioFlux system, a microtiter assay was used. In this setting PA biofilms were grown in 96 well plates under static conditions and crystal violet staining was performed to measure biomass (O'Toole, 2011). As QS systems and biofilm formation are closely interconnected QS molecules produced by PA biofilms generated in X-Vivo 15 and LB were also analysed (Jensen et al., 2010, Sakuragi and Kolter, 2007). A slight reduction in biofilm generation (biomass and average thickness) and QS molecules production was seen using the X-Vivo 15 media compared to LB broth. Nevertheless X-Vivo 15 media was found suitable for PA biofilm development as it allowed consistent and reproducible biofilm generation compatible with myeloid cell viability.

### 3.5.3. GM-CSF and IFN- γ do not have direct effect on PA biofilm development and quorum sensing regulated virulence factors

Macrophages play an important role in regulation of inflammation. The response of macrophages towards PA infection can be modulated by cytokines like IFN-γ and GM-CSF, which synergise to promote the pro-inflammatory response of macrophages during PA infection. Macrophages stimulated by IFN-γ produced more TNF-α and less IL-10 against PA infection and GM-CSF co-operated with IFN-γ in reducing IL-10 production (Singh et al., 2015). Thus it was postulated that GM-CSF and IFN-γ activated macrophages might be beneficial in controlling PA infection most likely by recruiting neutrophils and by activating them at the

site of infection. It was also hypothesised that cytokines might themselves have a role in controlling PA growth pattern by triggering different immunological responses. Also previous laboratory results showed smaller PA microcolonies associated with macrophages treated with GM-CSF and IFN-γ compared to untreated macrophages. Microcolonies associated with untreated macrophages appeared to increase over time from 2 hours post infection to 4 hours post infection but those associated with macrophages treated with GM-CSF and IFN-γ remained constant (Singh Sonali PhD thesis, University of Nottingham). It was proposed that these microcolonies might represent the earlier stages of PA biofilms and their development might be affected by the presence of activated immune cells. Thus within our model we tried to analyse whether these two cytokines could directly affect PA biofilm development. For this, two different stocks of cytokines were used (1) GM-CSF (10 µg/ml) and IFN-γ (100µg/ml) prepared in RPMI-1640 with 10 % AB human serum and (2) GM-CSF and IFN-γ (100µg/ml) in X-Vivo 15. A significant reduction in surface coverage was observed in response to GM-CSF and IFN-γ using the stock prepared in presence of 10 % AB human serum. No such reduction was observed when the main stock of cytokine was prepared in X-Vivo 15. It was mentioned earlier that IFN-γ has been found to bind the outer membrane protein OprF resulting in altered expression of PA QS regulated virulence determinants such as PA-Lectin (Wu et al., 2005). Within the present study we were unable to detect any differences among biofilms grown in presence or absence of cytokines. This might be related to the lower concentration of IFN- γ (20ng/ml) used in our assays compared to the published report (IFN-

γ 100 µg/ml was used to measure the binding with PA). Similarly our preliminary results showed a dose dependent binding of GM-CSF to PA when using a very high concentration of GM-CSF (1.6µg/ml highest), compared to the concentration used in our assay (GM-CSF 50ng/ml), and stock prepared in the presence of Human AB serum, but no such binding as observed when GM-CSF was diluted in PBS-BSA (Yi-Chia, unpublished). Furthermore the present study showed that both GM-CSF and IFN-γ do not have any effect on QS molecules produced by PA biofilms is agreement with the fact that the QS system has been shown to play an important role in the development of PA biofilms (Williams and Camara, 2009, Davies et al., 1998). It was speculated that PA survival by acquisition of different survival strategies might include the ability to modify/cleavage cytokines. PA secretes a number of toxins such as Elastase and Alkaline protease (AprA) which are able to cleave a number of host defense proteins and tissue constituents (Matsumoto, 2004). AprA produced by PA degrades various host immune system components including complement C1q and C3 and cytokines such as IFN-γ and TNF-α (Parmely et al., 1990). It has been shown that the activity of PA Elastase was markedly reduced in the presence of the serum component α2 macroglobulin which is a protease inhibitor found in high concentration (1-3mg/ml) in human serum. Treatment of IFN-γ with Elastase in the presence of a suboptimal concentration of AprA resulted in an Elastase dose-dependent inactivation of IFN-γ which suggests that the destruction of small, biologically significant peptides by Pseudomonas proteases can involve synergistic effects between proteases which promote their activity even in the presence

of the serum protease inhibitor α2 macroglobulin (Horvat et al., 1989). Besides toxins PA biofilm matrix plays an important role by protecting them from antibiotics and host immune response. Alginate is an important matrix component of mucoid PA has been observed to scavenge hypochlorite, inhibit complement activation and reduce phagocytosis of planktonic mucoid bacteria. In presence of IFN-γ leukocyte killing of (algD-) biofilm bacteria was increased compared to wild type controls, and addition of exogenous alginate to the (algD-) biofilm restored resistance to phagocytosis (Leid et al., 2005). Thus it would appear that these cytokines do not have direct effect on PA biofilm development but act as potent regulators of immune response that promote and activate immune cells at the site of infection.

### 3.5.4. Effect of human serum on PA biofilm development

An important early event in PA biofilm formation is adherence of planktonic cells to surfaces. Approaches directed towards the development of new therapeutic agents to prevent bacterial adhesion and prevent further infections are highly desirable (Hendricks et al., 2000, Sanni et al., 2015, Hook et al., 2013). A published report suggests that serum has the capability to inhibit adhesion of certain bacterial strains to biomaterials (Benson et al., 1996). Our present study confirmed that HS reduced PA biofilm development. Confocal images and COMSTAT analysis of biofilms generated in the presence of HS showed 25 % reduction in surface coverage in the presence of 0.05 % HS which was similar to the reduction observed with cytokines dissolved in RPMI-10% HS. Due to the clear inhibitory effect seen using the BioFlux system it was proposed that HS

might have effect on earlier PA attachment and serum components might be causing this inhibition. Furthermore within the present study all the experiments were performed in X-Vivo 15 media which already contain high levels of human serum albumin, thus it is unlikely that the effect of HS is due to unspecific blocking by serum proteins. Therefore a different assay was developed to analyse the effect of HS at different time points from the same PA culture. The result showed HS inhibit bacterial adhesion significantly at 4 hours at all concentrations tested (0.2, 0.1, and 0.05 %), a similar trend but mild reduction was also observed after 24 hours incubation. Our results were similar to a published report showing that HS inhibits PA biofilm formation by blocking the direct interaction between PA and substrates and by enhancing twitching motility (Hammond et al., 2010). Similarly a reduction up to fivefold of bacterial initial attachment was observed in response to bovine or human serum at 0.5% or higher concentrations in the medium. Further investigation of this phenomenon suggested that Apo-Transferrin (Apo-Tf) is the predominant inhibiting factor and affects early bacterial adhesion (Ardehali et al., 2003). We aimed to determine the potential serum component inhibiting the early PA attachment. Heat activation of HS was performed in order to disrupt the complement activity because complement system can rapidly detect and kill gram negative bacteria (Laarman et al., 2012). We discovered that even after heat inactivation HS (0.2, 0.1, and 0.05 %) is able to restrict earlier PA attachment. Furthermore ACN precipitation of serum was performed to remove all large proteins. Albumin, IgG, transferrin, IgA, haptoglobin and alpha1-anti-trypsin constitute up to 85% of the total protein mass in serum

(Martosella and Zolotarjova, 2008). Thus these large abundant proteins can mask the function of numerous proteins of lower abundance. Addition of 2 volume of ACN is able to remove most of the albumin and other large abundant proteins of more than 20 kDa. After ACN precipitation similar concentrations of both fractionated HS vs non-fractioned HS (0.2, 0.1, and 0.05 %) in X-Vivo 15 was analysed and it was observed that fractionated serum lost the ability to inhibit PA biofilm development. Thus it was suggested that probably the large and abundant protein(s) is/are responsible for inhibition of early PA attachment. Among these serum components Apo-transferrin (Apo-Tf), a 80 kDa iron deficient form of Tf, has been shown as a potent inhibitor of PA adhesion to several biomaterials. Apo-Tf belongs to a family of iron binding monomeric glycoproteins, with a broad spectrum of antimicrobial properties (Jurado, 1997). Apo-Tf is known to limit bacterial growth by sequestering iron, an action that makes this essential nutrient unavailable to an invading pathogen. At a concentration of 20 µg/ml which is lower than the concentration present within different body fluids, Apo-Tf is able to reduce bacterial adhesion to all tested biomaterials more than fourfold (Ardehali et al., 2002, Ardehali et al., 2003). A similar mechanism was described for Lactoferrin which inhibit PA biofilm formation by curbing the available iron. Interestingly it was observed that Lactoferrin at concentrations 20 µg/ml is able to inhibit PA biofilm development but do not affect the growth rate of planktonic PA (Singh et al., 2002). The exact mechanism by which serum inhibits initial PA attachment in this study is still unknown. Similar to many bacteria PA require iron for infection success, PA utilize iron to control bacterial

behaviour by influencing cell–cell communication (quorum sensing) and virulence factor expression (Smith et al., 2013, Vasil and Ochsner, 1999). Low iron availability activates the Las system (Bollinger et al., 2001, Duan and Surette, 2007). The relationship between iron and PQS system is complex. PQS itself acts as an iron chelator and thus controls the activation of the Rhl system through iron limitation (Bredenbruch et al., 2006). It has been showed that biofilm formation by PA strains including PAO1 and CF sputum isolates from chronically infected patients was reduced significantly upon iron removal under aerobic conditions (O'May et al., 2009). Also it was shown that addition of iron to cultures can reverse the negative effect of synthetic chelators effect on biofilm formation, whereas the biological chelator lactoferrin has found to increase anti-biofilm activities upon addition of iron. Thus it was proposed that lactoferrin might use a more complex mechanisms compared to the synthetic chelators to impair biofilm development (O'May et al., 2009). Thus it might be interesting to identify iron regulated bacterial systems involved in biofilm formation.

## 3.6. Summary

Results of the present chapter demonstrate that GM-CSF and IFN-γ at the tested concentration are unable to restrict PA biofilm development and QS molecule production in absence of human serum. Although the presence of human serum would make the assay physiologically more relevant it was necessary to exclude serum to determine the specific effects of cytokines. Also no direct effect of both GM-CSF and IFN-γ was seen on PA

growth(Singh et al., 2015).Because we were unable to identify any effect of these cytokines on PA biofilm we continued our work into the effect of innate immune cells on PA biofilm in context of inflammation. For this an *in vitro* model was developed by generating PA biofilm in the BioFlux system which was found able to provide clear and reproducible results, also PA biofilm development was optimized using a cell culture media X-Vivo 15 which will allow us to explore the role of other innate immune components specially different immune cells such as monocytes and macrophages by adding them over a PA biofilm and thus different parameters could be analysed such as measuring adhesion of these cells on PA biofilm, measuring different parameters of PA biofilm such as biomass, average thickness after incubating them with immune cells, measuring different PA virulence factors including Rhamnolipid, QS molecules which will give a clear idea about the effect of these immune cells on PA biofilm as well as on PA virulence factors. Also it would be interesting to explore other cytokines that might have role in PA biofilm development. Thus future work could involve the analysis of other cytokines including IL-17A produced from Th17 cells in PA biofilm development. As it has been mentioned previously Th17 cells and their effector cytokine particularly IL-17A provide host defensive against different extracellular bacterial infections and able to induce the expression of a variety of pro-inflammatory cytokines, thus provide early recruitment of neutrophils at the site of infection (Ouyang et al., 2008). Some of our preliminary lab work has already shown that IL-17 enhances neutrophil- mediated killing of PA under non opsonic condition (performed by Msc student Asante

Afrakoma). Also special emphasis can be placed on the analysis of the effect of specific combinations of effector cytokines that modulate the microbicidal capacities of immune cells but would promote less tissue damage.

# Chapter 4

## Effect of immune cells on *Pseudomonas.aeruginosa* biofilms in the context of inflammation

### 4.1. Introduction

Bacteria growing in biofilms often display a variety of phenotypic differences compared to planktonic cultures. The biofilm mode of growth provides many advances to bacteria compared to the planktonic lifestyle (Jefferson, 2004). During infection the mechanism of resistance against phagocytes is not completely understood but likely depends on a number of biofilm-specific factors including slow growth and the sticky matrix which may contain DNA and other polymers, predominantly exopolysaccharides.

Biofilm matrix performs an important role within biofilms by maintaining cell-to-cell contact and acting as a scaffold. The EPS contributes to overall biofilm architecture and to the resistant phenotype of bacteria in biofilms (Branda et al., 2005, Ramsey and Wozniak, 2005, Sutherland, 2001). The matrix in PA biofilms is mainly composed of exopolysaccharides, proteins and extracellular DNA, although composition depends on the growth conditions, the age of the biofilm, and the PA strain (Tolker-Nielsen, 2014).PA expresses three different types of exopolysaccharides that help in EPS formation: alginate, Pel, and Psl (Ryder et al., 2007). These polysaccharides varied in chemical structure and also in their biosynthetic mechanisms (Franklin et al., 2011). The contribution of each exopolysaccharide to the biofilm matrix varies among strains. Alginate facilitates the persistence of biofilms in CF patients and is commonly found in

mucoid PA strains (Wozniak et al., 2003). Non mucoid strains such as PAO1 and PA14 were found fully capable of producing biofilm lacking alginate which suggests that there were one or more polysaccharides, in addition to alginate, that contribute to biofilm formation (Ryder et al., 2007). The Psl polysaccharide is composed of a repeating pentamer containing D-mannose, L-rhamnose, and D-glucose (Franklin et al., 2011). Psl can be detected using labelled mannose-specific lectins. It displays a helical distribution that surrounds the cell surface of PAO1 and enhances cell to cell and cell surface interactions during biofilm formation (Ma et al., 2009). PA appears to deposit a Psl trail while moving on a surface, thus under these conditions Psl facilitates bacteria motility and favour encounter with subsequent cells that follow the trails leading to a positive feedback loop (Zhao et al., 2013). Rugose small colony variants (RSCVs) of PA isolated from CF sputum are characterized by hyper adherence and hyper aggregation that result from elevated expression of Pel and Psl (Colvin et al., 2012, D'Argenio et al., 2002, Irie et al., 2010). Thus it was proposed that Psl might have an important role in the formation of biofilms within the CF lung. Psl is considered to play an important role in PA pathogenesis. Psl is required for adherence to a variety of surfaces including mucin-coated surfaces and airway epithelial cells and overproduction of the Psl is associated with enhanced cell surface and intercellular adhesion of PAO1 (Ma et al., 2006). Moreover it has been shown that production of Psl is associated with initial attachment and promotion of NF-κB Activation in A549 cells (Byrd et al., 2010). Additional studies showed that Psl could reduce neutrophil reactive oxygen species (ROS) production and phagocytosis by reducing complement-mediated opsonisation (Mishra et al., 2012). Thus Psl

provides a survival advantage *in vivo* and therapeutic measures aimed at Psl could facilitates clearance of persistent PA infections. Psl also promotes antibiotic resistance, Psl-mediated formation of tight micro-colonies increase resistance towards antibiotic treatment (Yang et al., 2011) and Psl provides a generic first line of defence against antibiotics with different biochemical properties during the earlier stages of biofilm development (Billings et al., 2013). Psl-mediated protection extended to *Escherichia coli* and *Staphylococcus aureus* in biofilm co-cultures (Billings et al., 2013). Another key aspect of Psl is that it stimulates the production of a secondary messenger molecule c-di-GMP by signalling the production of two diguanylate cyclases; SiaD and SadC and provides a positive feedback circuit that potentiates Psl production, and maintains biofilm growth (Irie et al., 2012). A monoclonal antibody that bound to one particular epitope of Psl facilitates potent phagocytic killing of PA *in vitro* and also limits bacterial attachment to cultured lung epithelial cells, and provides prophylactic protection in different animal models of PA infection. (DiGiandomenico et al., 2012). Thus Psl can be targeted as a novel protective antigen and antibodies against Psl might provide an effective mean for prophylaxis against PA infections in CF patients.

The innate immune system constitutes the first line of defence towards pathogens and performs an important role in the early recognition and triggering subsequent response to invading pathogens (Medzhitov and Janeway, 2000). The response of the innate immune system mainly relies on recognition of evolutionarily conserved structures on pathogens named pathogen/microbe-associated molecular patterns (P/MAMPs) using germ line-encoded PRRs , which are expressed on innate immune cells including DCs,

macrophages and neutrophils (Akira et al., 2006, Medzhitov and Janeway, 2000, Kawai and Akira, 2010). Till now not many studies have been performed addressing the innate immune response to biofilm-associated infections. Recent studies involved exposure of PA biofilm to human neutrophils and macrophages and assessment of respiratory burst, penetration, phagocytosis and killing of bacteria within the biofilm (Bjarnsholt et al., 2005, Jesaitis et al., 2003, Jensen et al., 2007, Leid et al., 2005). It has been shown before that biofilm mode of growth of PA which is regulated by QS facilitates increase tolerance to antibiotics and also protect them from bactericidal activity of PMNs, thus an intervention with conventional antimicrobial, PMNs along with QS inhibitors would be an option to eliminate biofilm PA before establishing chronic infection (Bjarnsholt et al., 2005). It has been shown that the infected CF airway is dominated by endobronchial mucoid biofilms surrounded by numerous PMNs (Bjarnsholt et al., 2009, Kolpen et al., 2010). Alginate which is a major matrix component involved with PA biofilm development has been demonstrated to protect PA biofilm from IFN-γ mediated macrophages killing (Leid et al., 2005). However the recruitment and activation of immune response may vary throughout the lung. The upper part of the lungs termed the conductive zone (trachea, bronchi and bronchioles) is important for capturing inhaled particles and microbes, which are expelled into the oropharynx by the mucociliary activity and cleared by swallowing or coughing. In CF patients, function of cilia is severely affected due to the reduced volume of the airway surface liquid (ASL) which facilitates microbe adherence in the larger airways (Knowles and Boucher, 2002). Pathogens trapped within the mucus can be transferred to the respiratory zone (respiratory bronchioles and alveoli) where

gas exchange takes place. This region of the lung containsAM, alveolar epithelial cells and the majority of the pulmonary DCs which recognize the P/MAMPS within microbes through their PRRs (Craig et al., 2009, Schmiedl et al., 2010, Christophersen et al., 2012).

Macrophages are major components of the lung innate cellular surveillance system (Hartl et al., 2012). Different PPRs have been implicated in PA recognition by macrophages (Chapter 1) but PRRs specific for PA biofilms have not been identified (Speert et al., 1988, Heale et al., 2001, Descamps et al., 2012, Xaplanteri et al., 2009). Phagocytosis of unopsonised PA by human monocyte-derived macrophages is facilitated by MR which is expressed in abundance by monocyte-derived macrophages and tissue macrophages (including alveolar macrophages) but not monocytes (Speert et al., 1988). MR has been involved in recognition of unopsonised PA and thus contributes together with to TLR2 to pro-inflammatory cytokine production by human monocytes in response to PA infection (Xaplanteri et al., 2009). MR has been implicated in the modulation of cellular activation, and also in the promotion of antigen presentation (Martinez-Pomares, 2012). Structurally, MR contains distinct regions, capable of binding to different carbohydrates (Martinez-Pomares, 2012). The MR cysteine-rich domain (MR-CR) binds to sulphated carbohydrates such as $SO_4$-3-galactose, the fibronectin type II domain (MR-FNII) binds to collagen, and the C-type Lectin-like domains (CTLDs) bind to mannosylated sugars and several other carbohydrates (for example those that terminate with fucose) (Martinez-Pomares, 2012) (showed in Figure 4.1). PA Slime-(Glyco lipoprotein) GLP has been suggested as a potential ligand for

MR and is able to induce TNF-α production by macrophages (Xaplanteri et al., 2009).

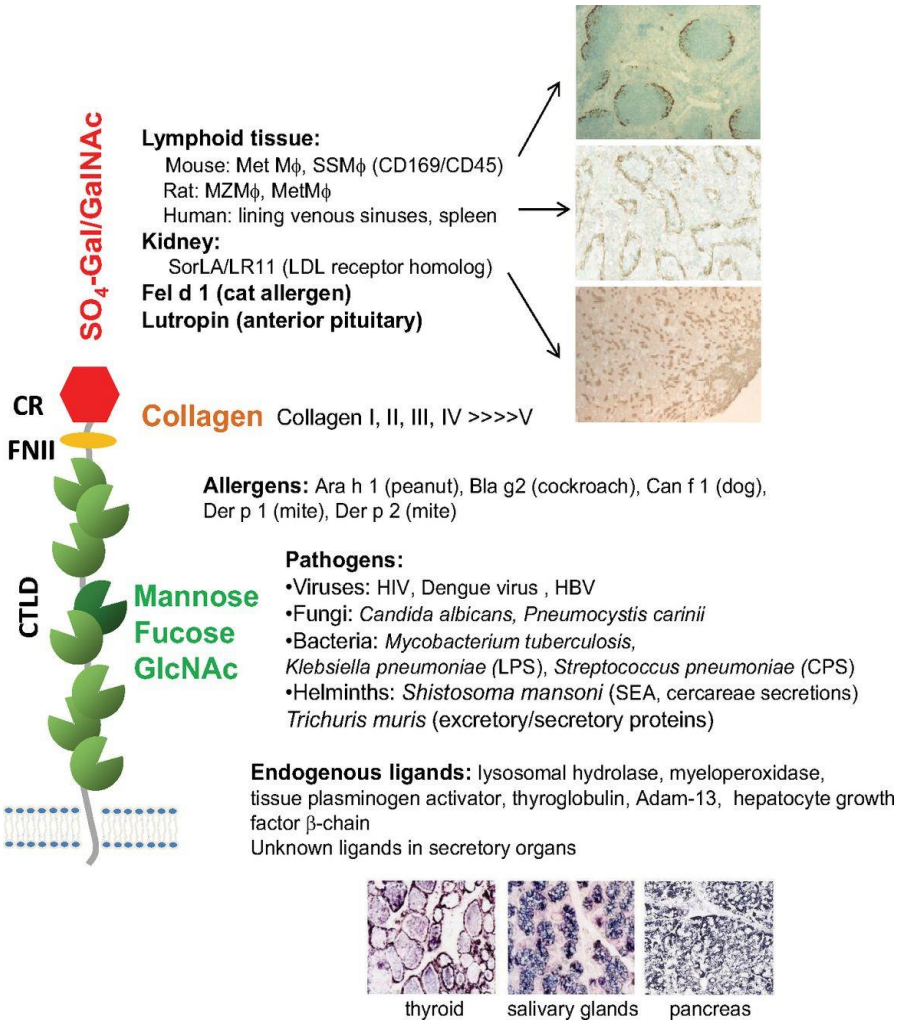



Fig: 4.1 Structural properties of MR

MR has three types of extracellular domains. 1. The CR domain binds sulfated glycans that can be found in lymphoid tissues and kidney and can also be found in the major cat allergen Fel d 1 and lutropin. The FNII domain binds collagens. The CTLDs bind to mannosylated sugars and several other carbohydrates (for example those that terminate with fucose) in endogenous and exogenous molecules, including allergens and microbial products.Macrophage; HBV, hepatitis B virus; CPS, capsular polysaccharide; SEA, secreted egg antigen; Adam-13, a disintegrin and metalloprotease 13. This figure was adopted from **(**Martinez-Pomares**,** 2012**)**

## 4.2. Hypothesis

The present study aimed at understanding the interaction between PA biofilm and human monocytes and macrophages with the underlining hypothesis that human immune cells could interact with PA biofilms through the recognition of mannosylated carbohydrates by lectin receptors expressed by these cells.

## 4.3. Aims

1. Analysis of the adhesion of human primary phagocytic cells (macrophages and monocytes) to PA biofilms generated using the BioFlux system.
2. Measure the binding of PA biofilm to MR.
3. Measure the binding of purified Psl to MR
4. Explore the use of MR-Fc chimaeric protein in modulating neutrophil activation in response to PA and zymosan
5. Study the effect of lectins on PA biofilm development

## 4.4. Results

### 4.4.1. Optimization of monocytes labelling with PKH26

After optimising the generation of PA-GFP biofilms in a culture media (X-Vivo 15) compatible with eukaryotic cell viability using the BioFlux system (Chapter 3) it was necessary to develop a co-culture strategy to investigate the interaction of human immune cells and PA biofilms. A first step towards this aim was the optimisation of a procedure to fluorescently label the cells. A red fluorescent dye named PKH26 suitable for monitoring cell movement and location was chosen (Chawla et al., 2006). Optimisation of the labelling procedure was performed to explore whether the label had any effect on cell

surface receptors expression and cellular activation. For this surface marker expression and ROS production of labelled and unlabelled monocytes was compared. Monotypes purified from fresh blood were labelled with PKH26 and characterised by flow cytometry (Fig 4.2A) and observed by confocal microscopy (Fig. 4.2B). Preliminary flow cytometry analysis showed similar levels of surface markers in unstained and stained cells. Labelled monocytes could be clearly observed by confocal microscopy. To measure the functional activity of PKH26-labelled monocytes, ROS production in response to Zymosan (50 particles/cell) and live PA (10 PA/cell) was compared between monocytes unlabelled and labelled with two different concentrations of PKH26 (2 and 4 µM). ROS produced by monocytes labelled with 2 µm PKH26 dye was closer to that of unlabelled cells (Fig 4.3) and was used in future experiments.

