THE ROLE OF C-TYPE LECTIN RECEPTORS IN THE RECOGNITION OF *PSEUDOMONAS AERUGINOSA*

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Abstract

Cystic fibrosis patients endure serious lung infection caused