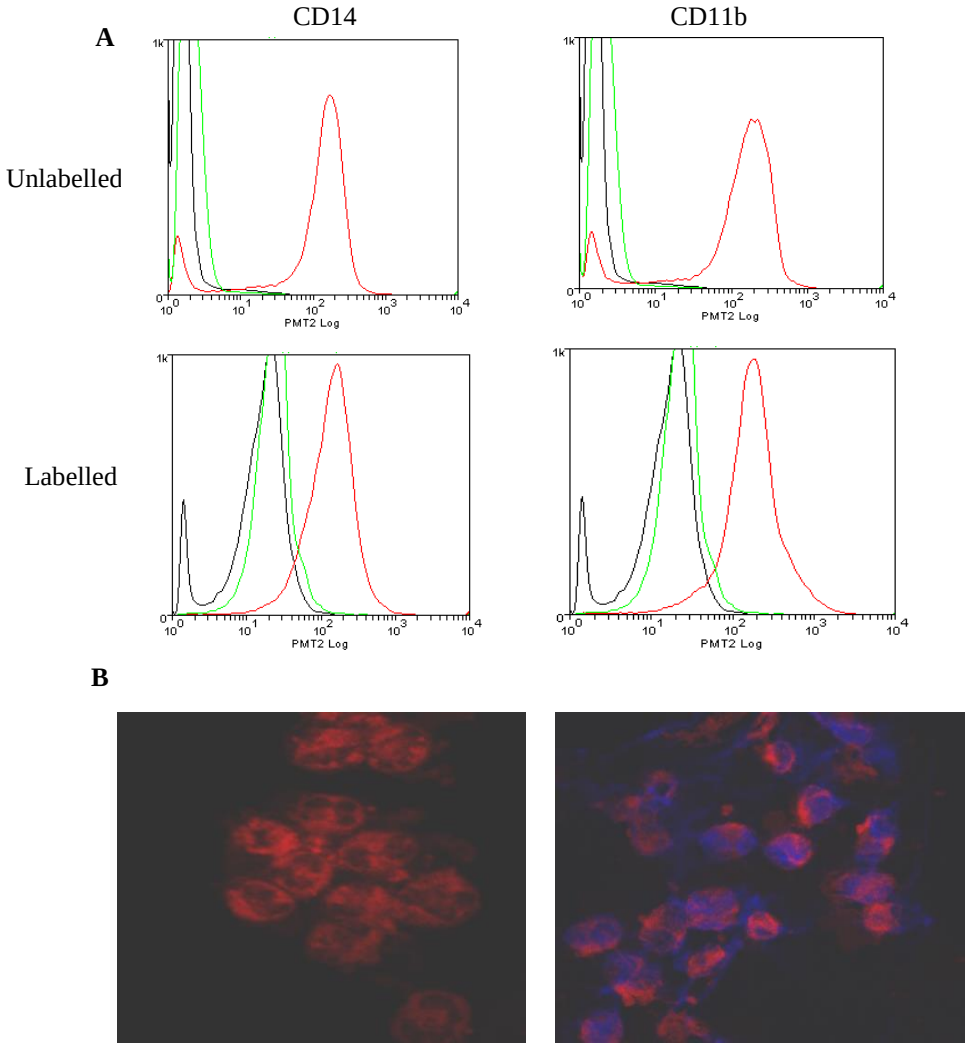



Fig: 4.2 Characterisation of PKH26-labelled monocytes

**A.** Histograms depicting expression of CD14 and CD11b on unlabelled and PKH 26-labelled monocytes. Black lines represent unstained cells, green lines represent cells stained with isotype control antibody, and red lines represent specific staining for the respective markers. **B.** Confocal images of PKH26-labelled monocytes with magnification 60x without (left) and with nuclear stain (blue colour, right)

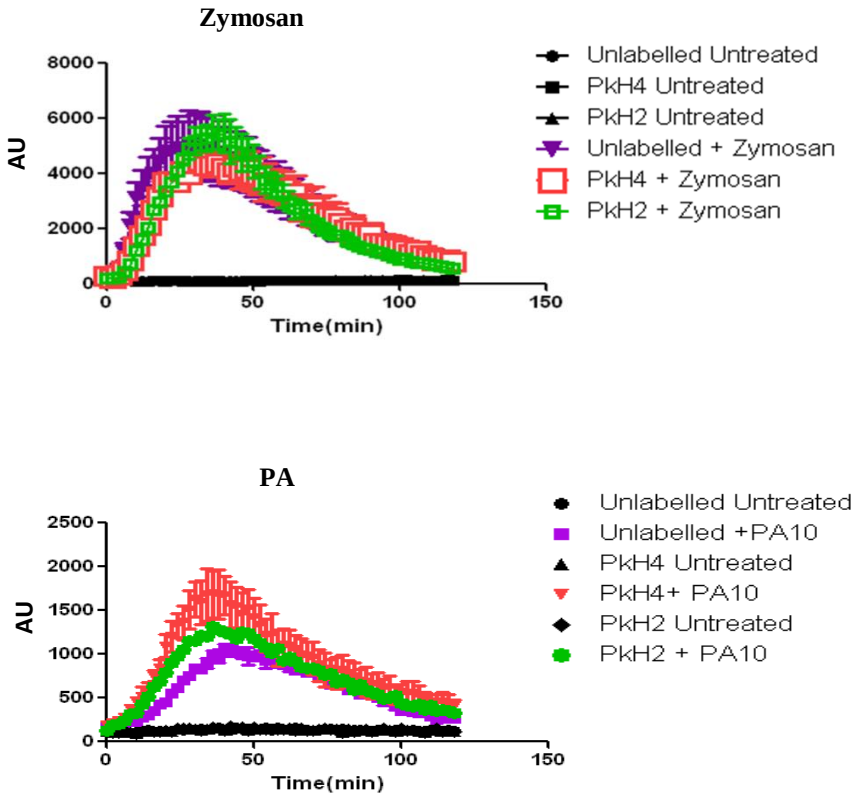



Fig: 4.3 ROS production by unlabelled and PKH26-labelled monocytes

For labelling two different concentrations of PKH 26 dye were used PKH2= 2µm and PKH4= 4µm. 50,000 monocytes/well were added to a 96-well microtitre plate that already contained luminol and zymosan (50 particles/cell) (Top panel) or live PA (10 PA /cell) (Bottom panel). Luminescence was measured every 2-5 min for 2 h. All conditions were carried out in triplicate.

### 4.4.2. Analysis of the interaction between Monocytes and Macrophages with PA biofilms

To explore the possibility of using the BioFlux system to investigate the interaction of monocytes with PA biofilms, 50,000 PKH 26-labellled monocytes were added to a 15 hours grown PA biofilm established within a BioFlux channel using a shear pressure of 1 dyn/cm$^2$. After one hour of incubation two different behaviours of monocytes were seen. One set (more than half) remained motile and did not adhere to the PA biofilm. The second set were adherent but it was not clear if they were adhered to the substrate or the biofilm. Indeed most monocytes got trapped within the hinges of the BioFlux channels and were unable to reach the site of PA biofilm. Nevertheless

a fraction of monocytes were in contact with PA biofilm and remained over the surface (Figure- 4.4, 4.5), n=1.

In a different set of experiments PHK26-labelled macrophages prepared from monocytes in the presence of M-CSF (50,000 macrophages/ channel) were used. In this occasion the cells were incubated for 1 and 3 h using separate channels. Macrophages showed a tendency to form clusters that attached to PA biofilm (Fig 4.6), n=1.But due to the initial design of the experiment the fate of this cluster wasn't followed after 3 hours of incubation. On Both occasion labelled monocytes and macrophages were counted and viability was assessed before adding them on Bioflux channels. No cell clumps were observed at that stage.

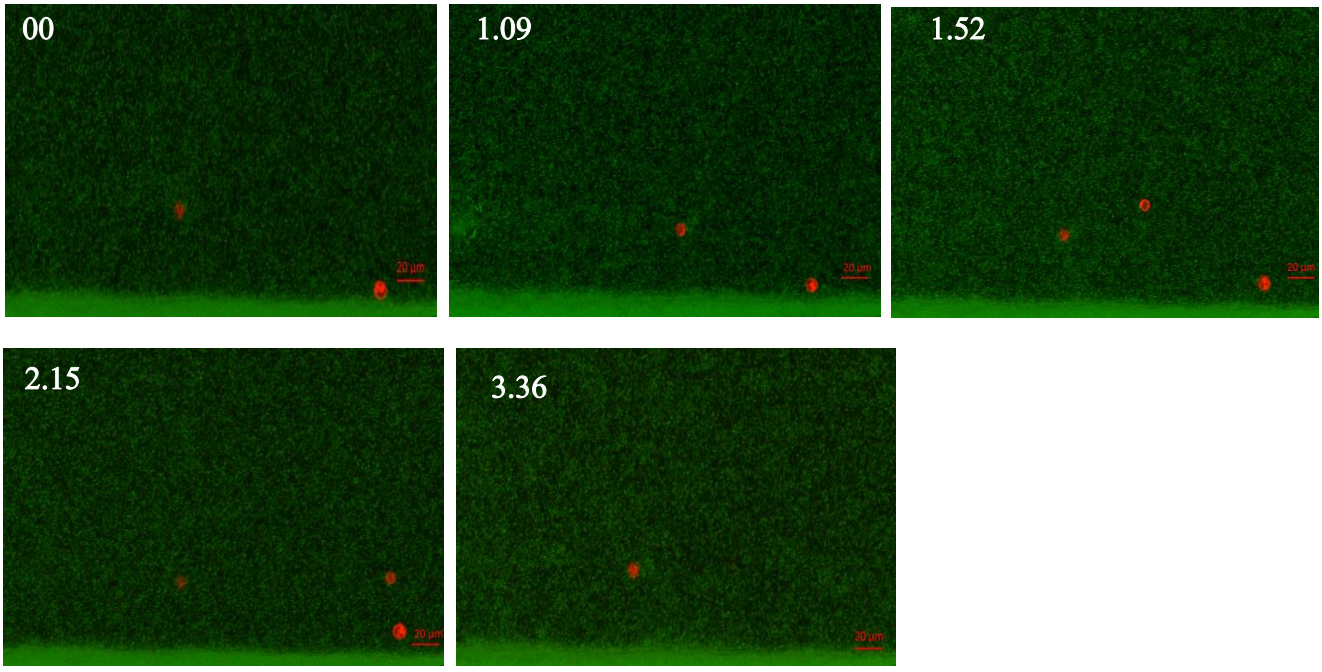



Fig: 4.4 Interaction of Monocytes with a PA biofilm over time

Monocytes (Red) were incubated with PA biofilm (Green) for 1hour, after that their movement was observed by confocal microscopy. A sequence of events at 0, 1.09, 1.52, 2.15 and 3.36 min within a selected area is depicted in images showing monocytes movement over PA biofilm.

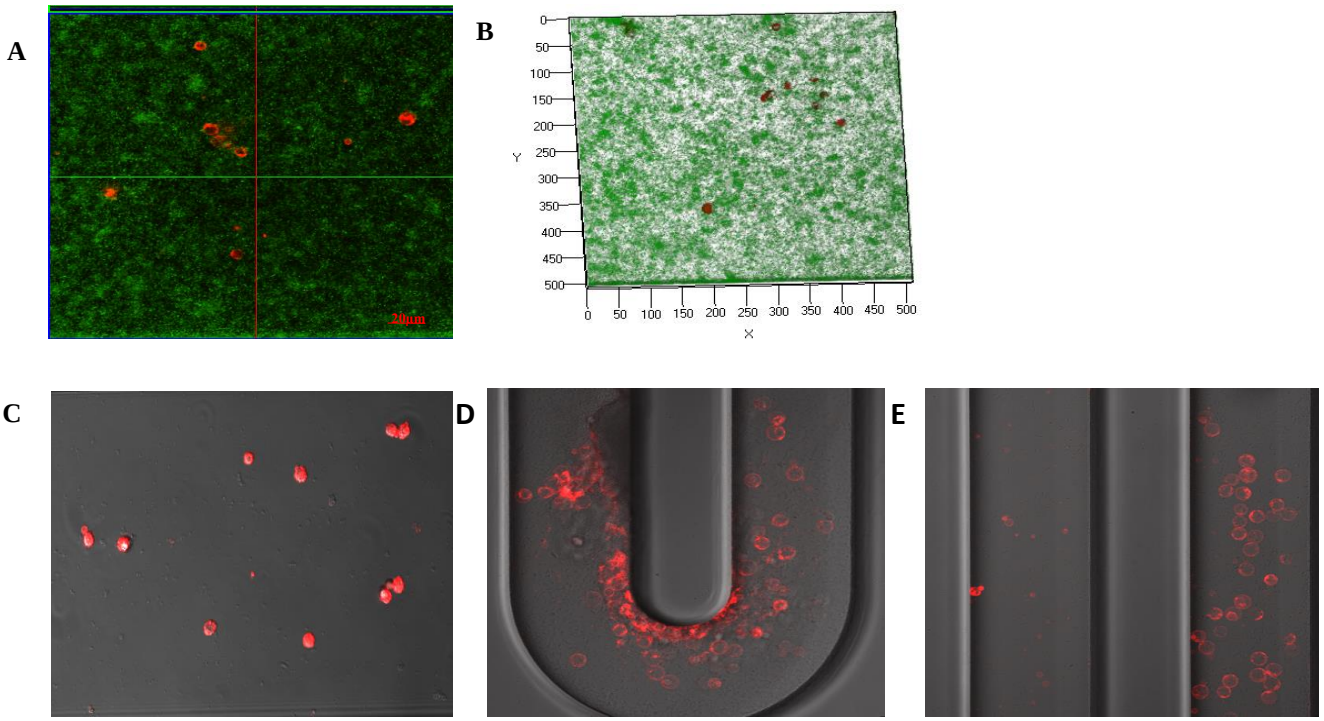



Fig: 4.5 confocal images showing interaction between monocytes and PA biofilm after 1 hour incubation

A and B Adherent monocytes remained over PA biofilm. C. Channel containing monocytes only. D. Monocytes remained adherent to different sites of the BioFlux channel. Images were acquired using 20 x magnifications.

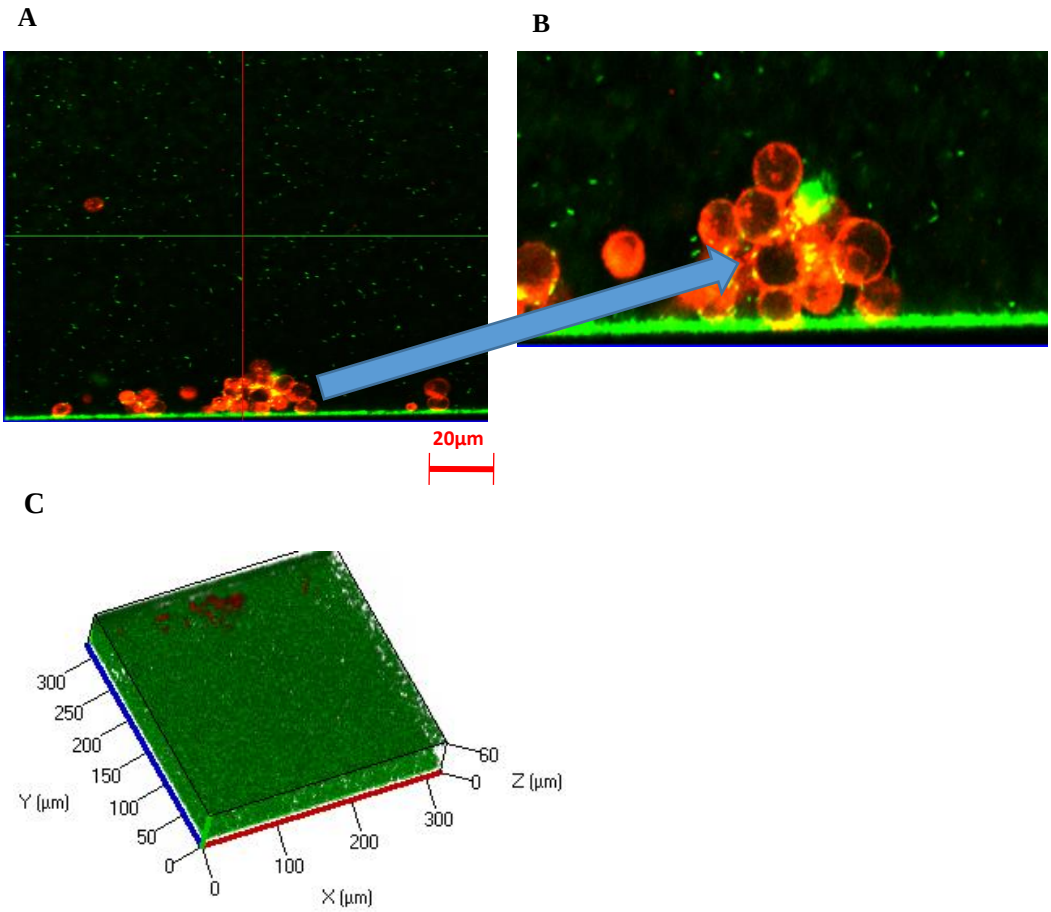



Fig: 4.6 Interaction of macrophages with PA biofilms

Confocal images of PKH26-labelled macrophages after incubation of 3 hours with a PA biofilm grown for 15 h. Images Were acquired using 20 x magnifications.

Flow cytometric analysis of cell surface markers on freshly isolated monocytes and day 7 differentiated M-CSF macrophages were compared. By Day 7 the macrophages displayed elevated expression of particular cell surface markers such as, MR and CD16 and a modest increase in CD14 and CD11b. Macrophages were also more autofluorescent (Fig 4.7).

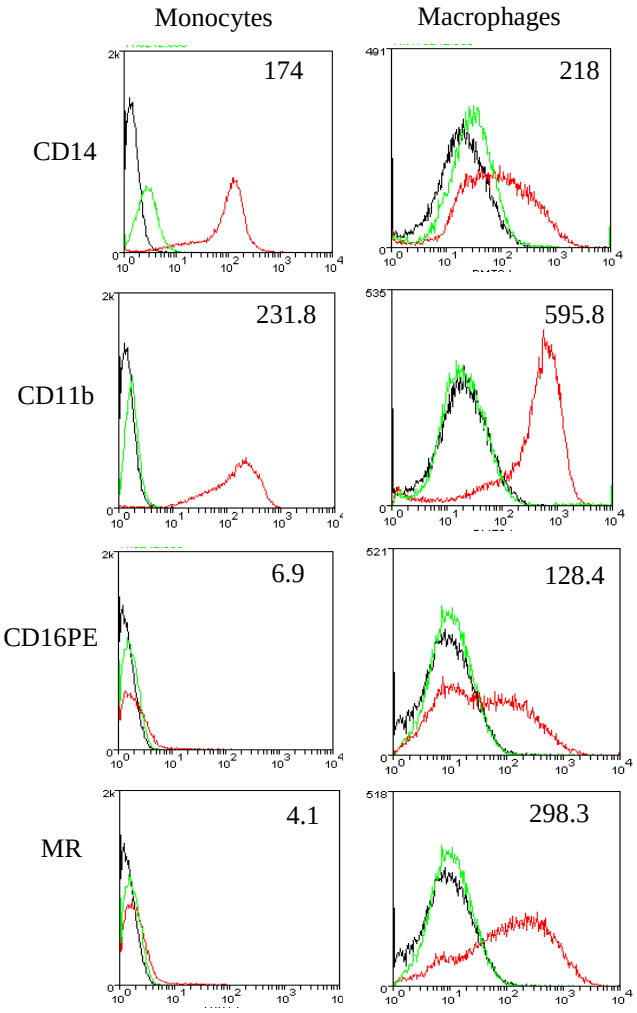



Fig: 4.7 Flow cytometry analysis of human monocytes and macrophages

Representative histograms denoting expression of CD14, CD11b, CD16 and MR on monocytes and macrophages generated from leucocyte cones in RPMI complete medium with 50 ng/ml rhM-CSF. Black lines represent unstained cells, green lines represent cells stained with isotype control antibody, and red lines represent specific staining for the respective markers. MFI for the markers is noted in the top right hand corner of each histogram.

### 4.4.3. Potential contribution of MR to the recognition of PA biofilm by macrophages

Previous results suggest that human monocyte-derived macrophages attached and formed clusters over PA biofilm. Flow cytometric analysis of cell surface receptors expression showed MR expression in macrophages is higher compared to monocytes as already described in several studies before (Shepherd et al., 1982, Speert and Silverstein, 1985, Singh et al., 2015). It has been already reported that MR is involved in macrophages phagocytosis of unopsonized PA (Speert et al., 1988). Therefore it was hypothesized that macrophages could interact with PA biofilms through recognition of mannosylated carbohydrates in the extracellular matrix by MR. To test this hypothesis we utilized two strains, PAO1 (Parsek) and a mutant Δ*wspf* Δ*pel* PA 105 (Parsek) which overproduces Psl but lacks Pel (Kirisits et al., 2005). Before testing their ability to bind MR, characterization of these two strains was performed to confirm their ability to form biofilm and whether Δ*wspf* Δ*pel* PA 105(Parsek) is able to produce more Psl as expected.

#### 4.4.3.1. Overexpression of Psl correlates with improved biofilm formation

To visualize Psl on the PA biofilm we utilized a fluorescently labelled lectin HHA (from Hippeastrum hybrid) which detects mannose-rich structures, in particular Psl within PA biofilms (Ma et al., 2009). Both PAO1 (Parsek) and Δ*wspf* Δ*pel* PA 105(Parsek) biofilm were grown in the BioFlux and stained with HHA Lectin (20µg/ml). Confocal images showed that Δ*wspf* Δ*pel* PA 105 (Parsek) produced more Psl than PAO1 (Parsek) (Figure 4.8). To determine whether increased Psl production correlates with improved biofilm formation, biofilms of both PAO1 (Parsek) and Δ*wspf* Δ*pel* PA 105(Parsek) were

generated on 96 well plates at 37°C in the presence of $CO_2$ for 4 and 24 hours. Crystal violet staining was performed to measure biofilm biomass. Results at 4 hours showed more biomass in the case of Δ*wspf* Δ*pel* PA105 (Parsek) compared to PAO1 (Parsek). A similar trend was observed after 24 hours but difference was more clear after 4 hours of incubation (Figure 4.8) (n=2).

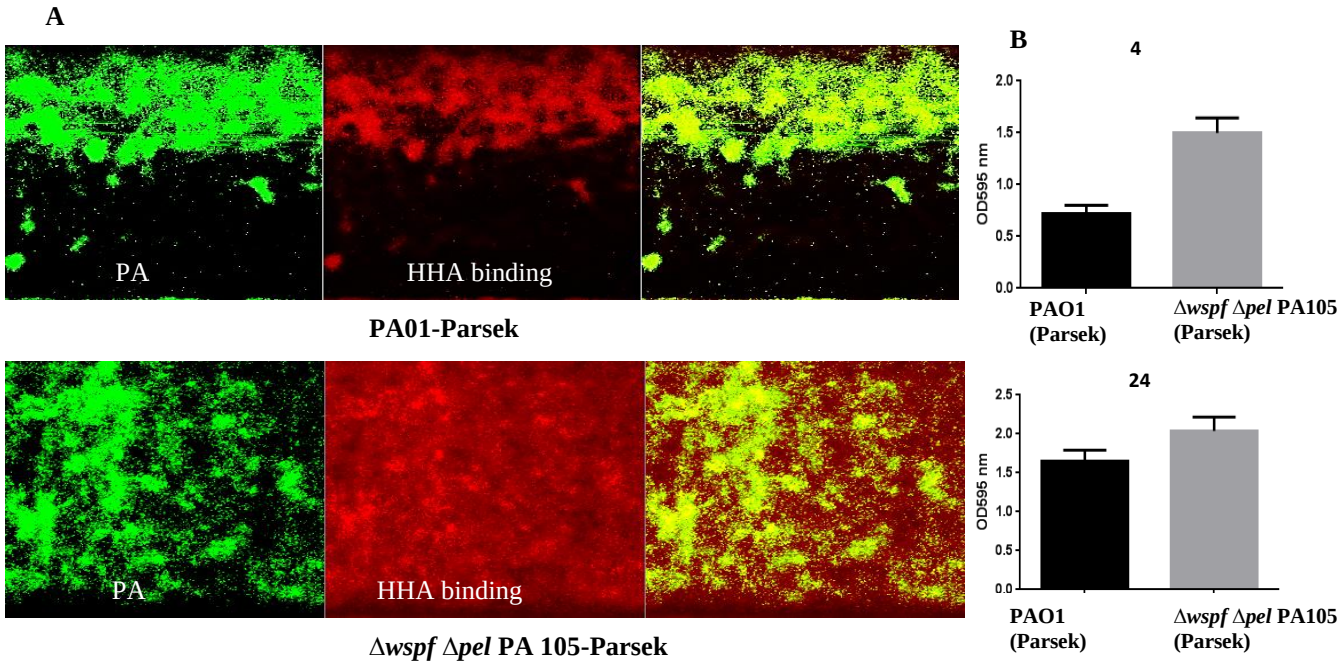



Fig: 4.8 Overexpression of Psl correlates with increased HHA binding and more biofilm formation

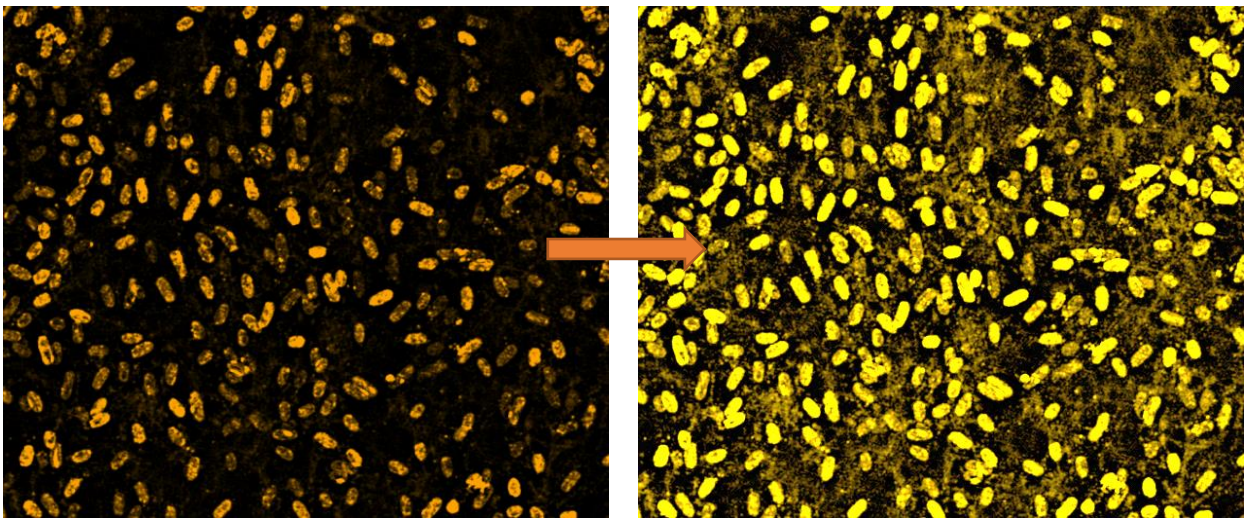
A. Confocal images (20x magnification) showing binding of HHA (red) to both PA01-Parsek and Δ*wspf* Δ*pel* PA 105-Parsek biofilms. Biofilm were generated inside a BioFlux system and Psl was detected using HHA Lectin (20 µg/ml) conjugated with Texas red. Biomass was detected using sytox 9 (green). B. Crystal violet assay was performed to measure biomass in PA01-(Parsek) and Δ*wspf* Δ*pel* PA 105 cultures at two different times (4 and 24 hours), N=2 .

### 4.4.3.2. Analysis of Psl distribution within biofilms using super resolution microscopy

To better understand the distribution and organization of Psl within the PA biofilm, Δ*wspf* Δ*pel* PA 105(Parsek) was grown on a coverslip using 6 well plates at 37°C in presence of 5 % $CO_2$ for 72 hours. Psl was stained using HHA conjugated with Texas Red (20µg/ml) and samples were visualised using super

resolution microscopy. As seen in Figure 4.9 Psl matrix material was also detected in areas where there were no bacterial cells, n=1.

**Δ*wspf* Δ*pel* PA 105(Parsek)**



**Original image** **Image after reconstitution**

Fig: 4.9 Super resolution images showing Psl anchored to the PA surface and forming a extracellular matrix

Biofilm of Δ*wspf* Δ*pel* PA 105(Parsek) was generated on cover slips using 6 well plates (72 hours). Psl was detected using HHA Lectin (20µg/ml).

### 4.4.3.3. Psl was detected in PA supernatants

Based upon the above results we hypothesized that Psl could be released from the bacterial cells and thus promote cell-cell and cell-surface interactions. To detect secreted Psl, supernatants from both PAO1 (Parsek) and Δ*wspf* Δ*pel* PA 105 (Parsek) were prepared in X-Vivo 15 and tested for the presence of Psl using the HHA lectin in a dot blot assay. A clear signal in both undiluted and diluted samples of Δ*wspf* Δ*pel* PA 105 (Parsek) could be detected. A faint signal was seen in the case of the PAO1 (Parsek) supernatant (Figure 4.10),n=1.

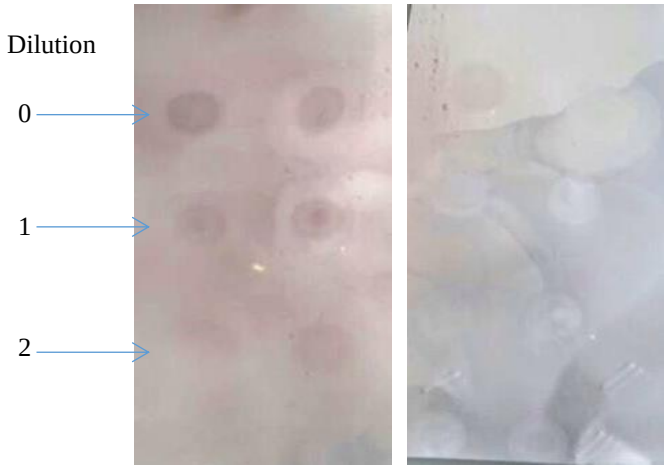



Fig: 4.10 Psl was detected on PA supernatant

Supernatants were prepared from PAO1-Parsek and Δ*wspf* Δ*pel* PA 105 (Parsek) overnight cultures in X-Vivo 15 and Psl was detected using a dot blot assay and HHA- Lectin (20 µg/ml).

### 4.4.4. The CTLD4-7 region of MR binds to PA biofilms

To assess whether the MR was able to bind directly PA biofilms, and in particular Psl, a plasmid encoding for the recombinant protein CTLD4-7-Fc was isolated (Martinez-Pomares et al., 1996, Linehan et al., 2001) and the purified protein resulted from transfecting human cells was used to test binding to PA biofilms grown in 96 well plates. Results showed that CTLD4-7-Fc binds to PA biofilms. The average OD was high when CTLD4-7-Fc (10 µg/ml) was added to both Δ*wspf* Δ*pel* PA105 (Parsek) and PA01 (Parsek) biofilm (average=1.04276 and 0.844625 respectively, n=2). This was substantially higher than the average OD when 2 µg/ml CTLD4-7-Fc was used was added to Δ *wspf* Δ *pel* PA105 (Parsek) and PAO1 (Parsek) biofilms (average=0.419225 and 0.3077, n=2). We also compared the binding of CTLD-4-7-Fc with a negative control CR-FNII-CTLD1-3-Fc which contains the regions of MR responsible for binding sulphated sugars and collagen. As expected a higher

binding was observed with CTLD 4-7-Fc compared to CR-FNII-CTLD1-3-Fc (average OD=0.3861 for 10 µg/ml and 0.2268 for 2µg/ml with Δ *wspf* Δ *pel* PA105 (Parsek) and 0.3103 for 10 µg/ml and 0.1931 for 2µg/ml with PAO1 (Parsek)). Also the binding appeared substantially higher with CTLD4-7-Fc than the average OD for both Δ *wspf* Δ *pel* PA105 (Parsek) and PA01 (Parsek) strains when just anti-human IgG and the substrate were added (0.1972 and 0.172 respectively) (Figure 4.11). However pre incubation with mannose (10 and 2 mM) and EDTA (5mM) was unable to inhibit CTLD 4-7-Fc binding completely (Figure 4.12, n=2).

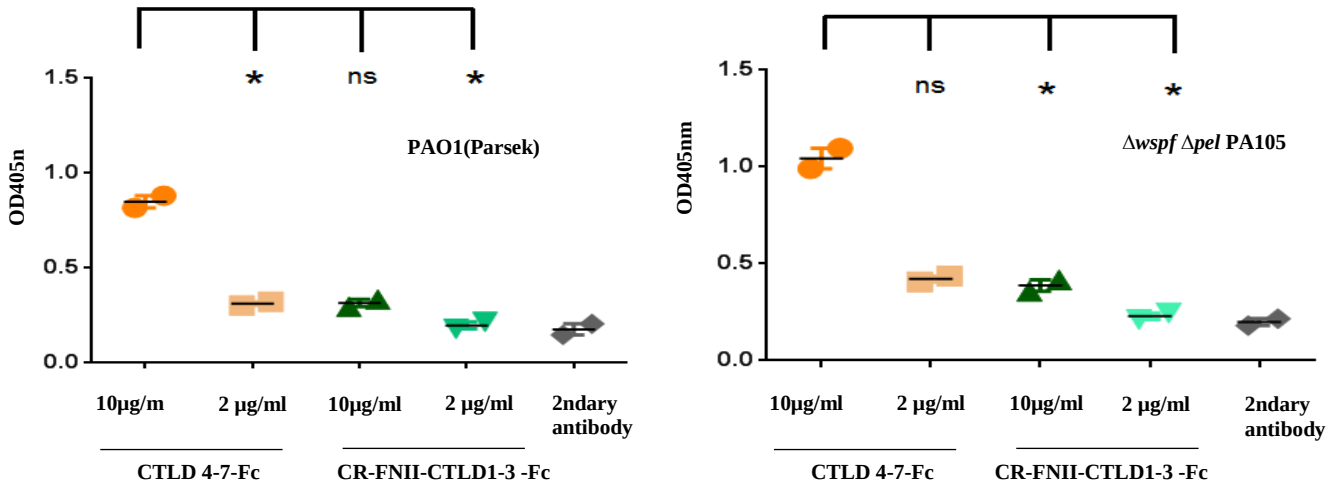



Fig: 4.11 The CTLD4-7 region of MR binds to PA biofilms

Binding assay demonstrates dose dependent, higher binding of CTLD4-7-Fc to Δ *wspf* Δ *pel* PA105 (Parsek) and PA01 (Parsek) compared to CR-FNII-CTLD1-3-Fc. Binding assay was performed by growing biofilm in 96 well plates (24 hours). After fixation with 2 % formalin biofilm was incubated with CTLD 4-7-Fc or CR-FNII-CTLD1-3-Fc. Binding was detected using anti-human IgG Fc-specific antibody conjugated to alkaline phosphatise. Data points represent Mean ± SEM from two different experiments. Statistical significance was calculated by one way ANOVA (Dunnett's multiple comparisons test) * = $p \leq 0.05$,

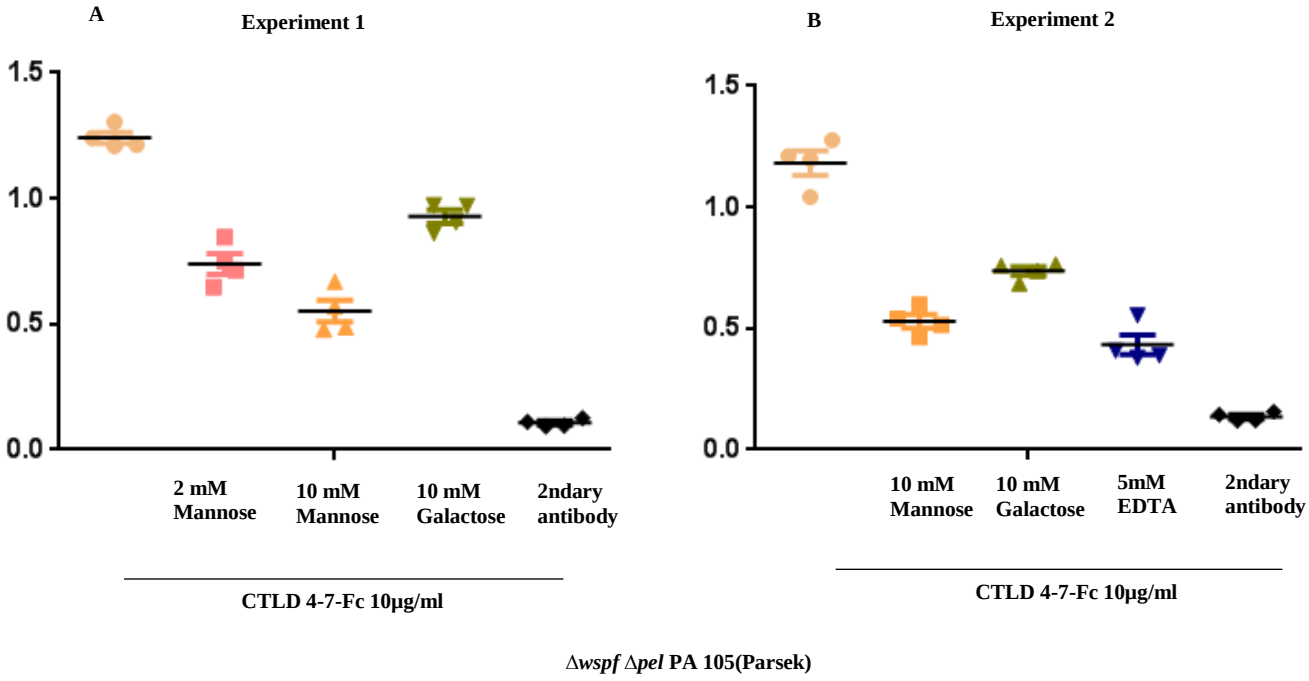



Fig: 4.12 Pre-incubation with Mannose partially inhibits CTLD4-7-Fc binding to PA biofilms

Competitive binding assay was performed by pre incubating CTLC 4-7-Fc (10 µg/ml) in presence or absence of Mannose (2, 10 mM), Galactose (10mM) in experiment 1 **(A)** and Mannose (10 mM), Galactose (10mM), EDTA (5nM) in second experiment (B). Biofilm of *Δ wspf Δ pel* PA105 (Parsek) was grown on 96 well plates for 24 hours. Binding was detected using anti-human IgG Fc-specific conjugated to alkaline phosphatise. Data points represent Mean ± SEM from four plates of one experiment.

To confirm these initial findings biofilms of Δ *wspf* Δ *pel* PA105 (Parsek) grown in the BioFlux were incubated with CTLD 4-7-Fc (10 µg/ml) for 1.5 hours. The binding was detected using anti-human IgG Fc-specific conjugated with Alexa fluor 647 (secondary antibody-red). Confocal 3D images showed binding of CTLD 4-7-Fc to PA biofilm (Shown in red Figure 4.13). Interestingly the binding of CTLD4-7-Fc appeared more in and around the biomass of PA (shown in green). Within the same experiments HHA-Texas red (20 µg/ml) staining was performed on a separate BioFlux channel and the labelling pattern observed with HHA lectin was similar to that obtained with CTLD-4-7-Fc. However a high background was seen when the biofilm was incubated with secondary antibody (Figure 4.13). It was predicted that binding without fixation of Δ *wspf* Δ *pel* PA105 (Parsek) biofilm might be the reason for high background. To determine whether fixation was able to minimize the background with the secondary antibody, Δ*wspf* Δ *pel* PA105 (Parsek) biofilms were grown on cover slips in 6 well plates at $37^{o}$C in presence of 5 % $CO_2$. After 72 hours incubation biofilms were fixed with 4 % paraformaldehyde and the binding assay was performed. After 2 hours of incubation a clear binding was observed when biofilms were incubated with CTLD 4-7-Fc (10 µg/ml) compared to negative control CR-FNII-CTLD1-3-Fc (10µg/ml) (Figure 4.14). It was difficult to perform replicates as biofilm generation in coverslips is very unreliable.

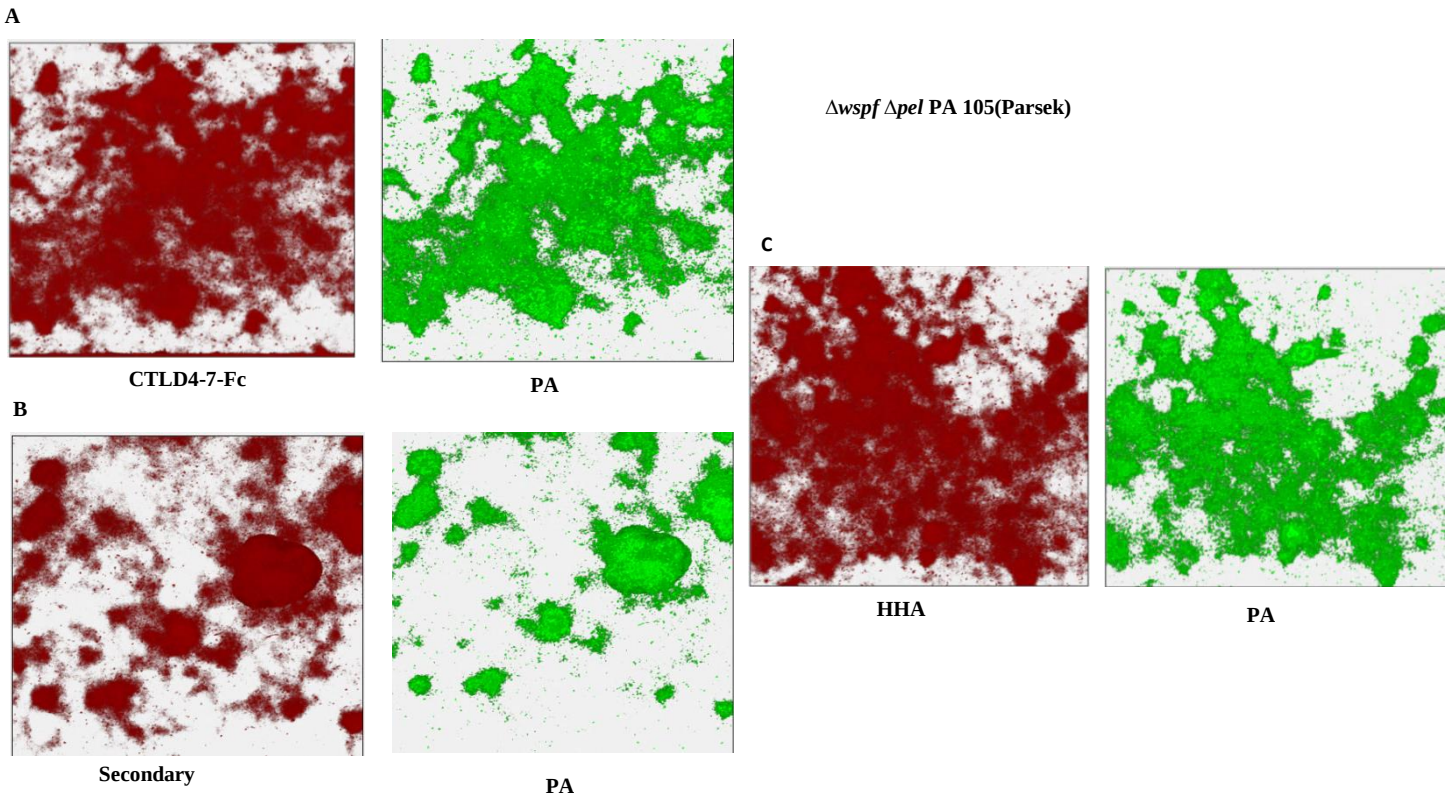
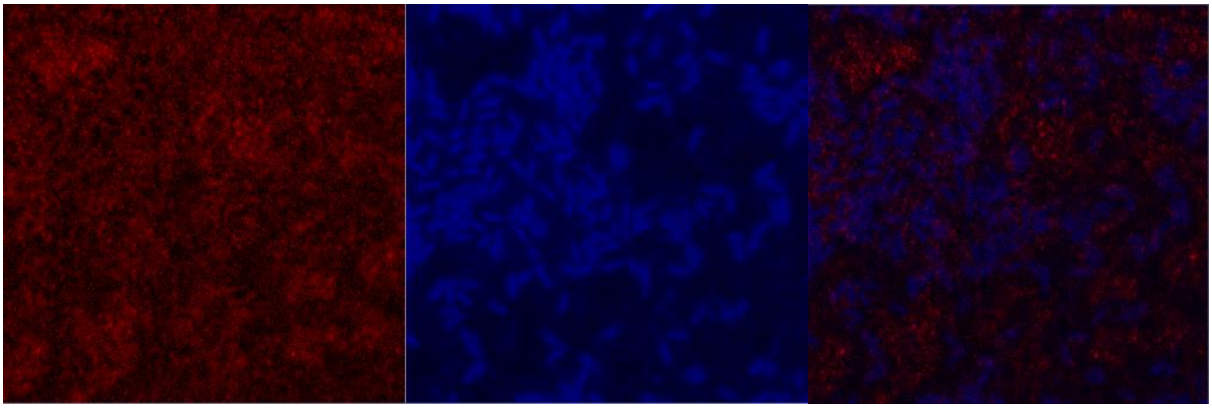
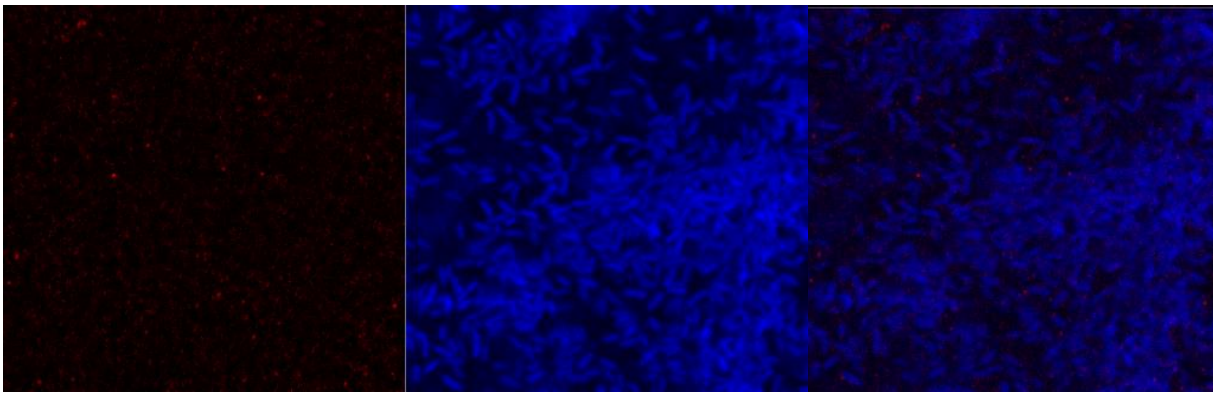



Fig: 4.13 The CTLD4-7 region of MR binds to non-fixed PA biofilms

Confocal images showing binding of CTLD 4-7-Fc (red) to PA biofilm (green) (20x magnification). Biofilm of Δ*wspf* Δ*pel* PA 105(Parsek) was generated within the BioFlux channel and was incubated with CTLD-4-7-Fc (10µg/ml). The binding was detected using anti-human IgG Fc-specific conjugated to Alexa Fluor 647(secondary antibody-red). Biomass (Live bacterial cells) within the biofilm was detected using sytox 9 (green).To detect Psl biofilm of Δ*wspf* Δ*pel* PA105 (Parsek) was incubated with HHA Lectin (20 µg/ml) conjugated with Texas Red.



**CTLD 4-7 Fc 10 µg/ml+ secondary antibody 5µg/ml** **Δ*wspf* Δ*pel* PA 105(Parsek)**



**CR-FNII-CTLD1-3-Fc 10 µg/ml+ secondary antibody 5µg/ml**

Fig: 4.14 The CTLD4-7 region of MR binds to fixed PA biofilms

Confocal images showing binding of CTLD 4-7-Fc (red) to PA biofilm (60x magnification). Biofilm were generated on coverslips using 6 well plates (72 hours). Binding of CTLD4-7-Fc or CR-FNII-CTLD1-3-Fc were detected using anti-human IgG Fc-specific conjugated with alexa fluor 647(red)**.** Biomass in biofilms was detected using HOECHST (1 ug/mL in PBS).

### 4.4.5. CTLD 4-7-Fc binds crude EPS preparation from Δ *wspf* Δ *pel* PA105 (Parsek)

The ability of CTLD 4-7-Fc to bind EPS of Δ *wspf Δ pel* PA105 (Parsek) was examined. For this a crude preparation of EPS was prepared as described in (Chapter 2) based on the procedure described by (Byrd et al., 2009). Maxisorp plates were coated with the extracted crude EPS overnight at a concentration of 500µg/ml. Results showed that CTLD 4-7-Fc binds this crude EPS extract and a higher binding was observed with 10 µg/ml (average OD= 0.665) compared to 2 µg/ml (average OD=0.20). Whereas binding of the negative controls appeared lower (CR-FNII-CTLD1-3-Fc 10 and 2 µg/ml average OD=0.13, 0.13733 respectively) (Figure- 4.15).

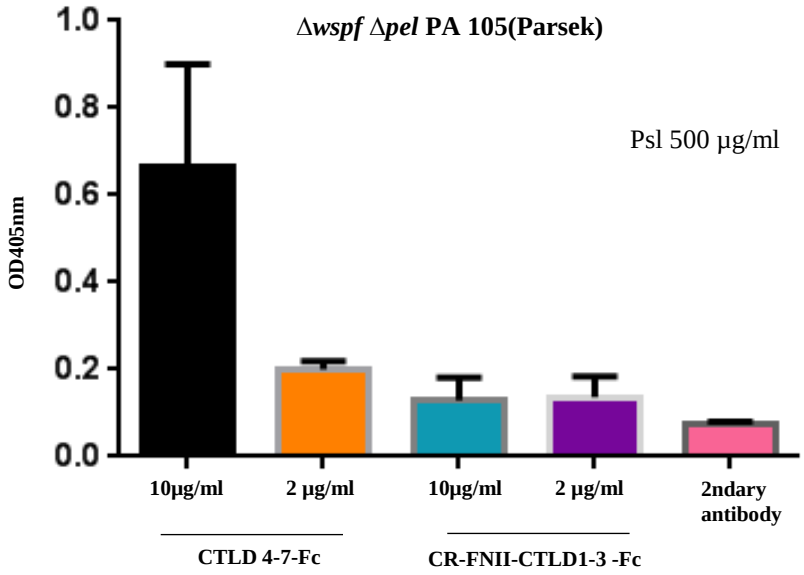



Fig: 4.15 CTLD4-7-Fc region of MR binds to crude EPS extracted from Δ*wspf* Δ*pel* PA 105(Parsek) culture

Binding assay demonstrated dose dependent and higher binding of CTLD4-7-Fc to crude extract of EPS compared to CR-FNII-CTLD1-3-Fc. Binding assay was performed by coating wells with crude EPS overnight at a concentration of 500µg/ml. After that EPS was incubated with CTLD 4-7-Fc or CR-FNII-CTLD1-3-Fc. Binding was detected using anti-human IgG Fc-specific conjugated to alkaline phosphatase. Each bar represents Mean ± SEM of one experiment.

### 4.4.7. CTLD4-7-Fc was unable to modulate neutrophil ROS production in response to Zymosan and PA

The present study indicates that MR can bind to the mannosylated polysaccharide Psl on PA biofilms. It was proposed that this binding could have therapeutic merit in the future, as the recombinant protein consist of mannose binding site of mannose receptor (CTLD4-7) on one end and an Fc fragment at the other which could be utilized to activate and up-regulate various immune cells through Fc receptors. It was considered useful to test whether pre incubation of PA or Zymosan with CTLD4-7-Fc could modify ROS production by human neutrophils. Result showed that CTLD4-7-Fc was unable to modify neutrophils ROS production in response to both PA and Zymosan (Figure 4.16). Initially the assay was performed using bacteria cultured for 3 hours but a second experiment was done using an overnight culture of PA in the presence of increased concentration of $Ca^{2+}$. It was expected that overnight cultured PA would be producing more Psl compared to 3 hours culture, increased amount of $Ca^{2+}$would improve CTLD4-7-Fc binding to the particles. Positive controls showing that antibody optonisation increased ROS production would have been desirable.

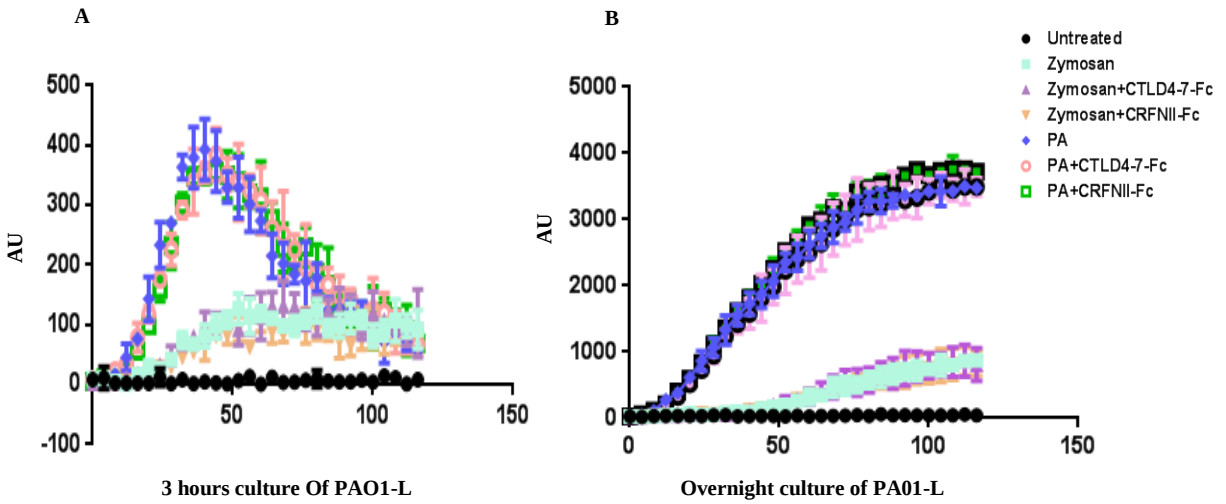



Fig: 4.16 CTLD4-7-Fc was unable to promote neutrophils ROS production in response to zymosan and PA

50,000 neutrophils/well were added to a 96-well microtitre plate that already contained luminol and zymosan (50 particles/neutrophil) or live PA (10 PA/neutrophil) prepared from either 3 hours culture (A) or overnight culture (B) incubated with or without CTLD4-7-Fc or CR-FNII-CTLD1-3-Fc. Luminescence was measured every 2-5 min for 2 h. All conditions were carried out in triplicate.

### 4.4.8. Presence of CTLD4-7-Fc does not affect PA biofilm formation

Within the present study it was investigated whether CTLD4-7-Fc was able to block PA biofilm formation by recognising mannose-rich Psl as its ligand. Confocal image (5X magnification) showed CTLD4-7-Fc (10µg/ml) doesn't have any effect on PA biofilm formation (both live and dead cells). Furthermore the result was also confirmed by performing crystal violet assay where both CTLD4-7-Fc and CR-FNII-CTLD1-3-Fc (10 and 20µg/ml) were found unable to inhibit PA biofilm development after 24 hours of incubation.

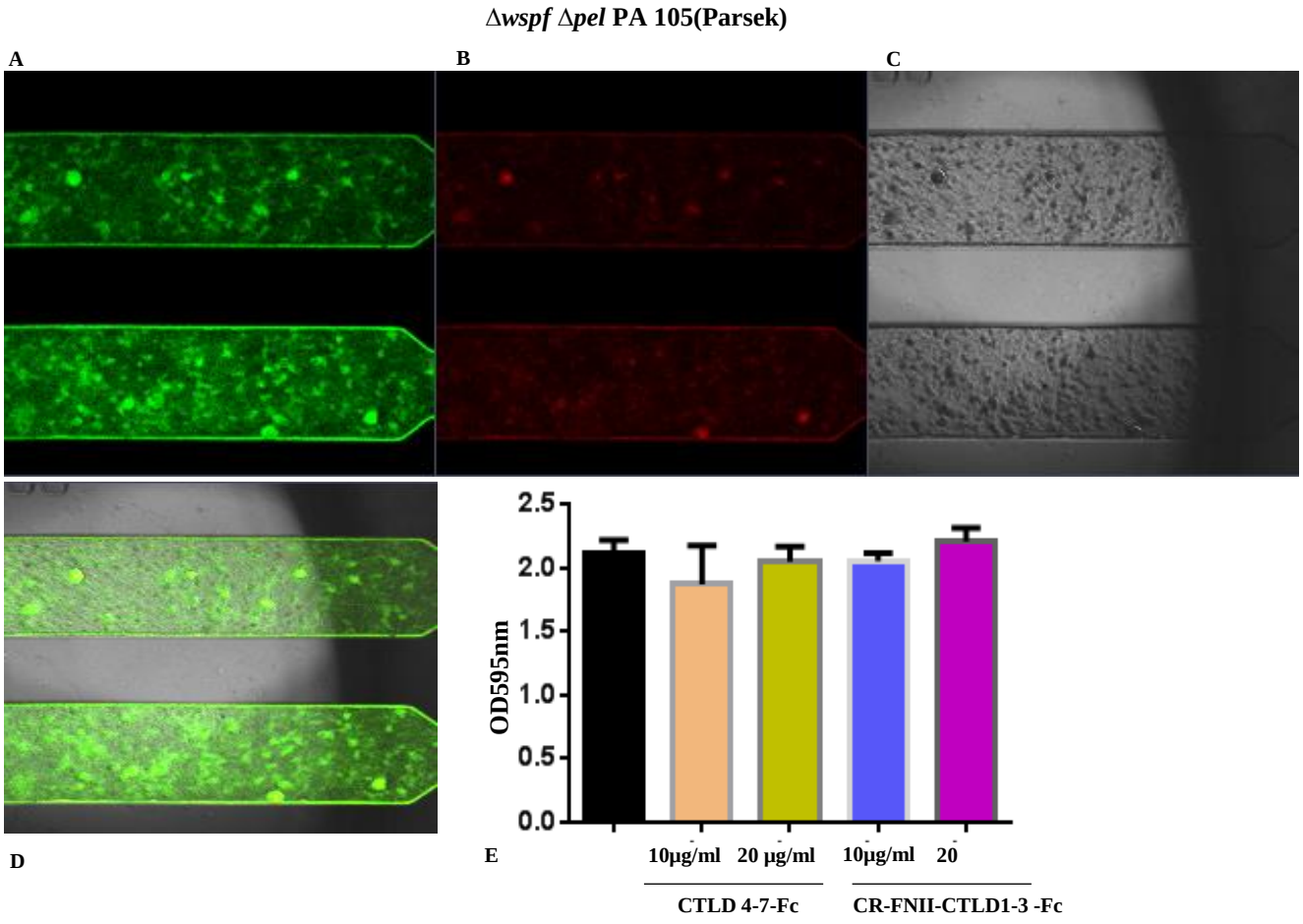



Fig: 4.17 CTLD4-7-Fc does not affect Δ*wspf* Δ*pel* PA 105(Parsek) biofilm formation

Images from two adjacent BioFlux channels containing PA biofilm incubated with or without CTLD 4-7-Fc (10µg/ml)**A.** Sytox 9 labelling showing live cells **B.** PI staining showing dead cells **C.** Bright field microscopy image **D.** Merged image of both Sytox9 and PI (5X magnification) . **E.** Crystal violet assay was performed to measure biomass of PA biofilm incubated with or without lectins CTLD 4-7-Fc (10 and 20 µg/ml).

## 4.5. Discussion

### 4.5.1. Role of innate immune cells in recognition of PA biofilm

The present study aimed to investigate the recognition of PA biofilms by immune cells (monocytes and macrophages) using an *in vitro* model. Although a number of studies have addressed the ability of both human and murine macrophages to phagocytose and regulate inflammation by producing cytokines and chemokines in response to PA and its products, there is limited information about the role of macrophages in PA biofilm-associated infection (Speert et al., 1988, Speert and Gordon, 1992, Kannan et al., 2008, Raoust et al., 2009, Power et al., 2007). Several types of PRRs and their corresponding ligands have been already identified for PA (see Chapter 1 for details), but PRRs capable of recognising biofilm-growing microorganisms have not yet been determined. It has also been shown that bacterial factors are able to modify immune response after PRRs recognition. Under non-opsonic conditions presence of pili and functional flagella (a TLR5 ligand) are believed to render PA more susceptible to phagocytosis (Amiel et al., 2010, Mahenthiralingam and Speert, 1995, Speert et al., 1986). Moreover, it has been shown that TLR2 and TLR4 are involved in the activation of monocytes by mannuronic acid polymers (poly-M) which is the major component of alginate EPS (Flo et al., 2002). Similar to alginate, other EPS components such as Psl and Pel within PA biofilms may also trigger immune responses (Ryder et al., 2007). Psl is involved in both initial bacterial attachment to surfaces and PA biofilm maintenance throughout the different stages (Ryder et al., 2007, Ma et al., 2009, Byrd et al., 2010). Some of the preliminary experiments performed in the present study showed that macrophages showed a tendency to cluster over

PA biofilms whereas monocytes were unable to associate to these structures. It was speculated that macrophages might have different cell surface receptors expression pattern compared to monocytes and thus could recognise particular components of PA biofilm. As expected analysis of cell surface markers expression showed an elevated expression of MR in macrophages compared to monocytes from the same donor which was similar to other published reports (Shepherd et al., 1982, Speert and Silverstein, 1985, Singh et al., 2015). MR is an integral membrane protein expressed, among others, on tissue macrophages and immature dendritic cells. MR contains multiple domains in the extracellular region, including $Ca^{2+}$-dependent lectin-like carbohydrate recognition domains (CTLD) region that bind to D-mannose, L-fucose, and N-Acetyl-glucosamine expressed on different pathogens and endogenous molecules (Reading et al., 2000, Martinez-Pomares, 2012). Psl is a mannose-rich polysaccharide which led to the hypothesis for this study; that the mannose-rich nature of Psl would allow binding to a mannose-binding lectin (Ma et al., 2009).

### 4.5.2. Involvement of MR in recognition of PA biofilm and contribution to PA infection

In addition to using the wild type strain of PAO1 (Parsek) a Δ*wspf* mutant of PAO1 (Parsek) with a deletion in the Pel operon was used in this study (Kirisits et al., 2005). Before analysing the role of MR in recognition of PA biofilm, characterization of both WT PAO1 (Parsek) and Δ*wspF* Δ*pel* PAO1 mutant (Parsek) strains was performed. This study showed: (i). Δ*wspf* Δ*pel* PA105 (Parsek) produce more Psl compared to PAO1 (Parsek) based on HHA lectin staining (Figure- 4.8). (ii). *Δ wspf* Δ *pel* PA105 (Parsek) produce more biofilm

compared to PAO1 (Parsek) based on crystal violet assay after 4 and 24 hours incubation but the difference appeared more obvious after 4 hours incubation (Fig-4.8). It was predicted that the difference should have been maintained even after 24 hours but because cultures were incubated in 96 well plates it is possible that this culture condition might fail to provide enough space for biofilm formation or that the OD reading was above the linear scale. For future work dilutions of the solubilised crystal violet before measuring OD to obtain more accurate results was considered. (iii) High resolution microscopy images of HHA lectin staining of Δ *wspf* Δ *pel* PA105 (Parsek) biofilms showed that Psl covered individual bacterial cells and formed a spider web like structure which connect bacterial cells and also attach them to the surface as described (Ma et al., 2009). Interestingly in some places the Psl matrix fibre was seen without any bacterial cells which was similar to a previous published report (Ma et al., 2009). It was predicted that PA might be able to secrete Psl and it might be possible to detect Psl in supernatants. In this study we were able to detect Psl in Δ *wspf* Δ *pel* PA105 (Parsek) supernatant using HHA lectin in a dot blot assay. The reason for considering Δ *wspf* mutants was to keep c-di-GMP levels consistent among all strains as it was shown that the intracellular concentration of c-di-GMP also increases when Psl is over expressed (due to the positive feedback nature of Psl) (Irie et al., 2012).

The present study showed that MR is a potential functional receptor for PA biofilm. We found that a recombinant protein (CTLD4-7-Fc) comprising the mannose binding region of MR can bind to PA biofilms, possibly through Psl. A higher binding of CTLD-4-7-Fc towards both PAO1 (Parsek) and Δ *wspf* Δ *pel* PA105 biofilm was observed compared to all negative controls including

CR-FNII-CTLD1-3-Fc and secondary antibody only, this observation was confirmed by three individual repeats. The protein CTLD4-7-Fc was produced by plasmid transfection of HEK293T cells. Once purified this protein was tested using different carbohydrates including mannose as described (Linehan et al., 2001) to ensure it functioned correctly (data not shown). The binding assays performed towards PA biofilm indicated that the recombinant protein performed similarly to the way that the mannose binding domain of MR would be expected to. Binding of CTLD-4-7-Fc towards both PAO1 (Parsek) and Δ *wspf* Δ *pel* PA105 biofilm was confirmed and a higher binding was observed when CTLD-4-7-Fc was added at a concentration of 10µg/ml compared to 2 µg/ml. CTLD-4-7-Fc binding to Δ *wspf* Δ *pel* PA105 does not probably involve recognition of alginate because alginate production is associated to a mucoid phenotype and these strains did not displayed a mucoid phenotype. Also alginate and Psl have distinct chemical structures (Franklin et al., 2011). Alginate is a high molecular weight acidic polysaccharide composed of non-repeating subunits of selectively O-acetylated d-mannuronic acid and its C5 epimer l-guluronic acid whereas Psl polysaccharide is composed of repeating pentamers containing D-mannose, L-rhamnose, and D-glucose. Also these two polysaccharides utilize different biosynthetic mechanisms, Psl requires oligosaccharide subunit assembly on a lipid carrier prior to transport across the inner membrane whereas alginate do not require this isoprenoid lipid carrier. Rather, alginate is believed to be polymerized and use a glycosyltransferase termed Alg8, in conjunction with Alg44, a bis-(3–5)-cyclic-dimeric guanosine monophosphate (c-di-GMP) binding protein to transport directly across the inner membrane (Franklin et al., 2011) (Figure- 4.19). To confirm our

preliminary observations a binding assay was performed by growing Δ*wspf* Δ*pel* PA105 (Parsek) biofilms within BioFlux channels. Although high background was observed in the negative control channels (secondary antibody only), confocal images showed binding of CTLD-4-7-Fc (10 µg/ml) to PA biofilm in a manner similar to that of the mannose-specific lectin HHA. To minimise the background biofilms were fixed to minimize non-specific binding. The binding assay was performed by growing Δ*wspf* Δ*pel* PA105 (Parsek) biofilm on a cover slips and fixation was performed with 4 % paraformaldehyde in PBS. Normal donkey serum was added during the incubation with the secondary antibody to block unspecific binding. The reason behind growing biofilms on coverslips is that the binding assay requires long incubations and the main drawback of BioFlux system is dispersion which was always observed when the shear flow was stopped. Using this new protocol confocal images showed CTLD-4-7-Fc (10 µg/ml) binding to intercellular spaces within PA biofilm, probably to Psl. Also much reduced background was seen with the negative control (CR-FNII-CTLD1-3-Fc, 10 µg/ml). Binding was observed only on one occasion because coverslips do not readily support biofilm formation. Therefore to support these observations a crude preparation of Psl was prepared (Byrd et al., 2009) from Δ*wspf* Δ *pel* PA105 (Parsek) overnight culture and a binding assay was performed by coating 96 well plates with the extracted crude EPS at a concentration of 500µg/ml. A higher binding of CTLD4-7-Fc (10µg/ml) towards extracted EPS was observed compared to negative controls (CR-FNII-CLD1-3-Fc or secondary antibody only). A relatively lower binding of CTLD4-7-Fc (2µg/ml) was observed which was found similar to rest of the negative controls. It was speculated that the

extracted EPS might still contain protein which might interfere with the binding of CTLD4-7-Fc or of the polysaccharide of the plate. For future work treatment with 20% TCA was considered to remove all protein instead of 5% TCA. Also to remove all LPS, 95% Ethanol extraction was proposed (Bales et al., 2013). In addtion to all these observations further experiments have been performed which showed that CTLD4-7-Fc is able to bind Δ*wspf* Δ *pel* (Parsek) biofilm whereas lower binding was observed to Δ*wspf* Δ *psl* (Parsek) biofilm. In addition CTLD4-7-Fc was found unable to bind planktonic PA regardless of the presence of Psl or Pel, which would indicate that the reason CTLD4-7-Fc did not promote ROS productin by neutrophils in response to PA might be that the protein did not bind PA. Having said that zymosan is a CTLD4-7-Fc ligand and no enhancement of ROS was observed either.

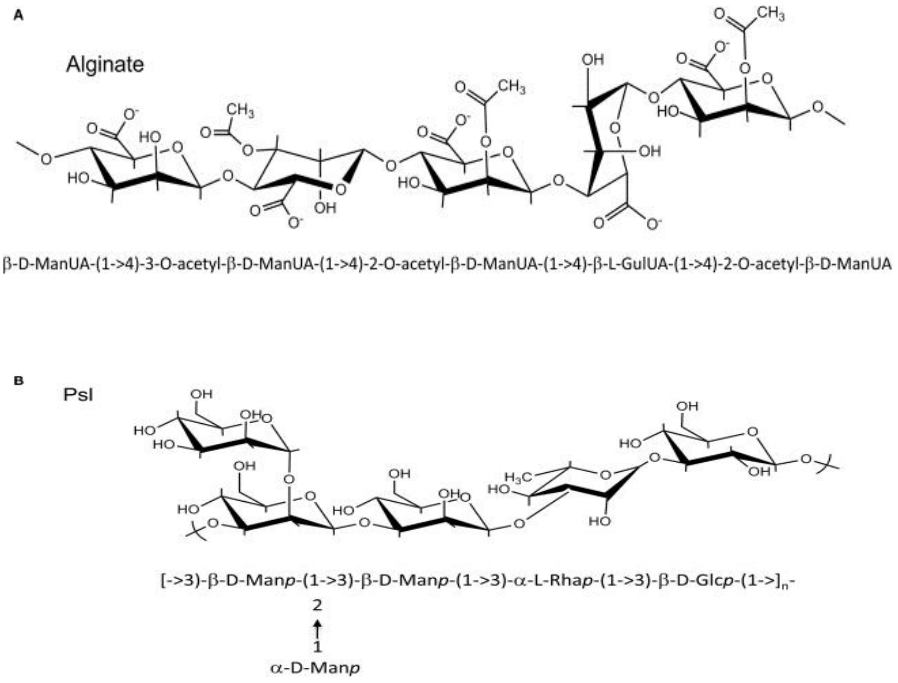



Fig: 4.18 Structures of Alginate and Psl polysaccharides. (A) PA alginate is consists of D-mannuronic acid residues interspersed with L-guluronic acid residues. The hydroxyl groups of the D-mannuronic acid residues may be O-acetylated at the C2′ and/or C3′ positions.(B) Psl polysaccharide is consists of a repeating pentamer composed of D-mannose, L-rhamnose, and D-glucose residues.(Franklin et al., 2011)

### 4.5.3. Contribution of MR and other C- type lectins in determination of infection outcome

The present study showed that CTLD 4-7-Fc region of MR is able to bind PA biofilm utilising Psl as a possible ligand. The outcome of this binding is yet to be determined. Immune cells recognition of PAMP may result in different outcomes: 1) an adequate response that leads to the eradication pathogen or 2) an inappropriate aggravated inflammatory response that may lead to chronic inflammation as well as illnesses such as sepsis and shock (Medzhitov and Janeway, 2000, Girardin et al., 1988, Xaplanteri et al., 2009). Several studies have shown that MR binds and internalises ligands via receptor-mediated endocytosis (Stahl et al., 1978, Stahl et al., 1980). MR also participates in phagocytosis of mannosylated particles and pathogens (Linehan et al., 2001, Kang and Schlesinger, 1998, Fraser et al., 1998). Also MR has been involved in macrophages microbicidal mechanisms during fungal infection, such as respiratory burst, and induction of pro-inflammatory cytokines including IL-1β, IL-6 and GM-CSF (Yamamoto et al., 1997, Marodi et al., 1998).

MR expressed on DCs enhances the ability of these cells to target mannosylated antigens for presentation to T cells by MHC class II molecules (MHCII) and CD11b (Sallusto et al., 1995, Caux et al., 1997, Prigozy et al., 1997). DCs are important immune cells that play an important role in induction of an adaptive immune response. Because of these characteristics DCs remain as an attractive target for many pathogens including mycobacteria that modulate their function (van Vliet et al., 2007, Pearce et al., 2006, van Kooyk et al., 2004). Thus it would be interesting to look at the consequences of PA biofilm or Psl on DCs. Besides it might be interesting to explore other C type lectins and what will be their role in recognizing PA biofilm, for example DC-

SIGN is a C-type lectin receptor that recognizes N-linked high mannose oligosaccharides and branched fucosylated structures (Feinberg et al., 2001, Appelmelk et al., 2003, Guo et al., 2004). It has been reported that initial interaction of T cells with DCs is mediated by the binding of the T cell surface receptor intercellular adhesion molecule 3 (ICAM-3) to the DCs surface receptor DC-SIGN and thus initiate primary immune response (Feinberg et al., 2001). Binding to high mannose sugar underlies the interaction of DC-SIGN with many viruses including HIV, Hepatitis C and Ebola virus (van Kooyk et al., 2003, van Kooyk and Geijtenbeek, 2003). It has been shown that binding of HIV to DC-SIGN on DCs facilitates the infection of T cells (Geijtenbeek et al., 2000, Pohlmann et al., 2001). DC-SIGN is expressed by dermal DCs and also in the mucosal tissues by interstitial DCs in lungs, intestine, rectum, cervix, placenta and in lymph nodes (Geijtenbeek et al., 2000, Kammerer et al., 2003). Both MR and DC-SIGN have shown to recognize mannose containing carbohydrates but with different specificity depending on branching of the mannose structures and also on the spacing of the carbohydrate structures on a glycoprotein (Geijtenbeek et al., 2000, Frison et al., 2003, Feinberg et al., 2001, Mitchell et al., 2001). DC-SIGN binds with carbohydrate structures including mannose-containing glycoconjugates and fucose containing Lewis blood-group antigens ($Le^x$, $Le^y$, $Le^a$ and $Le^b$) which indicate that the carbohydrate specificity of DC-SIGN cover over a broad range of ligands (Mitchell et al., 2001, Feinberg et al., 2001, Appelmelk et al., 2003, Geijtenbeek et al., 2003, Colmenares et al., 2002, Alvarez et al., 2002). Although DC-SIGN functions as a C-type lectin receptor on DCs and target antigens to lysosomal compartments for presentation to T cells, certain

pathogens for example viruses target DC-SIGN for transmission and non-viral pathogen such as mycobacteria regulate DC-induced immune activation using DC-SIGN (van Kooyk and Geijtenbeek, 2003). Future work will require analysis of how Psl recognition by mannose-specific lectins expressed by immune cells affect infection outcome which will give us a comprehensive insight of role of Psl in PA pathogenesis. Although it has been shown that MR is involved in different pathogen recognition and cellular activation (van de Veerdonk et al., 2009) the outcome of MR engagement is still unclear because most of the reports showed involvement of MR in cellular deactivation receptor that promotes Th2 responses. (Martinez-Pomares, 2012). Intriguingly a Th2 bias and high MR expression has been observed in CF patients (Murphy et al., 2010). MR could play a role in evasion of host defence by *P. carinii* by shifting the host-pathogen balance in favour of the pathogen (Fraser et al., 2000). Similar to Psl, *P.carinii* glycoprotein A is rich in mannose which facilitates *P.carinii* binding with macrophages (Linke et al., 1989, Lundgren et al., 1991, Pesanti and Shanley, 1988) .

Thus it could be predicted that the mannose rich nature of Psl might be an advantage for the bacteria and enables evasion from host immune responses. A recent study showed that increased numbers of dendritic cells expressing CD209 are associated with biofilm-positive nasal polyps compared to control (biofilm-negative) specimens (Karosi et al., 2013). Besides MR other lectins also need to be taken into consideration including Dectin-1, Dectin-2 and mannose binding lectin (MBL). Dectin-1 differs from receptors like DC-SIGN as it does not contain a standard Ca2+-dependent CRD, but it is more similar to the CLRs expressed by NK cells that binds to MHC class I (MHC-I) and

MHC-I-like counter-receptors (McQueen and Parham, 2002) (details in Chapter -1). Also Dectin-2 plays an important role in inducing immune responses against *C.albicans* by inducing Th17 cell differentiation (Saijo and Iwakura, 2011). Similarly MBL is a serum protein participating in innate immune defence. The Carbohydrate-recognition domain (CRD) of MBL binds ligands containing high mannose and N-acetylglucosamine oligosaccharides expressed by a variety of pathogens (Bouwman et al., 2006). It has been reported that MBL variant alleles generating low MBL serum levels are related with an increased risk of infections that occur primarily in children but also can be found in adults (Sumiya et al., 1991, Garred et al., 1995, Garred et al., 1997, Summerfield et al., 1995, Summerfield et al., 1997). In CF patients presence of MBL variant has been associated with reduced lung function compared to normal homozygotes. This negative impact of MBL variant alleles on lung function was observed especially in patients with chronic PA infection. MBL does not provide protection against chronic colonisation of PA because no significant differences were found between patients with normal and defective alleles of MBL with respect to the prevalence of chronic infection or age at onset. Thus it was proposed that MBL might not have a direct role in PA killing or opsonisation in vivo, rather MBL deficiency may be related with viral respiratory infections that render the lungs more susceptible to the damage caused by chronic PA colonization (Garred et al., 1999).

A recombinant protein that can target mannose rich molecules such as Psl and also contains a domain which can elicit an immune response by activating immune cells may have therapeutic value. In this study we did not observe increased ROS production by neutrophils exposed to PA or zymosan treated

with CTLD4-7-Fc. Recently work in our laboratory has demonstrated strong binding of DC-SIGN to biofilms with this binding being higher than that observed with CTLD4-7-Fc (Figure- 4.20). Also the affinity of DC-SIGN binding towards purified Psl appeared stronger than that of CTLD4-7-Fc (Figure- 4.21). There is a potential for using DC-SIGN-Fc to opsonise (coat) the bacteria in biofilms which could result modulation and activation of immune cell activity.

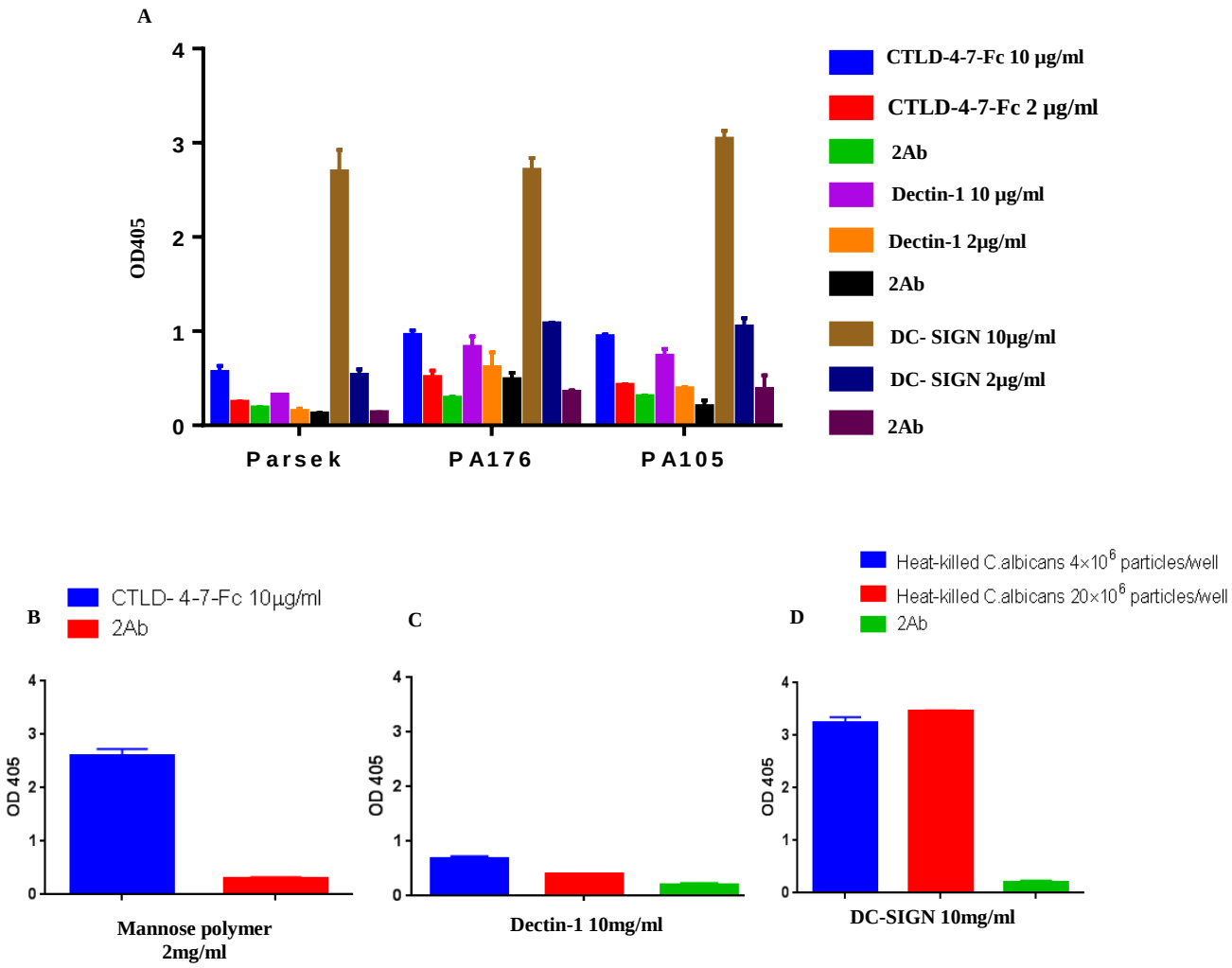



Fig: 4.19 Binding of different lectins to PA biofilms

**(A)** Binding assay demonstrated dose dependent and higher binding of DC-SIGN compared to CTLD4-7-Fc and Dectin1 to Δ wspf Δ pel PA105 (Parsek)biofilm. Binding assay was performed by growing biofilm using 96 well plates (24 hours). After fixation with 2 % formalin biofilm was incubated with CTLD 4-7-Fc or DC-SIGN or Dectin-1. Binding was detected using anti-human IgG Fc-specific conjugated to alkaline phosphatise. B, C, D showing binding of CTLD-4-7-Fc, Dectin-1-Fc and DC-SIGN-Fc towards their respective positive control, (Mohammad Alshahrani, unpublished).

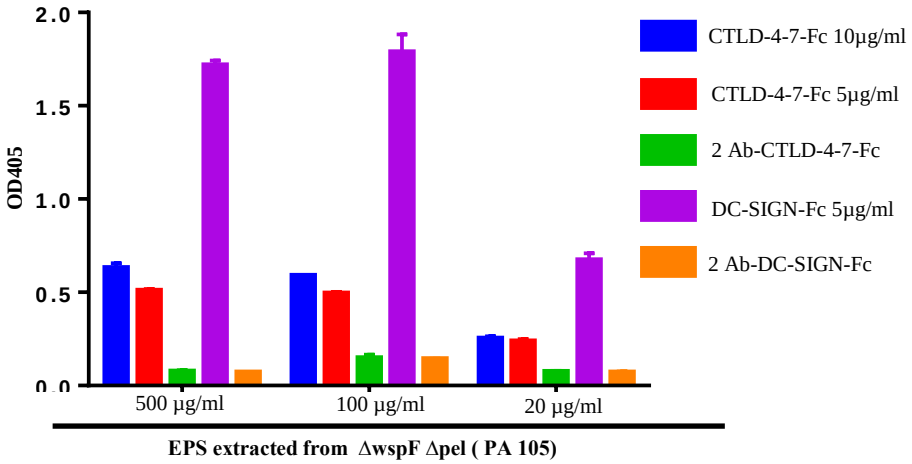



Fig: 4.20 CTLD4-7-Fc region of MR and DC-SIGN bind to crude EPS extracted from Δ*wspf* Δ*pel* PA105 (Parsek) culture

Binding assay demonstrated dose dependent and higher binding of DC-SIGN-Fc to crude extracted EPS compared to CTLD-4-7-Fc. Binding assay was performed by adding crude EPS 96 well plates overnight at a concentration 500,100 and 20µg/ml. After that EPS was incubated with DC-SIGN-Fc or CTLD 4-7-Fc. Binding was detected using anti-human IgG Fc-specific conjugated to alkaline phosphatise, (Mohammad Alshahrani, unpublished).

### 4.5.4. Future work

The present study showed that a recombinant protein (CTLD4-7-Fc) comprising of the mannose binding region of MR can bind to Psl within PA biofilms and to extracted EPS. This work will be continued further and several approaches have been considered to investigate the role of Psl in pathogenesis within the host. Till now different strains of Δ *wspf* of PA have been utilized and results confirm that CTLD4-7-Fc is able to bind Psl on PA biofilms (Fig-4.19, 4.20) (Mohammad Alshahrani, unpublished).

#### 4.5.4.1. Purification of Psl confirm binding to lectins and study its effect on the activation of human macrophages

Although binding of CTLD4-7-Fc to Psl directly on PA biofilm is clinically relevant CTLD4-7-Fc binding to highly purified Psl would provide a definitive result. Within the present study binding of CTLD 4-7-Fc was observed towards crude EPS generated from Δ*wspf*Δ*pel* PA105 (Parsek) cultures indicating that CTLD-4-7-Fc does not require Pel for binding to biofilms. But the binding appeared very low compared to CTLD4-7-Fc binding directly to Δ*wspf* Δ *pel* PA105 (Parsek) biofilm. It was speculated that the EPS preparation still might have contaminants which may be affecting the CTLD4-7-Fc binding. A modification of the current EPS extraction protocol has been now included which comprises addition of 20% TCA to remove extra protein and an ethanol extraction step to precipitate carbohydrate and remove LPS from bacterial supernatant (Bales et al., 2013). With the new preparation of extracted EPS the binding assay was performed and showed a higher binding of CTLD4-7-Fc compared to previous results (Mohammad Alshahrani, unpublished).

#### 4.5.4.2. Test clinical isolates

For future studies clinical isolates from CF patients need to be considered which will increase the clinical relevance of the study. Also analysis of lectins expression (particularly DC-SIGN and MR) in infection sites would give a detailed knowledge about the role of these lectins during chronic PA infection, for these different clinical samples such as BALs from CF patients, tissue samples from wound, samples from critical care patients suffering from ventilator- acquired pneumonia could be considered to localise both PA and lectins.

#### 4.5.4.3. Identification of role of other C- type lectins on recognition of PA biofilm

After testing CTLD4-7-Fc, it was proposed to test other mammalian lectins such as DC-SIGN, Dectin-1 and MBL whether they are able to interact with PA biofilms. As mentioned above binding assays have been performed which showed that DC-SIGN-Fc is able to bind Psl on PA biofilm as well as extracted EPS. The binding appeared stronger than that observed with CTLD4-7-Fc. Dectin-1-Fc, a kind gift form Prof. G. Brown, has also been tested. Dectin-1 binding towards PA biofilm was lower compared to DC-SIGN (Figure-4.20 and 4.21, study performed by Mohammad Y Alshahrani). Previous studies showed already that DC-SIGN can discriminate among ligands and that this discrimination depends not only on its ability to recognize primary mannose groups but also via secondary sites which carry carbohydrates with distinctive geometry, which might be one explanation of the higher affinity of DC-SIGN to their ligand compared to other C-type lectins such as MR (Guo et al., 2004, McGreal et al., 2005). For future work dectin-2 has also been considered due

to its specificity for mannose so could it could potentially bind to Psl (McGreal et al., 2006).

#### 4.5.4.4. Exploiting Fc chimaeras to promote biofilm clearance

Different Fc chimaeric constructs with different Fc regions could be generated and could be utilized to analyse whether they are able to facilitate PA biofilm clearance. It was hypothesized that as CTLD4-7-Fc lectin contained the Fc region of human IgG1, it might be possible to use these constructs to activate immune cells by binding through their Fc receptors (FcR) or complement proteins (Nimmerjahn and Ravetch, 2008).The FcRs for IgG (FcγRs) play an important role by triggering signalling pathways and setting the threshold for different cellular activation and produce a balanced immune response (Ravetch and Lanier, 2000, Nimmerjahn and Ravetch, 2008). There are four different IgG subclasses in humans (IgG1–IgG4) and mice (IgG1, IgG2a, IgG2b and IgG3) that bind with different affinity and specificity to different FcγR receptors (Dijstelbloem et al., 2001, Nimmerjahn et al., 2005). These FcγRs are widely expressed throughout the haematopoietic system including certain T-cell subpopulations, different innate immune effector cells including monocytes, macrophages, DCs, basophils and mast cells. Thus it would be interesting to use other subclasses of IgG to test whether Fc chimeric constructs would bind better to FcγRs expressed on different immune cells. For example, in human both IgG1 and IgG3 have been shown as the pro-inflammatory IgG subclasses (Nimmerjahn, 2006, Dijstelbloem et al., 2001). It also has been shown that human IgG subclasses pattern of complement-mediated cytolysis depends on different factors including antigen concentration, epitope patchiness, and antibody affinity and complement concentration (Michaelsen et

al., 1991). Among human IgG subclasses IgG3 has been shown most efficient in inducing complement mediated cytolysis under different conditions, whereas IgG1 and IgG2 require a high antigen concentration in order to be cytolytic and IgG4 isotype was not found cytolytic under these conditions (Michaelsen et al., 1991). The present study showed that CTLD4-7-Fc protein was unable to modify neutrophil ROS production in response to both PA and Zymosan. It was speculated that PAO1 (Lausanne strain) was unable to produce enough Psl both from 3 hours and overnight culture to be utilized as ligand to modify neutrophils ROS production. Thus further work to analyse whether these chimeric lectins are able to modify innate immune cells function in response to PA infection should involve increasing antigen concentration by using Δ*wspf* Δ *pel* PA105 (Parsek) and use of different chimaeric constructs with different Fc regions such as IgG2 and IgG3.

## 4.6. Summary

PA biofilms are important bacterial survival strategies during chronic infection. As it has been discussed earlier, the host immune response varied in the presence of biofilms compared to planktonic bacteria and because biofilm infection often persists this can result in an odd situation with simultaneous activation of host immune response with failure to remove pathogens within biofilms. Understanding the detection of conserved PAMPs associated with PA biofilm by PRRs of innate immune cells will give an insight into the underlying pathology of biofilm-associated infection. The Psl produced by PA appears to be highly conserved in both clinical and environmental isolates (Mishra et al., 2012) (Wolfgang et al., 2003, Jackson et al., 2004). Given the importance of Psl in earlier colonization, biofilm formation and involvement in

of host defence mechanisms, Psl targeting may be a new therapeutic strategy for prevention of chronic PA infection in CF patients.

# Chapter-5

## Diffusible signal factors as potential immunomodulators

### 5.1. Introduction

Surface-associated communities formed by many pathogens known as biofilms play an important role in pathogenesis of chronic infections. Regardless of the site of infection all biofilms share some common features such as production of extracellular matrix which will hold bacterial cells together and favour resistance against host defence and antimicrobial agents compared to free living planktonic pathogen (Mah and O'Toole, 2001). Within the PA biofilm developmental cycle dispersal is a crucial stage contributes to pathogen survival and facilitates disease transmission. (Kaplan, 2010). Biofilm dispersion is often related to phenotypic modifications in the dispersing cells. Thus the new dispersal phenotypes not only differ from the cells present inside biofilm but also differ from the planktonic bacteria (Sauer et al., 2002, Allison et al., 1990). Similar to other stages of the biofilm cycle, dispersal is a complex process regulated by environmental signals, regulatory networks and effectors (Karatan and Watnick, 2009). In general the biofilm dispersal process can be either active or passive. Active dispersal is normally initiated by the bacteria whereas passive dispersal can be described as biofilm cell detachment initiated by external forces including fluid shear, abrasion (collision of solid particles with the biofilm) and man-made intervention (Choi and Morgenroth, 2003, Ymele-Leki and Ross, 2007, Kaplan, 2010). Specific changes in the physiochemical properties of the environment can induce biofilm dispersion.

These include sudden elevation in carbon substrate availability which is associated with an increased expression of flagella (*fliC*) and decreased expression of pilus (*pilA*) genes among dispersed cells. Thus biofilm cells respond to the external environment by phenotypic adaptation probably by involving different regulatory networks that control nutrient-operated dispersal processes (Sauer et al., 2004). The product of the *bdla* gene is involved in PA biofilm dispersion. Sequence analysis and phenotypic comparison between wild type and a *bdla* mutant suggest the Bdla protein acts as chemotaxis regulator and affect the intracellular levels of c-di-GMP (Morgan et al., 2006). As mentioned in a previous chapter high intracellular c-di-GMP levels upregulate matrix production and thereby biofilm formation while low intracellular c-di-GMP levels down regulate matrix production and promote planktonic lifestyle (Hickman et al., 2005, Gjermansen et al., 2006, Meissner et al., 2007). Changes in the concentration of carbon sources and nitric oxide signalling were suggested to induce the dispersal of PA biofilms by regulating intracellular c-di-GMP levels (Schleheck et al., 2009). It also has been shown that the chemotaxis regulator *bdla* promotes nitric oxide-mediated biofilm dispersal (Barraud et al., 2009). PA QS has been implicated in the regulation of different developmental stages of biofilms including dispersion. The *rhl* QS system increases production of the extracellular surfactant rhamnolipid which promotes bacterial detachment (Boles et al., 2005) and restores antibiotic sensitivity in dispersing bacteria. Thus it has been proposed that the promotion of detachment can be beneficial as treatment against established biofilms (Boles et al., 2005). It also has been suggested that rhamnolipid-mediated PA biofilm dispersion may involve c-di-GMP. The PA 2572 protein, which

regulates production of rhamnolipid, contains a degenerate inactive c-di-GMP phosphodiesterase domain and may play a regulatory role in biofilm dispersion (Ryan et al., 2009a, Harmsen et al., 2010). It has also been shown that addition of exogenous rhamnolipid can disperse earlier stage of PA biofilm development most likely by disrupting the interaction between cell to cell and cell to surface interaction(Davey et al., 2003). In addition to PA biofilm rhamnolipid can disperse biofilm formed by other pathogens such as *Bordetella.bronchiseptica* (Irie et al., 2005).

### 5.1.1. Role of diffusible signal factors (DSF) in biofilm dispersion

Identification of cell to cell communication systems involved in biofilm dispersion has been an area of interest of a number of researchers over past decades. The Diffusible Signal Factor known as DSF are part of a new QS family originally discovered in the plant pathogen *Xanthomonas campestris* (Xcc) (Guo et al., 2012). The DSF produced by Xcc has been characterized as *cis*-11-methyl-2-dodecenoic acid (Wang et al., 2004). DSF is also produced by *S. maltophilia* whereas *B. cenocepacia* produces a similar dispersion molecule termed *cis*-2-dodecenoic acid (known as BDSF)(Boon et al., 2008, Huang and Wong, 2007). DSF positively regulates expression of an extracellular enzyme in Xcc termed endo-beta-1,4-mannanase which has been involved in releasing bacteria from biofilm and promote planktonic lifestyle to favour further colonization within the vascular system (Dow et al., 2003). DSF has also been involved in different biological functions in addition to biofilm dispersal including virulence and antibiotic resistance (Barber et al., 1997, Wang et al., 2004, He and Zhang, 2008, Deng et al., 2009, Deng et al., 2010, Deng et al., 2011, Deng et al., 2012, Schmid et al., 2012, McCarthy et al.,

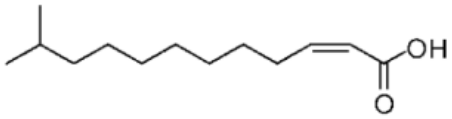
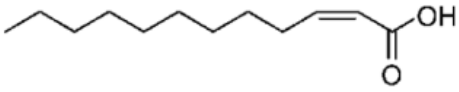
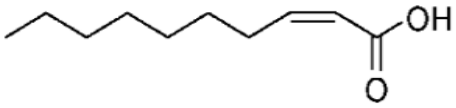
2010). The BDSF produced by Burkholderia activates DSF-dependent responses in *X. campestris* and limits germ tube formation in *Candida albicans*, which suggest involvement in interspecies and interkingdom communication. (Deng et al., 2011, Deng et al., 2012, Schmid et al., 2012, McCarthy et al., 2010, Boon et al., 2008, Ryan et al., 2009b). DSF signals produced by *Burkholderia cenocepacia* and *Stenotrophomonas maltophilia* regulate virulence and antibiotic resistance, during infection of CF transmembrane conductance regulator knockout mice. The presence of DSF promotes PA persistence (Ryan et al., 2008, Twomey et al., 2012). Furthermore DSF was detected in the sputum of CF patients who were colonized with *Stenotrophomonas maltophilia* and/or *Burkholderia cenocepacia* (Twomey et al., 2012).

### 5.1.2. CDA is responsible for PA biofilm dispersion

The DSF produced by PA is termed *cis*-2-decenoic acid (CDA). CDA is able to disperse PA biofilm as well as biofilms formed by other pathogens such as *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Streptococcus pyogenes, Bacillus subtilis, Staphylococcus aureus* (Davies and Marques, 2009). CDA is a medium-chain fatty acid chemical messenger; it does not only induce dispersion of mature biofilms but also prevents biofilm formation (Davies and Marques, 2009). Thus it was suggested that the biofilm preventive nature of CDA could be used as an adjunctivant therapy for prevention of biofilmassociated infection (Davies and Marques, 2009). CDA is structurally related to DSF where both have a double bond at position 2 but DSF contains a 12-carbon chain and a branched methyl group at the number 11 position (Barber et al., 1997) (Table-5.1). The synthesis of DSF depends on RpfF which

contains amino acid sequences related to enoyl coenzyme A (CoA) hydratase, and on RpfB which is predicted to have long-chain fatty acyl CoA ligase activity. In *X. campestris* DSF sensing is mediated by a two-component system consisting of a sensor kinase RpfC and a response regulator RpfG (Barber et al., 1997, Dow et al., 2003, Ryan et al., 2006, Ryan and Dow, 2011). No such Rpf gene cluster or proteins related to RPfF have been found in the PA genome. PA has 12 enoyl CoA hydratases with sequence homology to RpfF, which indicates their potential involvement in the synthesis of a DSF-related component. Further analysis showed that the PA protein PA0745 has greatest amino acid sequence homology to RpfF. Exogenous addition of CDA to a final concentration about 10 μM to mature PA biofilms promote disaggregation in 11 min with complete dispersal taking place within 30 min following exposure (Davies and Marques, 2009). Previous work in the laboratory showed that CDA have significant effect on PA biofilms developed using the BioFlux system; 1 μM CDA was able to significantly inhibit bacterial adhesion and attachment, 10 μM and 100 μM CDA resulted in undetectable biofilms (Ye Chan, 2014. Fatty acid signalling in Pseudomonas aeruginosa. Ph.D. University of Nottingham:UK).

Table 5.1 DSF- types and structures

| Name | Structure | Producer |
|---|---|---|
| DSF (11-Met-2-C12:1) |  | *X. campestris* *S. maltophilia* |
| BDSF (2-C12:1) |  | *B. cenocepacia* |
| CDA (2-C10:1) |  | *P. aeruginosa* |

### 5.1.3. Modulation of immune responses by QS molecules

The role of the QS system in PA pathogenesis has been demonstrated in different animal models including acute lung infection model in neonatal mice and chronic lung infection model utilizing adult rats (Pearson et al., 2000, Wu et al., 2001). Absence of las and rhl quorum-sensing systems affect expression of virulence factors and thus affect host immune response (Pearson et al., 2000, Rumbaugh et al., 1999, Tang et al., 1996, Wu et al., 2001). In support of an important role for AHLs in PA pathogeneses in patients AHLs have been detected in PA infected-CF sputum (Singh et al., 2000). During PA infection the production of 3O-C12-HSL has been reported to induce pro-inflammatory cytokine production from different cell types. In *in vitro* 3O-C12-HSL is a potent stimulator of human bronchial epithelial cells for the production of the neutrophil chemokine interleukin-8 (IL-8) which is regulated by NF-κB (DiMango et al., 1995, Smith et al., 2001). Also 3O-C12-HSL was found to induce higher levels of IL-6 production in the CF respiratory epithelial cells compared to the control cells and thus proposed to contribute in pathogenesis inside CF lungs (Mayer et al., 2011).

Based upon all these studies, it was considered that the dispersion molecule, CDA, like other QS molecules, might act as an immune modulator. Thus it was decided to include in this project an investigation of the ability of CDA to modulate the activation of immune cells.

## 5.2. Hypothesis

We hypothesise that CDA produced by PA modulates the activation of innate immune cells.

## 5.3. Aims

Analyse the effect of CDA on the activation of different cellular innate immune components; monocytes and macrophages.

## 5.4. Result

### 5.4.1. CDA modulates the expression of TNF-α in monocytes activated with LPS

To determine whether CDA has any direct immunomodulatory effect on monocytes, freshly purified monocytes were incubated for 1 and 3 hours (n=2) with or without CDA (10 μM) in the presence or absence of LPS (100 ng/ml). Controls included untreated and vehicle only-treated cultures (see Chapter 2). The transcription of the genes encoding TNF-α, GM-CSF, IL-1β, IL-6, and IL-10 was analysed by qRT-PCR. Both TNF-α and GM-CSF were detected after 1 and 3 hours of incubation in both unstimulated and LPS-stimulated monocytes whereas the rest of the genes remained undetectable. LPS induced increased transcription of TNF-α and GM-CSF at 1 hour (Fig. 5.1) but these transcripts were drastically reduced at 3 hours post-treatment except in the case of TNF-α production by one donor (Fig. 5.2). CDA did not induced cytokine production in isolation but consistently caused a reduction in the amount of TNF-α-

specific transcript in LPS-stimulated monocytes at 1 hour, while its effect on GM-CSF expression was variable (Fig 5.1 and 5.2). Interestingly when the levels of TNF-α transcript remained higher at 3 h, CDA still has an inhibitory effect (Fig 5.2). To improve understanding results have been represented separately for 1 and 3 hours respectively (Fig 5.1 representing both TNF- α and GM-CSF transcripts obtained from two donors after 1 hour incubation and Fig 5.3 representing both TNF- α and GM-CSF transcripts obtained from two donors after 3 hours incubation). Furthermore to summarize these results obtained in 1 h incubation experiments were normalised so that TNF-α transcripts in LPS-stimulated monocytes was considered 100 % and the other variables were calculated accordingly. This analysis (n=4) highlights the fact that the reduction in TNF-α transcription induced by CDA is statistically significant (Figure 5.3) when compared to the TNF-α transcripts detected from the LPS stimulated macrophages incubated in presence of diluent (calculated by paired student's t test) .

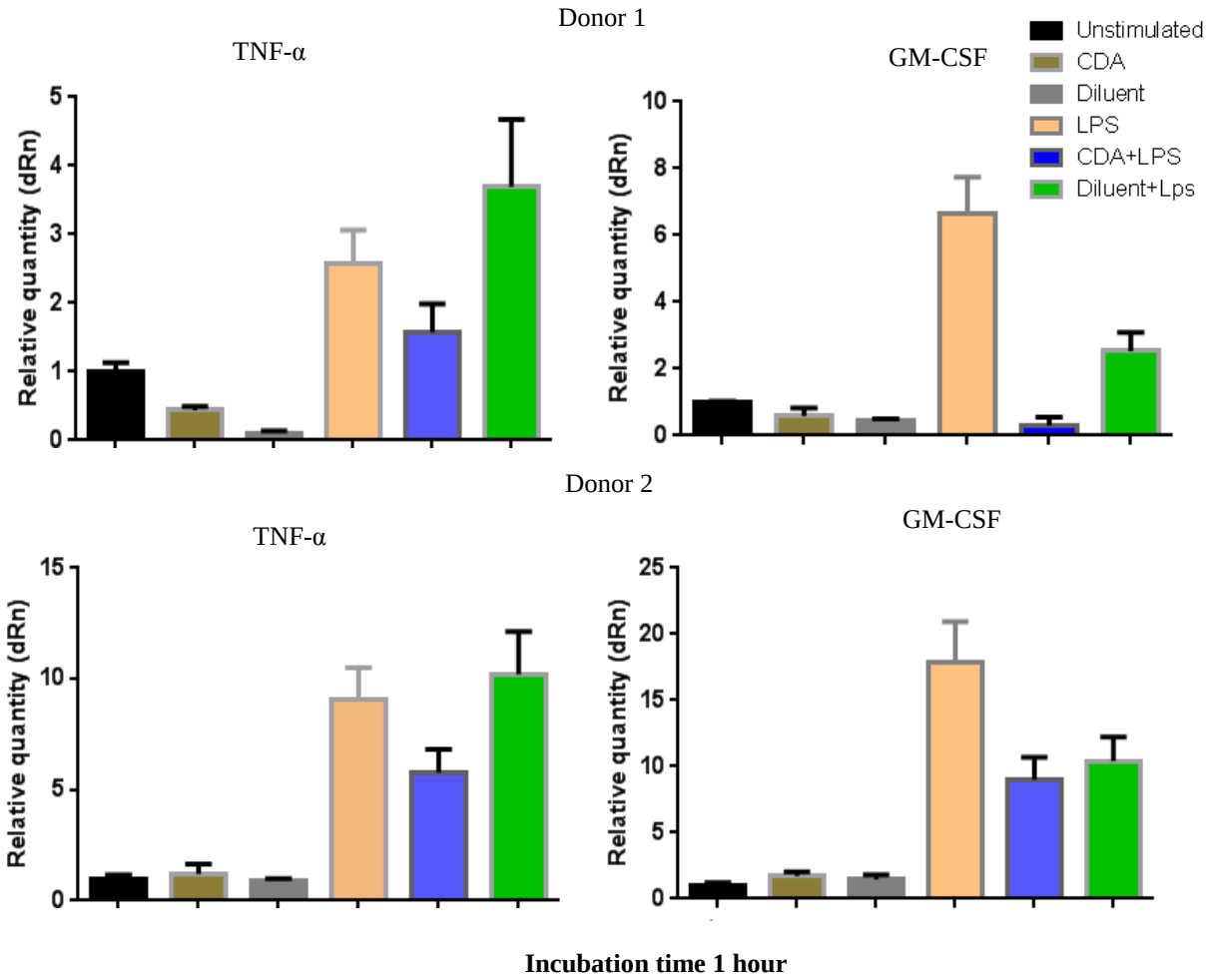



Fig: 5.1 Effect of CDA on expression of TNF-α in monocytes activated with LPS

Quantitative Real-Time-PCR (qRT-PCR) analysis showing expression levels of genes encoding TNF-α and GM-CSF after normalization to HRPT. Data represents relative mRNA expression comparing unstimulated controls with treated samples. . Each bar represents mean ± SEM of triplicate wells from that donor.

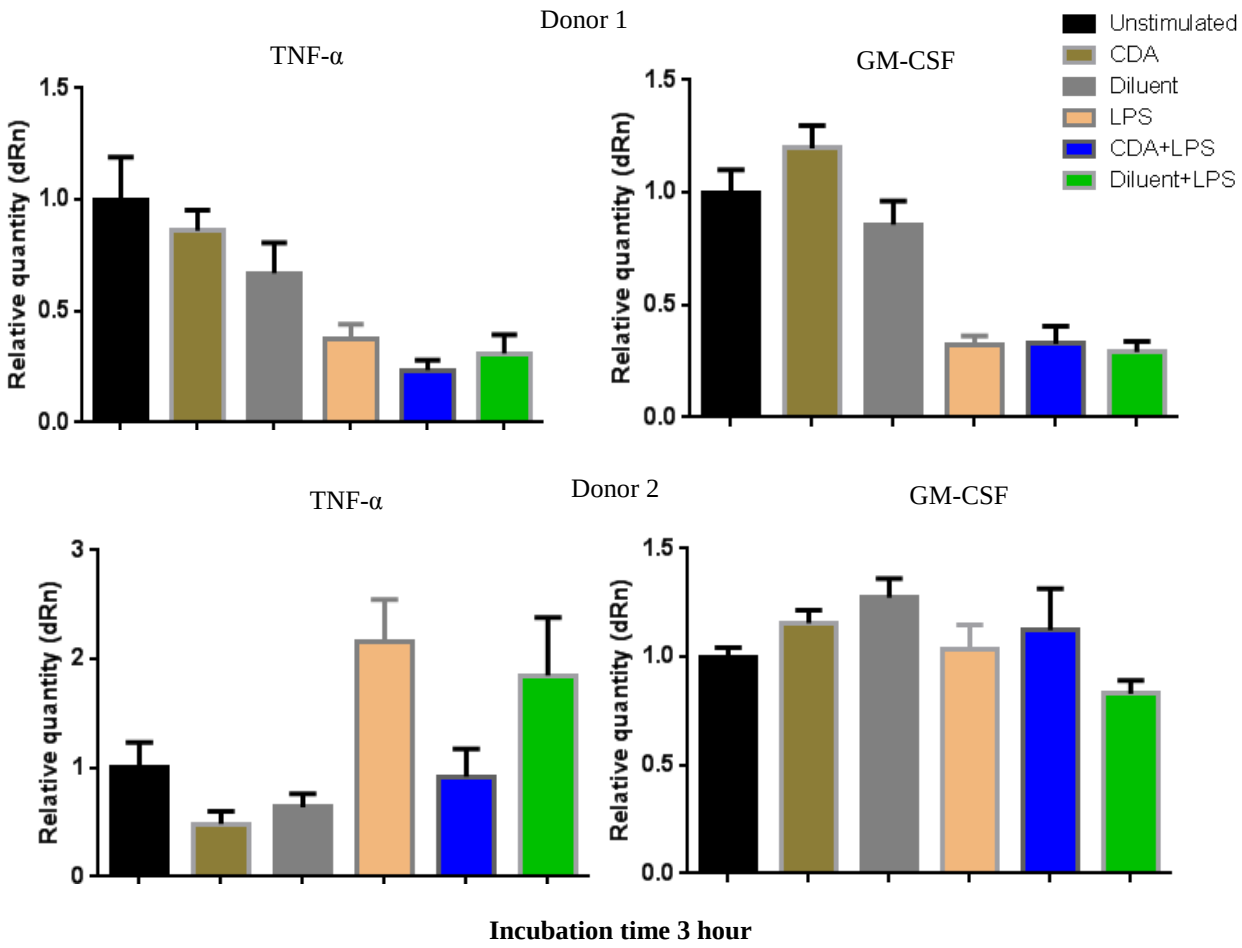
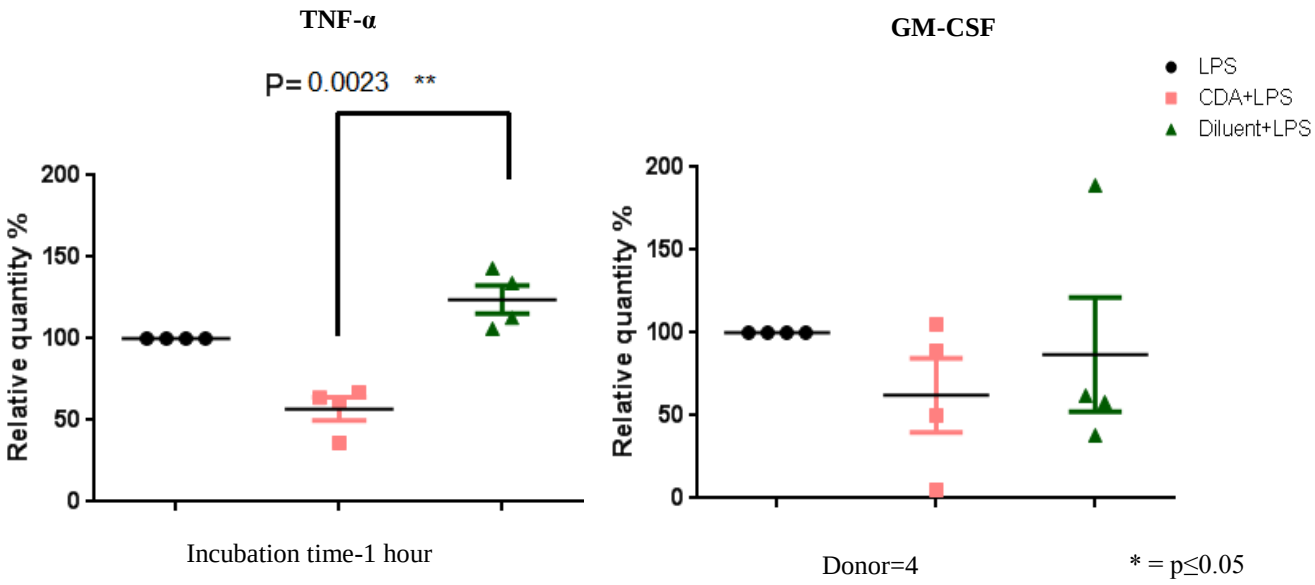



Fig: 5.2 Reduced transcription of TNFα and GM-CSF in human monocyte at 3 hour after LPS treatment

qRT-PCR analysis showing expression levels of genes encoding TNF-α and GM-CSF after normalization to HRPT. Data represents relative mRNA expression comparing unstimulated control with treated samples. Each bar represents mean ± SEM of triplicate wells from that donor.




Statistical significance was calculated by paired student's t test

Fig: 5.3 CDA significantly modulates transcription of TNF-α but not GM-CSF in monocytes in response to LPS

qRT-PCR analysis showing relative quantity (in percentage) of TNF-α and GM-CSF expression after normalization where LPS stimulated monocytes has been considered as 100 percentage and then the value of the other variants has been calculated accordingly. Data points represent mean ± SEM from 4 donors. Statistical significance was calculated by paired student's t test . * = p≤0.05

### 5.4.2. Dose dependent effect of CDA on the expression of TNF-α and GM-CSF in LPS-stimulated monocytes

To determine if the effects observed with CDA were dose-dependent, monocytes were stimulated with LPS for 1 hour in the presence of 10 or 1 μM CDA. In the presence of 10 μM CDA a reduction in TNF-α expression was found on LPS stimulated-monocytes in agreement with previous observations while with 1 μM CDA this inhibition was reduced (donor =2). As before CDA reduced TNF-α expression in response to LPS but its effect on GM-CSF expression was variable.

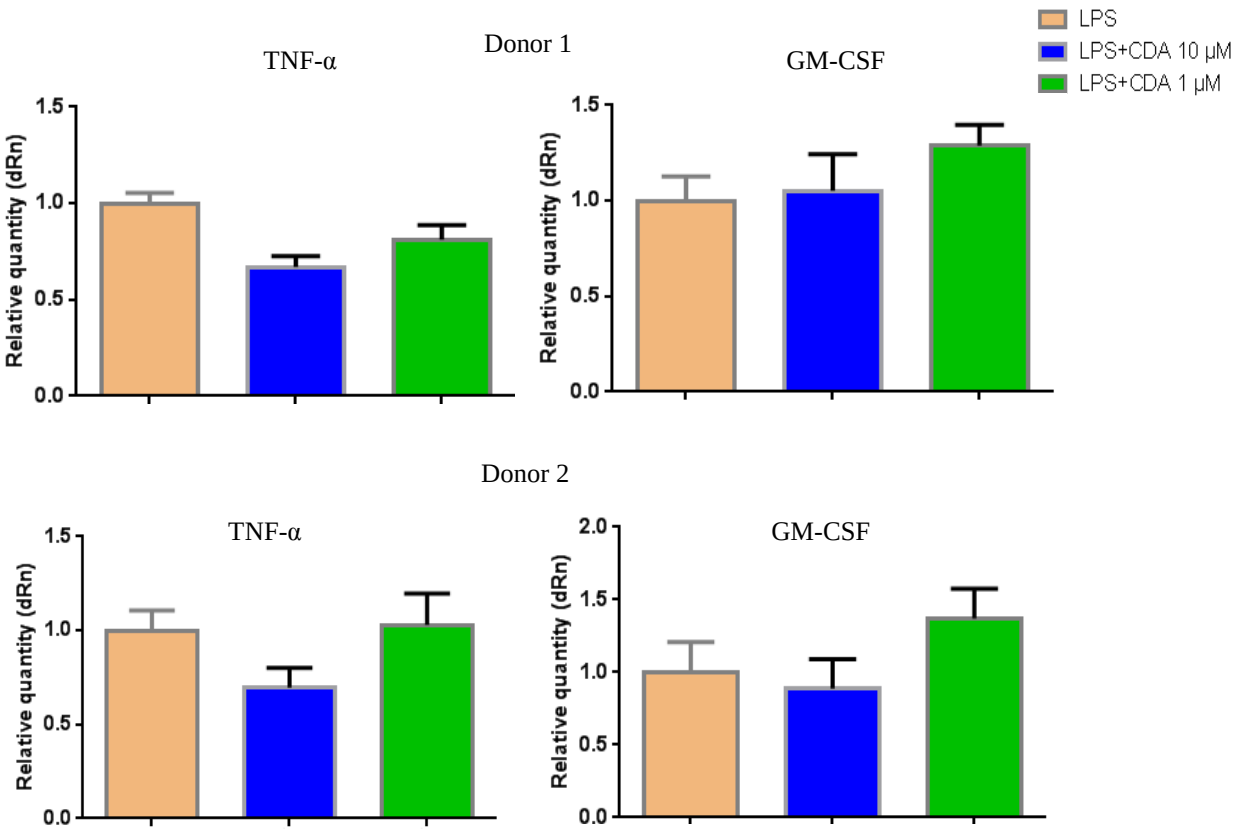



Fig: 5.4 CDA 10 µM inhibits TNF-α transcription by monocytes in response to LPS

qRT-PCR analysis showing expression levels of genes encoding TNF-α and GM-CSF after normalization to HRPT. Data represents relative mRNA expression comparing LPS stimulated monocytes incubated with CDA (10 and 1µM). Each bar represents mean ± SEM of triplicate wells from one donor.

### 5.4.3. Effect of CDA and its isomer TDA (Trans 2 Decenoic acid) on the expression of TNF-α and GM-CSF in LPS stimulated monocytes

To determine if a CDA analogue such as TDA has a similar effect on LPS-stimulated monocytes, monocytes were stimulated with LPS for 1 hour in the presence of 10 µM CDA or TDA. Similar to all previous results in the presence of 10 µM CDA a reduction in TNF- α expression was found on LPS-stimulated monocytes. A reduction in TNF- α transcript was also observed in response to TDA but it was less compared to CDA samples. In this experiment a reduction of GM-CSF expression was found in response to CDA, TDA and diluent although the effect was more obvious in the case of CDA (n=1).

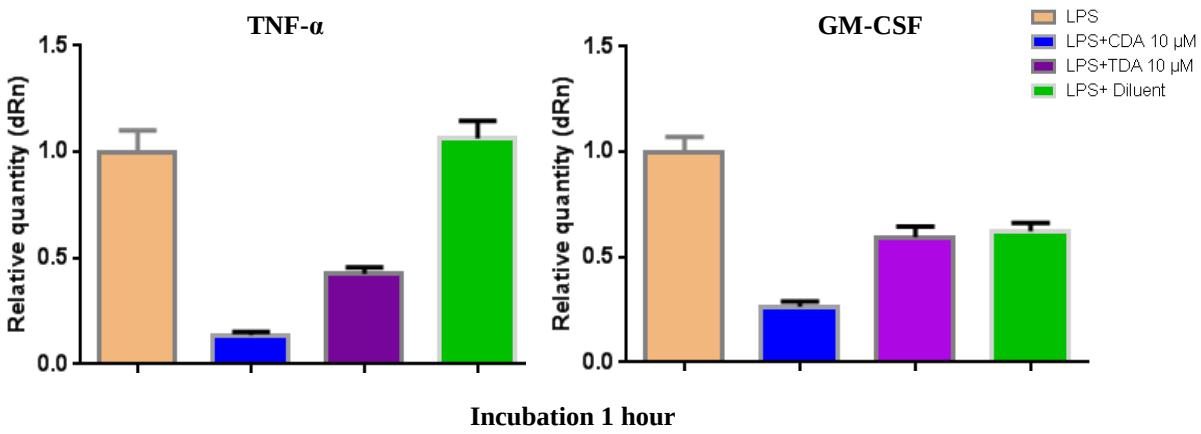



Fig: 5.5 Both CDA and TDA 10 µM inhibits TNF-α transcription by monocytes in response to LPS

qRT-PCR analysis showing expression levels of genes encoding TNF-α and GM-CSF after normalization to HRPT. Data represents relative mRNA expression comparing LPS stimulated monocytes incubated in presence and absence of CDA (10 µM), TDA (10 µM) or diluent. Each bar represents mean ± SEM of triplicate wells from one donor.

### 5.4.4. Effect of CDA on the expression of TNF-α by LPS-stimulated macrophages

#### 5.4.4.1. CDA modulates the levels of TNF-α transcripts in macrophages activated with LPS after 3 hours of incubation

To determine whether CDA has an immune-modulatory effect on macrophages, human monocytes were differentiated into macrophages in the presence of M-CSF and incubated for 3 and 6 hours (n=3) in the presence of LPS (100 ng/ml) with or without CDA (10 μM). Other controls included untreated control and diluent only and TDA 10 μM-treated samples. In this instance the induction of TNF-α, IL-8 and IL-10 at the mRNA levels were analysed. LPS induced increased transcription of TNF- α in macrophages after 3 hours of incubation (Fig. 5.6) (n=3) and this effect was reduced at 6 hours post-treatment (Fig. 5.7) (n=3). Compared to LPS-stimulated monocytes the level of TNF- α transcripts appeared higher in LPS-stimulated macrophages, although no direct comparison was performed. Also in LPS-stimulated monocytes TNF-α transcripts were reduced promptly within 3 hours whereas elevated level of TNF-α expression was observed in LPS-stimulated macrophages at 3 and 6 hours post-LPS stimulation although levels were drastically reduced at 6 hours post-LPS. Therefore the kinetics of TNF-α expression reduction from 3 to 6 hours appeared as an expected normal scenario (Fig 5.8) (Baer et al., 1998). Similar to the results observed with LPS-stimulated monocytes, the data trend showed that CDA caused a reduction in the amount of TNF-α transcript in LPS-stimulated macrophages at 3 hours post-treatment compared to LPS only and cultures treated with LPS and diluent. TNF-α transcripts observed in LPS stimulated macrophages in

presence of diluent appeared in the same level as LPS-stimulated macrophages only in two instances (Donors 2 and 3) but in the case of donor1 the level of TNF- α transcripts in the presence of diluent appeared lower than the transcripts found in LPS only-stimulated macrophages. TDA also affected the levels of TNF-α transcripts in response to LPS but this effect was not reproducible in all donors. After normalization of all the data (Donors=3) by considering TNF-α transcripts in LPS stimulated macrophages as 100%, it was evident that CDA is able to reduce TNF-α transcripts which was found statistically significance ( calculated by paired student's t test) when compared to TNF-α transcripts detected from LPS stimulated macrophages incubated in presence of diluent (Fig-5.9). Interestingly after 6 hours of incubation when the overall levels of TNF- α transcripts are reduced in all samples (Fig- 5.7 and 5.8) no such trend was observed except in Donor1 (Fig- 5.7). Two other genes IL-8 and IL-10 were also analysed. IL-10 transcripts were found undetectable but some IL-10 production was observed on supernatants at 6 hours. The transcription level of IL-8 was higher in LPS-stimulated macrophages compared to unstimulated macrophages, but the amount of the IL-8 transcripts was not affected by treatment with CDA, TDA or diluent at 3 hours (Fig 5.10).

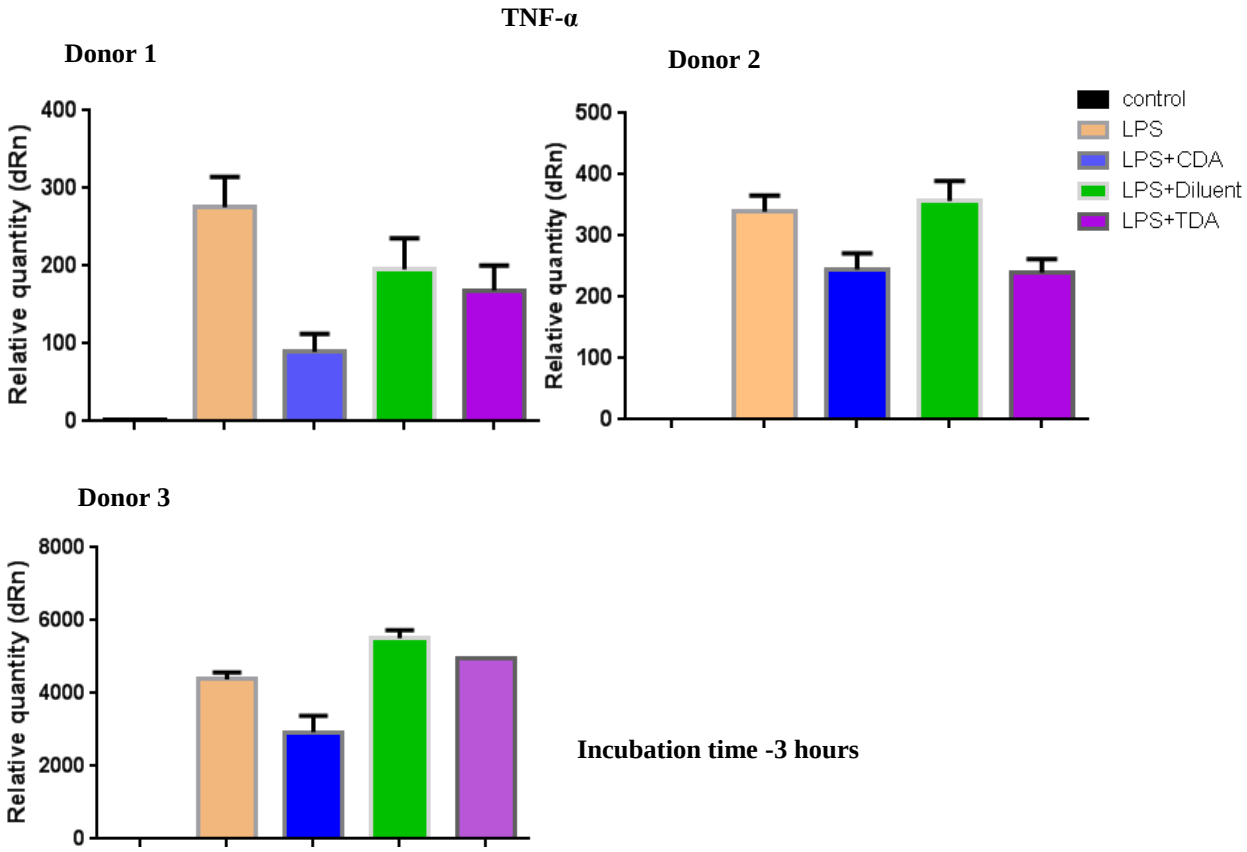



Fig: 5.6 CDA regulates expression of TNF-α in macrophages activated with LPS for 3 hours

qRT-PCR analysis showing expression levels of gene encoding TNF-α after normalization to HRPT. Data represents relative mRNA expression comparing unstimulated control to LPS-stimulated control incubated with or without CDA(10µM), isomer control TDA (10µM) or diluent. Each bar represents mean ± SEM of triplicate wells from one donor.

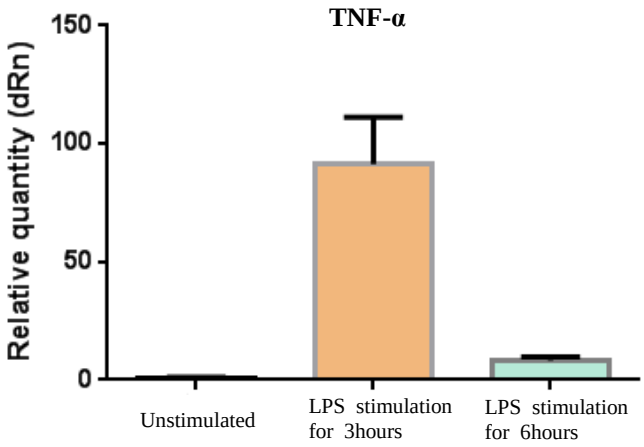



Fig: 5.8 Reduced transcription of TNFα in human macrophages at 6 hours after LPS treatment

qRT-PCR analysis showing expression levels of genes encoding TNF-α after normalization to HRPT. Data represents relative mRNA expression comparing unstimulated control plus LPS-stimulated cells incubated for 3 and 6 hours respectively. Each bar represents mean ± SEM. N=1.

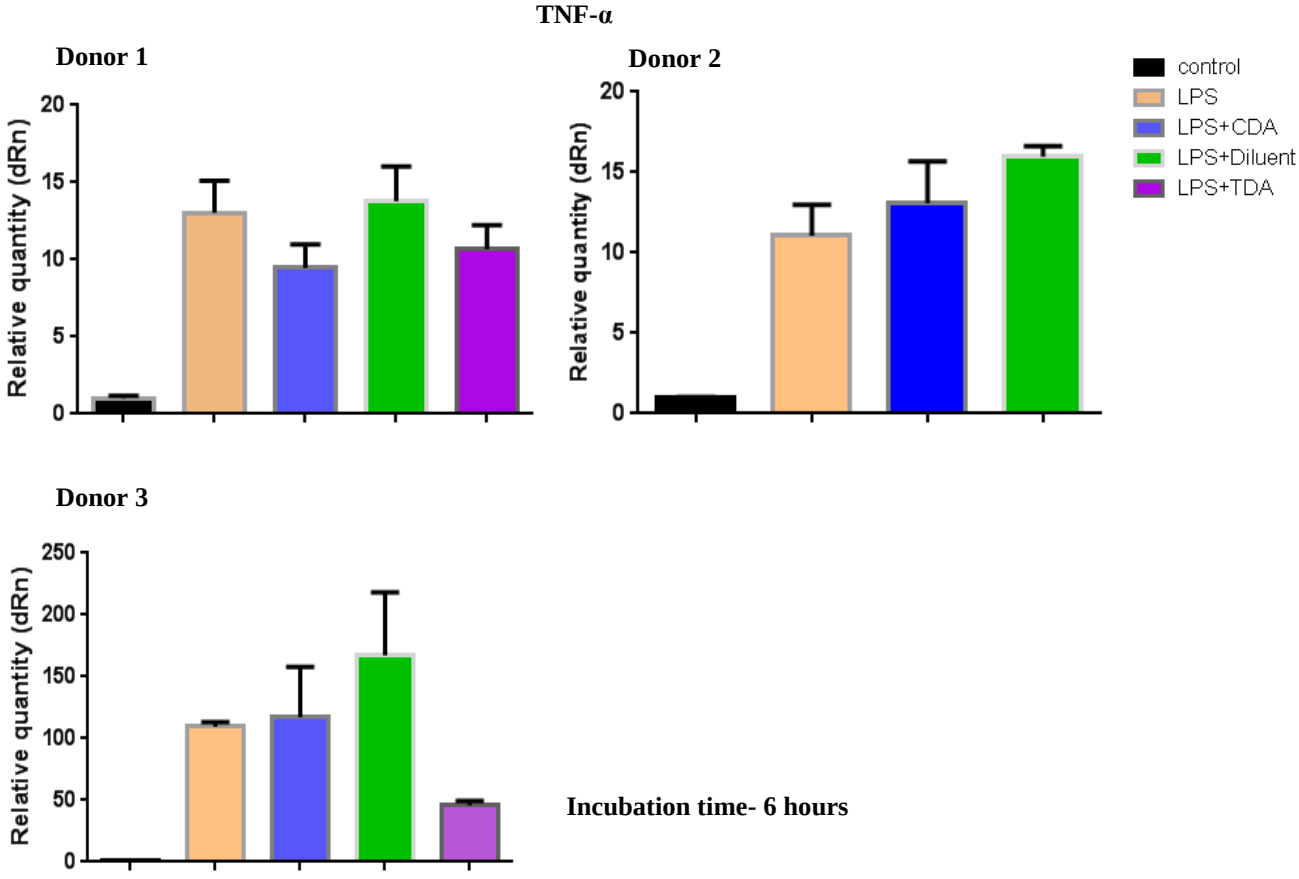



Fig: 5.7 CDA does not affect TNFα transcripts in human macrophages at 6 hour after LPS treatment

qRT-PCR analysis showing expression levels of gene encoding TNF-α after normalization to HRPT. Data represents relative mRNA expression comparing unstimulated control with LPS-stimulated control incubated with or without CDA (10μM), TDA (10μM) or diluent. Each bar represents mean ± SEM of triplicate wells from one donor.

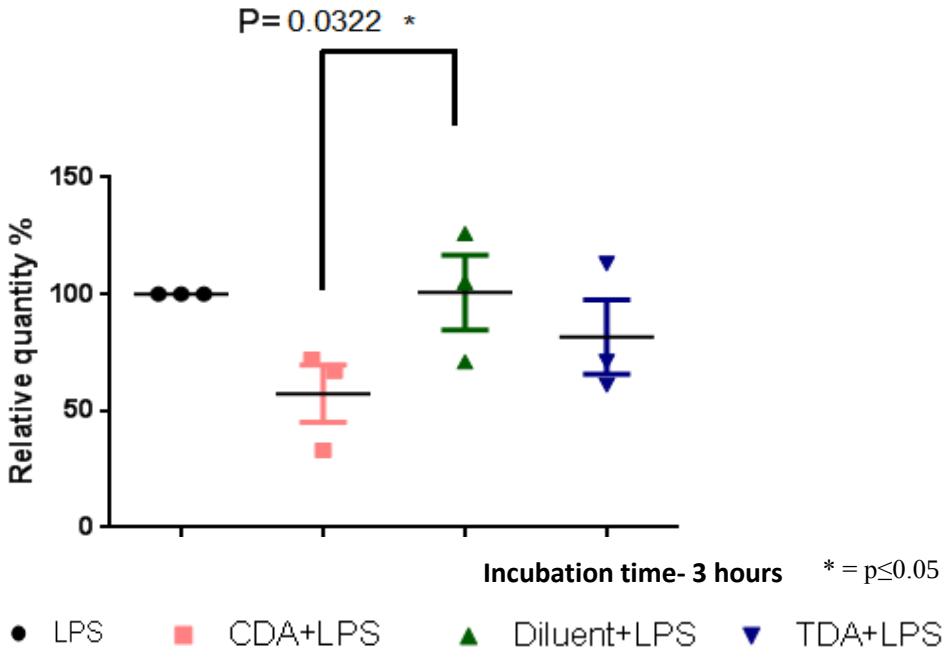



Fig: 5.9 CDA modulates transcription of TNF-α of macrophages in response to LPS at 3 hours post-treatment

qRT-PCR analysis showing relative expression levels TNF-α transcript after normalization where LPS stimulated macrophages has been considered as 100 percentage and then the value of the other variants has been calculated accordingly. Data points represent mean ± SEM from3 donors. Statistical significance was calculated by paired student's t test.* = p≤0.05

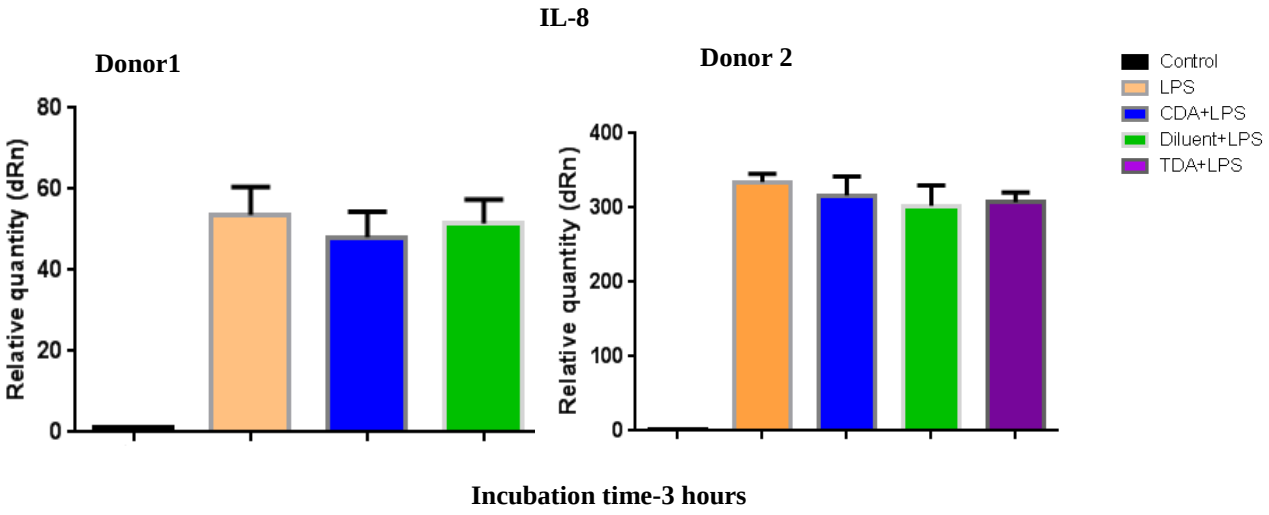



Fig: 5.10 CDA does not affect IL-8 transcription of macrophages in response to LPS after 3 hours of incubation

qRT-PCR analysis showing expression levels of IL-8 after normalization to HRPT. Data represents relative mRNA expression comparing unstimulated control and LPS-stimulated control incubated with or without CDA (10µM), TDA (10µM) or diluent. Each bar represents mean ± SEM of triplicate wells from one donor.

### 5.4.4.2. CDA modulates the production of TNF-α by macrophages at 3 hours after activation with LPS

Above results showed that CDA reduced the levels of TNF-α transcripts in LPS stimulated immune cells, in monocytes at 1 hour and macrophages at 3 hours post-treatment. To confirm these initial observations levels of TNF-α were quantified in supernatants collected from macrophage cultures stimulated with LPS (100ng/ml) incubated in presence or absence of CDA 10 µm, TDA 10µm or diluent. Similar to the results observed using qRT-PCR it was observed that CDA modulates TNF-α production by LPS-stimulated macrophages after 3 hours of incubation. In Donors 1 and 2 TNF- α production was reduced to 3.44 and 2.7 ng/ml compared to 6.8 and 5.35ng/ml respectively compared to LPS-only controls. Also CDA reduced TNF-α production compared to other variables including LPS stimulated macrophages incubated in presence of TDA (Donor 1=6.7, Donor 2 =5.4 ng/ml or diluent Donor 1=6.7, Donor 2= 5.8ng/ml). Similarly in Donor 3 a drastic reduction in TNF- α (2.9 ng/ml) production of LPS stimulated macrophages was observed in presence to CDA compared to TNF- α produced from LPS only stimulated macrophages (14.44 ng/ml), but in this case a reduction in TNF-α production in response to LPS was observed in response TDA (3.6 ng/ml) or diluent (6.6 ng/ml) (Fig-5.11). Therefore data summarized from these 3 donors showed that CDA is able to reduce TNF-α production significantly (calculated by paired student's t test) compared to TNF-α produced from LPS stimulated macrophages incubated in presence of diluent (Fig-5.12). At 6 hours a reduction in TNF-α production by LPS-stimulated macrophages was observed, in agreement with the reduced levels of mRNA. At this time no effect of CDA on TNF- α

production was observed (Fig- 5.13, Donor=3). Other cytokines including IL-10 and IL-8 were quantified to determine whether CDA may have any effect on their production. Supernatants collected at 3 and 6 hours were tested but no differences among treatments were observed. (Fig- 5.14 and 5.15).

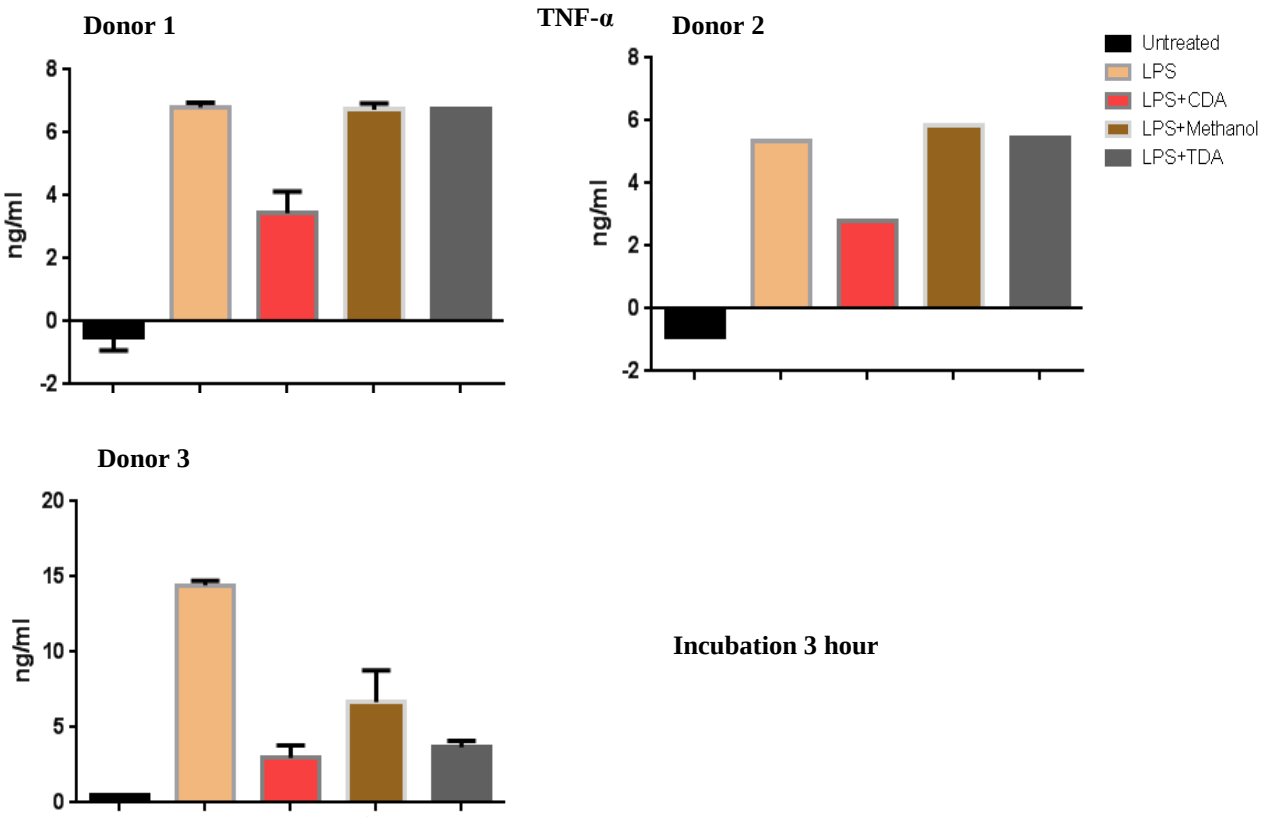



Fig: 5.11 CDA affects TNF-α production in macrophages activated with LPS after 3 hours incubation
Supernatant from unstimulated control or LPS-stimulated cultures incubated with or without CDA(10µM), TDA (10µM) or diluent were analysed for TNF-α. Each bar represents mean ± SD of duplicate wells except for donor 2.

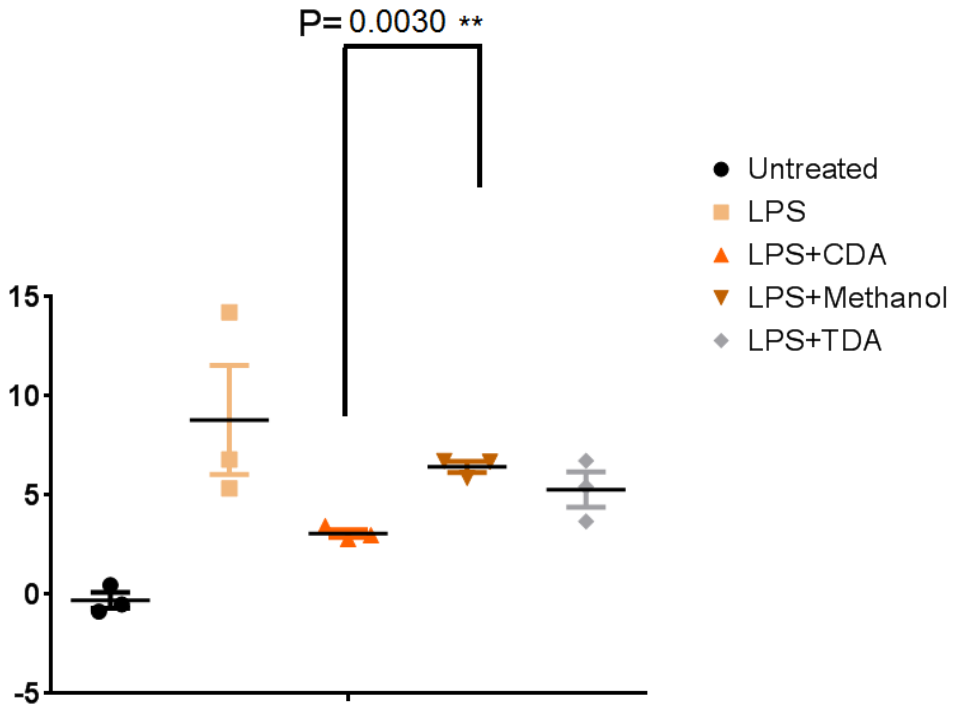



Fig: 5.12 CDA modulates TNF-α production of macrophages in response to LPS at 3 hours post-treatment

Supernatant from unstimulated control or LPS-stimulated control incubated with or without CDA(10µM), TDA (10µM) or diluent were analysed for cytokines TNF-α. Data points represent mean ± SEM from 3 donors. Statistical significance was calculated by paired student's t test . * = $p \leq 0.05$.

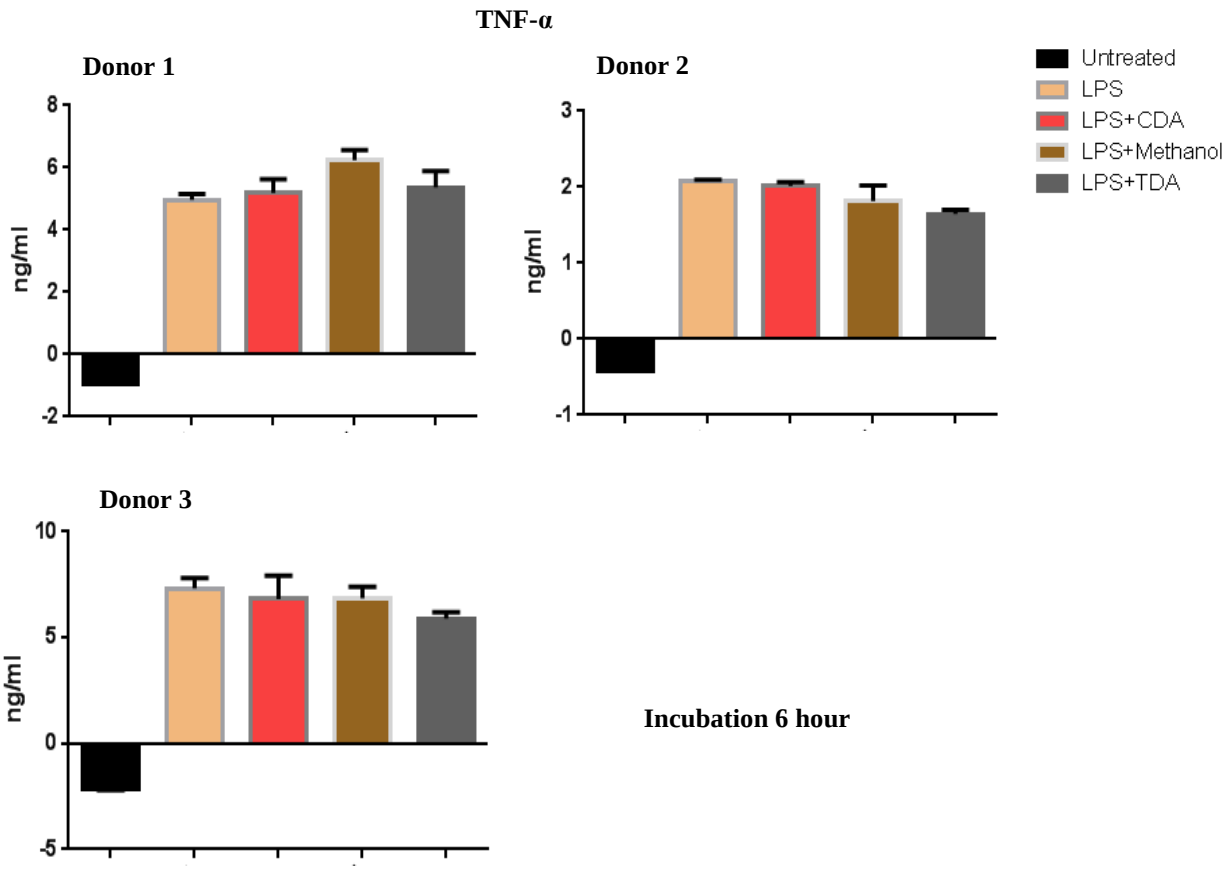



Fig: 5.13 Reduced production of TNF-α in macrophages activated with LPS after 6 hours incubation

Supernatant from unstimulated control or LPS-stimulated macrophages incubated with or without CDA (10µM), TDA (10µM) or diluent were analysed for TNF-α. Each bar represents mean ± SD of duplicate experimental wells.

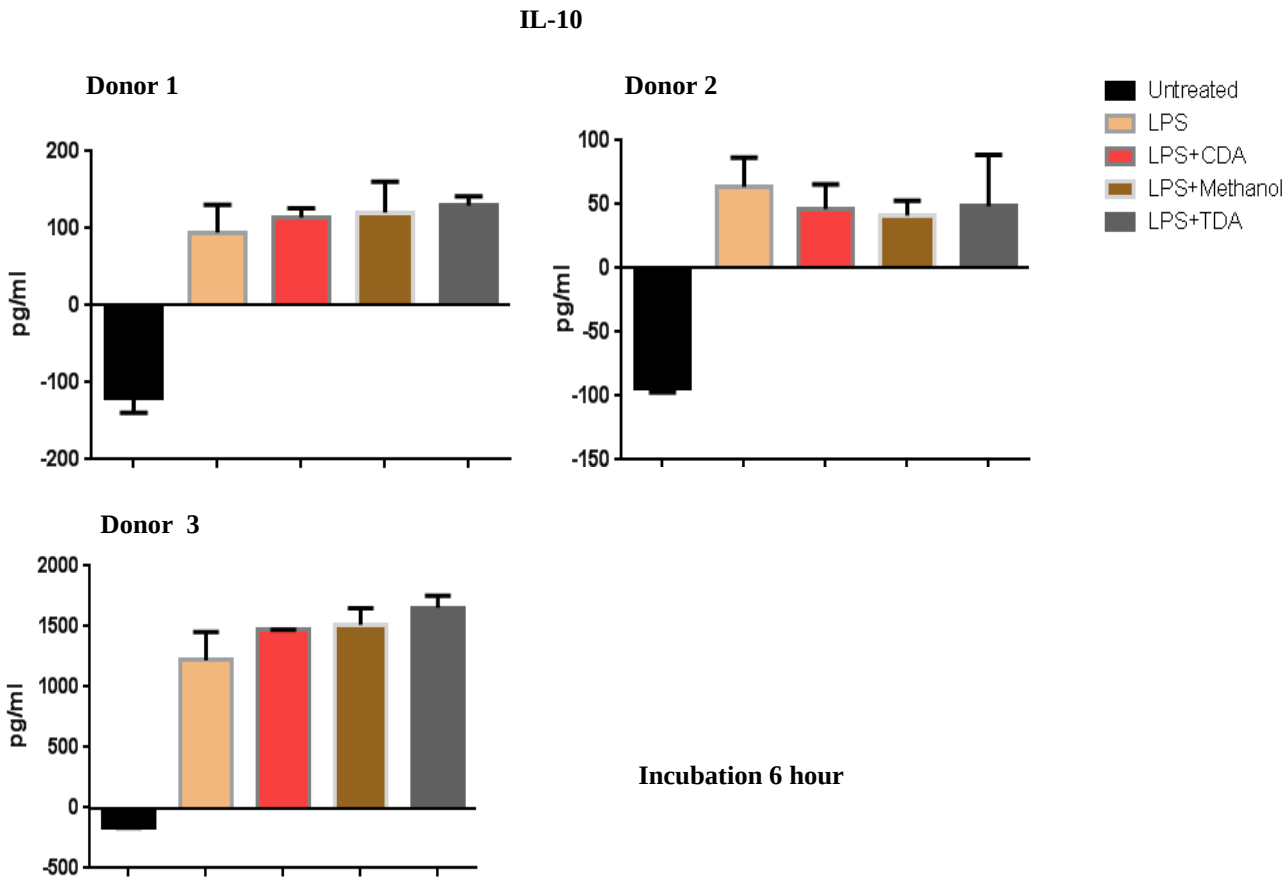



Fig: 5.14 CDA does not affect IL-10 production in macrophages activated with LPS after 6 hours incubation

Supernatant from unstimulated control or LPS-stimulated control incubated with or without CDA(10µM), TDA (10µM) or diluent were analysed for cytokines TNF-α. Each bar represents mean ± SD of duplicate wells.

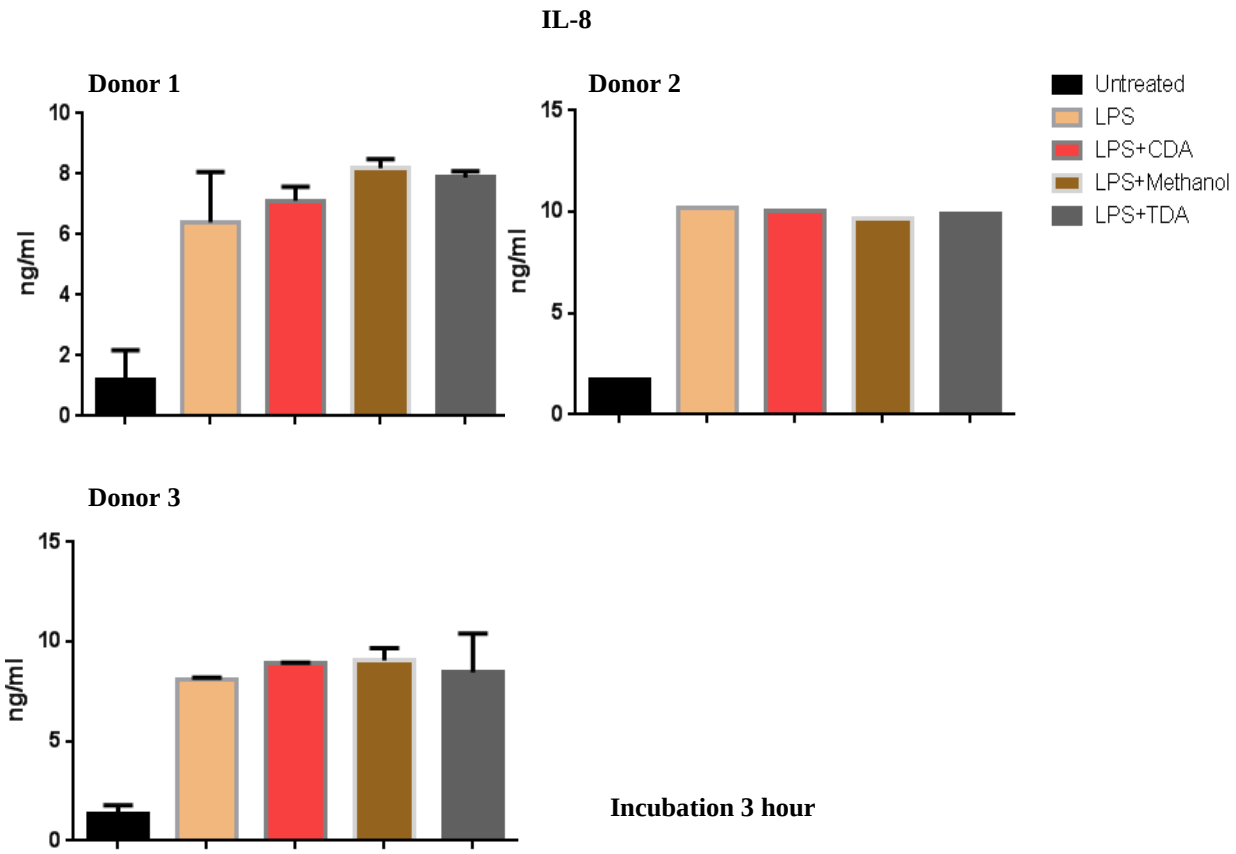



Fig: 5.15 CDA does not affect IL-8 production in macrophages activated with LPS after 3 hours incubation

Supernatant from unstimulated control or LPS-stimulated control incubated with or without CDA(10µM), TDA (10µM) or diluent were analysed for cytokines TNF-α. Each bar represents mean ± SD of duplicate wells except donor 2.

## 5.5. Discussion

### 5.5.1. Significance of the study

Dispersion is the final stage of the biofilm cycle and contributes to dispersal and transmission of pathogens to colonize new sites (Kaplan, 2010). The cells released during the dispersion process often showed different genomic expression and protein production pattern and are more susceptible to antimicrobial agents compared to their biofilm counterparts. Till now not many studies have focused on the role of dispersion in pathogenesis. It has been proposed that biofilm dispersion is an important part of an inherent strategy of many pathogens including PA which induce a disseminating phenotype to cause acute and periodic infections (Petrova and Sauer, 2012). It was already mentioned earlier that addition of exogenous CDA is able to disperse mature biofilm and inhibit biofilm formation of different gram negative and gram positive bacteria such as PA, *E. coli* and *S. aureus* (Davies and Marques, 2009). Previous experiments showed that CDA at a concentration of 10µM is able to disintegrate PA biofilm with the surface coverage being reduced to 40% in 15 minutes and 80% in 30 minutes (Ye Chan, 2014. Fatty acid signalling in *Pseudomonas aeruginosa*. Ph.D. University of Nottingham:UK). Also it was shown that CDA at 1 µM concentration is able to significantly inhibit PA adhesion and attachment and at 10 and 100µM concentrations is able to inhibit PA biofilm formation completely. CDA does not have any effect on bacterial growth even at the highest concentration. These results indicate that the impact of the CDA molecule on biofilm formation is caused by biologic modulation not by inhibiting bacterial growth (Ye Chan, 2014. Fatty acid signalling in *Pseudomonas aeruginosa*. Ph.D. University of Nottingham:UK).

CDA production starts in PAO1-L cultures after 9 hours of growth. At the onset of stationary phase CDA was detected at a concentration of 1 μM and at the late stationary phase after 25 hours of growth CDA accumulated up to 10 μM (Ye Chan, 2014. Fatty acid signalling in *Pseudomonas aeruginosa*. Ph.D. University of Nottingham:UK). It has been proposed a potential use of CDA in combination with antimicrobial agents to treat PA biofilm-associated infection (Davies and Marques, 2009). The present study was aimed at analysing the role of CDA in the inflammatory response to biofilms by assessing its effect on different immune cells including human monocytes and macrophages. We have observed that CDA modulates the inflammatory response of monocytes and macrophages by reducing transcription and production of TNF-α in response to LPS.

### 5.5.2. CDA modulates levels of TNF-α transcript and protein by LPS-activated monocytes and macrophages

The present study showed that CDA at 10μM concentration is able to reduce TNF-α expression by LPS-activated monocytes at 1 hour and macrophages at 3 hours post-treatment, respectively. The study was initiated to analyse how CDA could influence the inflammatory response of both unstimulated and LPS-stimulated monocytes. For this transcription of multiple genes including TNF-α, GM-CSF, IL-8, IL-10, IL-6 and IL-1β were analysed using qRT-PCR. Surprisingly, only TNF-α and GM-CSF expression was detectable in both unstimulated and stimulated monocytes. Furthermore the results showed CDA does not directly induce TNF-α expression in unstimulated monocytes but significantly reduces TNF-α expression in LPS-stimulated monocytes compared to the TNF-α expressed in LPS- stimulated monocytes in presence of

diluent (paired student's t test) after 1 hour of incubation although the effect was lost at 3 hours. No consistent effects were observed in the case of GM-CSF as there was high variation among donors. Due to these initial findings further experiments were designed to explore whether CDA has a similar effect on human macrophages. A reason not to continue work with monocytes was they were found to be short lived, even in the presence of M-CSF, with a high percentage of apoptotic monocytes detected in cultures after overnight incubation (F. Hussein, personal communication). Also it was found cumbersome to purify large number of monocytes from fresh blood for each experiment. In addition macrophages are the prime cells being involved in early recognition at the site of infection; they have a longer life span compared to freshly purified monocytes and thus provide a more robust system to characterise the role of CDA during the inflammatory response to infection. Similar to the initial results observed in monocytes it was shown that CDA reduced the TNF-α expression significantly (paired student's t test) in LPS-stimulated macrophages in presence of CDA comapared to the TNF-α expressed in LPS stimulate dmacrophages in presence of diluent at 3 hours. Thus it was speculated that CDA might be altering the kinetics of TNF-α production in response to LPS and is favouring a lower TNF-α production that could go on for longer. Thus a clear understanding can be achieved by doing a more detailed work especially time course experiments using LPS at different concentrations such as 100 and 10 ng/ml. The rest of the genes under investigation were undetectable except in the case of IL-8 but it appeared that CDA does not have any effect of IL-8 transcription. Although the transcript was undetectable, IL-10 was detected in the supernatants. Due to lack of a

positive control it was speculated that the primers used to detect IL-10 transcript did not work. Thus further experiments can be designed using a positive control such as plasmid DNA with the gene or samples that we know contain the transcripts. Furthermore TNF-α quantification in supernatants showed that CDA reduce TNF-α production of LPS-stimulated macrophages at 3 hours and no such effect was observed at 6 hours and also CDA does not affect cytokines IL-10 or IL-8 production by LPS stimulated macrophages. All these observations indicate that CDA appears to be affecting particularly the TNF-α pathway. TNF-α is an important component of the inflammatory response produced by activated monocytes, macrophages and T lymphocytes in response to variety of infectious stimuli (Johnston and Conly, 2006, Furst et al., 2005). The biological functions of TNF-α are numerous and include antiviral and antitumor activities, also TNF-α mediates systemic inflammatory response to several infections and sepsis (Aikawa et al., 1996). TNF-α plays a crucial role in host immune responses against a variety of pathogens, particularly against *Mycobacterium tuberculosis* and other intracellular oganisms (Algood et al., 2005, Zganiacz et al., 2004). TNF-α stimulates recruitment of inflammatory cells to the site of infection. In a murine tuberculosis infection models, mice deficient in TNF-α or TNF-α receptor are unable to recruit inflammatory cells to the infection site (Flynn et al., 1995, Algood et al., 2005, Roach et al., 2002). Multiple cellular and extracellular PA components including LPS, porins, pyocyanin, exoenzyme S and slime polysaccharide have been shown to stimulate TNF-α synthesis and release from human and mouse monocytes/macrophage (Otterlei et al., 1993, Staugas et al., 1992, Zughaier et al., 1999, Cusumano et al., 1997, Ulmer et al., 1990, Epelman et al., 2000,

Lagoumintzis et al., 2003, Simpson et al., 1988, Kooguchi et al., 1998), which suggest that TNF-α production plays an important role during the inflammatory response to PA infection. Thus it is important to understand the mechanisms through which CDA reduces TNF-α production. TNF-α transcription is induced in slime-GLP- stimulated monocytes through activation of the transcription factors NF-κB and AP-1 (Lagoumintzis et al., 2003). NF-κB/Rel is a family of transcription factors that regulates immune responses. Inactive NF-κB components are stored in the cytoplasm in association with inhibitory molecules called IκBs. When IκB get phosphorylated and subsequently degraded in response to appropriate stimuli, NF-κB translocates into the nucleus and activates transcription upon interaction with binding sites on DNA. A number of possible NF-κB binding sites are found in the promoter of the TNF-α gene. Similar to PA slime-GLP, it is important to analyse whether CDA exerts it regulatory function by affecting NF-kB activation and thus modulating the inflammatory response at the site of infection. Furthermore it has been shown that certain PRRs such as MR along with TLR2 play an important role in activating pro-inflammatory response including TNF-α production during PA infection. It was suggested that both MR and TLR2 contribute equally to pro-inflammatoty cytokine production from human monocytes in response to *in vitro* PA infection utilizing slime-GLP their legand (Xaplanteri et al., 2009). Thus it would be of interest to investigate if exogenous CDA could modulate the response to PA and/or its components; this will provide a better understanding of the role of this dispersal molecule in PA pathogenesis.

### 5.5.3. Future work

If this study was to continue, there are several approaches that could be taken to further investigate the interaction of CDA with the host. Firstly, using a particular PA mutant unable to synthesize CDA would help to determine whether CDA was definitely regulating TNF-α production during the course of PA infection. This mutant strain could be compared directly with its wild type which produces CDA. These studies should be done using a biofilm-forming strain that does produce CDA for the best direct comparison, as previous lab work showed that detectable CDA production started after 9 hours growth of PAO1-L, with a concentration about 1 µM at the onset of stationary phage which reached about 10 µM after 25 hours of growth (Ye Chan, 2014. Fatty acid signalling in *Pseudomonas aeruginosa*. Ph.D. University of Nottingham:UK). As mentioned earlier PA produces three EPS: Alginate, Pel, Psl which form the matrix of PA biofilm. It has been shown that EPS acts as a diffusional barrier for antimicrobial agents and regulate the access of the antimicrobial components (e.g. ciprofloxacin and ampicillin) to the biofilm interior (Anderl et al., 2000). Thus, planktonic bacteria that are sensitive to antibiotics become more resistant following biofilm formation (Stickler, 2008). A recent study showed that in catheter-associated urinary tract infections (CAUTIs) a combination treatment with CDA and antimicrobial agents was able to eradicate pre-existing biofilm (Rahmani-Badi et al., 2014). Therefore it would be interesting to investigate further the role of CDA itself in inflammation regardless of its involvement in biofilm development. Hence the effect of CDA in behaviour of other innate immune cells such as bronchial epithelial cells remodelling, barrier integrity, and paracellular permability

could be invegtigated. In addition to the role of CDA in the function of these immune cells the signalling machinary responsible for its effect need to be identified. Furthermore RNA expression analysis performed in our lab has shown that exogenous addition of CDA does not affect most of the *pel, psl* and *alg* related genes excepts *pslD* which is induced up to two fold (Ye Chan, 2014. Fatty acid signalling in Pseudomonas aeruginosa. Ph.D. University of Nottingham:UK). Thus it appears that CDA does not interfere with Psl polysaccharide production. Also as it has been mentioned in the previous chapter that Psl is the possible ligand for different C-type lectin receptor including MR, so further approaches can be taken to measure whether exogenous CDA is able to modify MR (and DC-SIGN) recognition of PA biofilms. Also it would be necessary to measure the effect of CDA in an infection model which can be performed by measuring killing of different PA strains by macrophages and neutrophils in the presence of exogenous CDA molecule.

Finally for the present study highly purified LPS has been used which will likely involved TLR-4 signalling pathway essential for the recognition of the lipid A moiety of gram negative bacterial LPS and provide effective host resistance against infection (Schnare et al., 2006, Pier, 2007). Similar to other gram negative bacteria, TLR4 and its accessory receptors CD14 and MD2, along with the soluble LPS-binding protein (LBP), are responsible for recognition of PA LPS. It has been shown that human TLR4 (hTLR4) is able to discriminate between lipid A structures of PA LPS. Human TLR4 responded to hexa-acylated LPS of PA isolates obtained from airways of CF-affected individuals and induce pro inflammatory response but not to penta-acylated

LPS of PA (Hajjar et al., 2002). Similarly it might be interesting to look at the effect of CDA on the response to both types of PA LPS. Furthermore, studies performed in MyD88-/- mice demonstrate that ligand/receptor pairs TLR4/LPS and TLR5/flagellin are important for the induction of KC, TNF-α, and IL-6 synthesis by PA activated cells including lung epithelial cells and AM (Raoust et al., 2009). Therefore it might be interesting to analyse whether CDA will be able to modify responses induced by TLR2 and TLR5 ligation.

### 5.6. Summary

A detailed understanding of the immune responses, inter-species communication and pathogens defence strategies during biofilm associated infection is important not only because knowledge of the immunopathology during biofilm related infections is lacking but because it is likely to provide important therapeutic tools to control chronic infections. Discovery of a dispersion molecule responsible for biofilm disintegration favours the transition from a biofilm growth mode to a planktonic state and promote subsequent pathogenesis. The persistence and chronic nature of biofilm-associated infections and the unusual resistance of biofilm bacteria towards antimicrobial agents could be reversed therapeutically if bacteria within biofilms could be forced into a planktonic phenotype. The present study showed that exogenous addition of CDA is able to modify some aspects of the inflammatory response by modulating production of the pro-inflammatory cytokine TNF-α but the impact of CDA on the outcome of inflammation during PA infection still needs to be determined.

# Chapter 6

## General discussion and future perspectives

Colonization of the lungs by PA is one of the major causes of morbidity and mortality in CF patients. In CF airways PA infections persist despite the use of aggressive antimicrobial therapy and are generally associated with the formation of biofilms. Within biofilms PA form micro-colonies embedded in an EPS matrix and are more resistant to antimicrobial therapy (Costerton et al., 1999, Drenkard and Ausubel, 2002). PA biofilm consist of several phases where production of EPS play a crucial role in the generation of biofilm structure and surface structures including pili and flagella also come into play (O'Toole and Kolter, 1998, Nikolaev Iu and Plakunov, 2007, Ma et al., 2009). Durring this developmental cycle dispersion of new phenotypic variants can be caused by environmental conditions (shear, chemical agents) or may be subject to microbial control. Thus a better understanding of each of these stages and their contribution towards immune recognition will provide a better understanding of PA pathogenesis and may unveil novel strategies for the control of chronic infections and their association with biofilm formation. Within the present study the effect of different innate immune components on PA biofilm recognition was evaluated.

The exact contribution of innate immune cellular components towards both planktonic and biofilm-associated PA infection is still unclear with most studies focussing on planktonic PA. For example selective depletion of AM by intranasal administration of liposome-encapsulated dichloromethylene diphosphonate showed no major increase in infectious bacteria or mortality

after low dose challenge with PA. Rather the evidence indicate a role for Alveolar macrophage in cytokine secretion and PMN recruitment rather than a direct role in mediating PA killing (Cheung et al., 2000). Human macrophages activated with IFN-γ in the presence and absence of GM-CSF were found unable to control PA growth or protect macrophages from PA-mediated cytotoxicity (Singh et al., 2015). Other studies showed that macrophages could potentially mediate phagocytosis, produce reactive oxygen species, release hydrolases and promote iron sequestration in order to control PA growth (Britigan et al., 2000, Speert et al., 1988, Kannan et al., 2008). In contrast there is limited information regarding the role of macrophages in the response toward PA biofilm (see details in Chapter 4).

Biofilm EPS contributes to the overall architecture and stability of biofilms (Sutherland, 2001, Branda et al., 2005). It has been suggested that after planktonic PA cells adhere to the substratum, non-motile PA cells initiate microcolony development which requires Pel and/or Psl polysaccharides, depending on the strain. For example the *Psl* genes are responsible for the formation of Psl EPS and thus required for the initiation and maintenance of biofilm in PAO1 and ZK2870. The *Pel* genes mediates synthesis of the Pel EPS and contributes to the development of biofilm in PA14 (Ryder et al., 2007, Ma et al., 2009, Friedman and Kolter, 2004a, Jackson et al., 2004, Friedman and Kolter, 2004b, Matsukawa and Greenberg, 2004). PA EPS provides the first line of defence against antimicrobial agents; by using fluorescent labelled antibiotics, the EPS was also found to protect PA biofilms from killing by reducing the penetration of tobramycin(Tseng et al., 2013). However the mechanism by which biofilm matrix is s protective against antibiotics and

interacts with these compounds is yet to be established. Similarly it was also shown that *Psl* can mediate resistance to the biofilm inhibitor Polysorbate 80 a non-ionic detergent and is able to inhibit PA biofilm formation at a very low concentration 0.001% which has been proposed well tolerated in human tissue (Zegans et al., 2012). EPS also provide protection from the innate immune system. For example alginate protects PA biofilm from macrophage killing (Leid et al., 2005). Furthermore addition of exogenous PA alginate limit the uptake and degradation of both viable and non-viable radiolabelled non-mucoid PA by resident mouse peritoneal macrophages(Simpson et al., 1988). Similarly Psl produced by PA reduces neutrophil phagocytosis by inhibiting complement-mediated PA opsonisation (Mishra et al., 2012). Thus further study into the role of EPS in biofilm formation and PA pathogenesis would be beneficial for the design of molecules targeted to the control of biofilm-mediated infection.

The present study investigated the effect of immune cells and mediators involved in innate immune responses on PA biofilm development. Initially the effect of inflammatory mediators on PA biofilm development was investigated. For this study GM-CSF and IFN-γ were considered. Both cytokines are important activators of inflammatory immune cells and are involved in other functions: for example promote antigen presentation, induction of phagocytosis, and induction of leukocyte chemotaxis and adhesion. A previous study showed that the response of macrophages towards PA infection can be modulated by IFN-γ and GM-CSF, which synergise to promote the pro-inflammatory response of macrophages during PA infection (Singh et al., 2015). Thus it was proposed that these two cytokines might directly affect PA

biofilm development. But the present study demonstrates that GM-CSF and IFN-γ at the concentration tested are unable to restrict PA biofilm development or affect QS molecule production in absence of human serum (Chapter 3).

Furthermore the present study mainly aimed at understanding the interaction between PA biofilm and human monocytes and macrophages with the hypothesis that human immune cells could interact with PA biofilms through the recognition of mannosylated carbohydrates by lectin receptors expressed by these cells.

Some of the initial observations showed that human monocyte-derived macrophages attached and formed clusters over PA biofilm; flow cytometric analysis of cell surface receptors showed MR expression restricted to macrophages compared to monocytes. MR is a multi-domain protein that internalises ligands that are recognised by three binding sites located in its extracellular region (Martinez-Pomares, 2012, Gazi and Martinez-Pomares, 2009). MR has a complex expression pattern and exhibits tissue dependent binding properties (Gazi and Martinez-Pomares, 2009). Thus given this complexity it is not surprising that besides being involved in homeostatic processes including clearance of endogenous products and cell adhesion, MR has been implicated in pathogen recognition and antigen presentation (Gazi and Martinez-Pomares, 2009).

The present study showed that a recombinant protein (CTLD4-7-Fc) comprising of the mannose-binding region of MR can bind PA biofilms and to extracted EPS (Chapter 4), probably through Psl. Besides MR other CLRs such as DC-SIGN have been found able to bind PA biofilm as well as extracted EPS (Mohammad Y Alshahrani, personal communication, Chapter 4). The

consequences of Biofilm/Psl-binding to human macrophages/DCs expressing different CLRs and how they contribute to PA pathogenesis are yet to be determined.

It is now clear that many CLRs are implicated in pathogen recognition. These include the MR, DC-SIGN and Dectin-1 (Taylor et al., 2005a). An effective immune response relies on the recognition of microbes by immune cells such as DCs through their PRRs. Since some these receptors are able to recognise pathogens but not to signal directly they can be exploited by pathogens for immune evasion. Thus a detailed investigation of the role of CLRs such as MR and DC-SIGN in the innate immune response to PA is necessary. In particular, DC-SIGN has emerged as a key player in the induction of immune responses against various pathogens by regulating TLR-induced activation. For example besides activating TLRs *Mycobacterium tuberculosis* interacts with DCs by binding DC-SIGN and this interaction induces production of immunosuppressive cytokines such as IL-10 (Geijtenbeek et al., 2003). After binding its specific ligands DC-SIGN activates an intracellular signalling pathway through the serine/threonine kinase Raf-1. Different pathogens including *Mycobacterium tuberculosis* and HIV-1 interact with DC-SIGN to activate Raf-1 which subsequently leads to acetylation of the NF-κβ subunit p65 and induction of specific gene transcription profiles (den Dunnen et al., 2009). Whereas interaction of fucose expressing extracellular pathogens including *Schistosoma mansoni* and *Helicobacter pylori* with DC-SIGN favours T helper cell type-2 (TH2) immune responses through activation of atypical NF-κB family member Bcl3 (Gringhuis et al., 2014). Similarly besides being involved in homeostatic processes MR participates in pathogen

recognition, and mediates immune responses by regulating the uptake of mannosylated antigens by DCs for processing and presentation to T lymphocytes (Taylor et al., 2005a). MR has also been involved in recognition of different pathogens and in intracellular signalling and regulation of gene expression in several studies (Zhang et al., 2004, Tachado et al., 2007, Yamamoto et al., 1997, Fernandez et al., 2005, Lopez-Herrera et al., 2005, Zhang et al., 2005) (Details has been described in Chapter 1). PA-infected CF patients exhibited upregulation of MR expression on CD11b+ cells with associated arginase activity in lung samples. These markers were found inversely related to FEV1. Both markers are linked with the alternative activation of macrophages. These observations might be related to the purposed deleterious role for type 2 immunity in CF patients which results in an imbalance between cellular and humoral immune responses (Murphy et al., 2010). In contrast to MR, no information is available regarding DC-SIGN expression in CF.

The relative contribution of MR and DC-SIGN to recognition of PA infection needs to be determined. Preliminary results show that DC-SIGN is able to bind both PA biofilms and extracted EPS and the binding affinity for DC-SIGN appeared higher compared to that of MR. Intriguingly binding of DC-SIGN to planktonic PA has been observed, possibly to a ligand independent from Psl and Pel (Mohammad Y Alshahrani, unpublished results). On the contrary MR appears more involved in binding PA biofilm utilizing Psl as a possible ligand, because MR was unable to bind with planktonic bacteria. This is in agreement with confocal images clearly showing that MR binds the PA biofilm matrix rather than bacteria. Thus it can be hypothesised that DC-SIGN is involved in

recognition of acute PA infection by detecting planktonic PA, modulating subsequent immune responses by altering TLR-mediated cellular activation. During chronic infection both DC-SIGN and MR would be involved in the recognition of PA biofilm matrix which could in turn, further influence the inflammatory response.

Thus detailed knowledge on factors involved in the innate immune recognition and PA clearance of both planktonic and biofilm-associated PA will provide an important tool for the factors affecting biofilm-associated infection.

The role of diffusible signal factors (crucial component involved in PA biofilm dispersion) on the activation of different cellular innate immune components was also studied (Chapter 5). As mentioned earlier PA produce DSF termed CDA which is a short chain fatty acid able to disperse biofilm formed by different pathogens (Davies and Marques, 2009). Exogenous addition of CDA is able to disperse preformed PA biofilm as well as inhibit PA biofilm formation (Davies and Marques, 2009). It was proposed that similar to QS molecules which contribute in PA pathogenesis and alter immune responses CDA might have a role in immune modulation. The present study showed that CDA acts as an immune modulator and reduces TNF-α production by LPS-activated human monocytes and macrophages. Also it was observed that CDA is affecting particularly the TNF-α pathways because no such effect was observed on other cytokines produced by these LPS-activated cells. Thus all the results from the present study showed that CDA is modulating immune response by regulating TNF-α but the subsequent outcome of this response is yet to be determined. For instance in the present study used *E.coli* LPS instead of PA LPS or whole PA. As mentioned in the previous Chapter TNF-α is

important for physiological immune function but also regulate pathogenesis of different autoimmune and inflammatory diseases including endotoxic shock, rheumatoid arthritis, ulcerative colitis, Crohn's disease, and multiple sclerosis (Feldmann et al., 1994, Strieter et al., 1993, Raine, 1995, Brynskov et al., 1994). Monocytic cells produce TNF-α in response to various stimuli such as bacterial LPS, TNF-α itself, superantigens and viral agents (Yao et al., 1997, Newell et al., 1994, Leitman et al., 1991, Trede et al., 1993, Geist et al., 1997). Regulation of TNF-α both transcriptional and post-transcriptional depends on the type of stimulus, cell type, and possibly differentiation (Swantek et al., 1997, Raabe et al., 1998, Udalova et al., 1998). TNF-α is considered to confer protection against PA infection in mice, depletion of TNF-α in DBA/2 mice and BALB/c mice using anti-TNF-α antibodies was associated with increased susceptibility to PA lung infection (Morissette et al., 1996, Gosselin et al., 1995, Sadikot et al., 2005). Similarly in a burn mouse model absence of TNF-α receptor induced an up regulation of PA-induced TLR-4, IL-1β expression, and lung microvascular dysfunction (Tsay et al., 2013). Hence it might be possible that CDA is facilitating lung injury and spreading of PA infection by reducing TNF-α production of activated immune cells and increasing TLR4 as well as IL-1β protein expression. IL-1β is a crucial inflammatory mediator with diverse physiological effects and performs an important role in pathogenesis of lung exacerbations in CF patients such as recruitment of inflammatory effector cells, induction of various pro-inflammatory cytokines including IL-6 and IL-8, fever and regulation of T cell responses (Dinarello, 2009, Tang et al., 2012, Dinarello, 2006, Ben-Sasson et al., 2009). IL-1β is generally produced in the cytosol as an inactive form pro-IL-1β. In response to various danger signals a

group of cytosolic protein termed inflammasomes assemble and initiate auto-activation of caspase-1 which therefore cleaves pro-IL-1β into their biologically active forms IL-1β for secretion (Dinarello, 2009, Lamkanfi, 2011). Several studies have supported the role for IL-1β in the pathogenesis of lung disease in CF patients (Douglas et al., 2009, Bonfield et al., 1995, Osika et al., 1999, Armstrong et al., 2005). As mentioned above PA is able to activate the NLRC4 inflammasome (Sutterwala et al., 2007).An eradication of PA infection was found associated with decrease production of IL-1β, and neutrophil elastase in bronchoalveolar lavage fluid (BALF) collected from CF patients (Douglas et al., 2009).The present study showed that CDA is able to regulate TNF-α production of LPS activated immune cells, but the specific signalling pathways affected by CDA are yet to be determined. Transcriptional activation of the TNF-α promoter in human monocytes remains controversial as expression depends on different stimuli. It has been observed that NF-κB and c-Jun/ATF-2 sites are adjacent in the TNF-α promoter, raising the possibility that they may promote transcription. For example in monocytic cells activated by stress, co-operation between NF-κB and c-Jun/ATF-2 sites was shown to induce TNF-α gene expression (Guha et al., 2000) while glucocorticoids reduce TNF-α expression by human monocytic THP-1 cells by inhibiting transactivation of NF-κB and c-Jun/ATF-2 at their binding sites in the TNF-α promoter (Steer et al., 2000), glucocorticoids exerted their inhibitory effect on TNFα production by reducing transcription of the TNF-α promoter which mainly occurred at a time between 90 minutes and 3 hours after LPS exposure in THP-1 cells (Steer et al., 2000). Thus understanding the

underlying signalling pathways modulated by CDA will offer many opportunities for development of drug with potential anti-inflammatory action.

In conclusion, a detail understanding of the innate immune recognition and PA defence mechanisms under conditions of biofilm associated infection is crucial not only because these processes are part of the immunopathology of biofilm associated infection but because it will also provide important treatment tools with potential anti-microbial action.

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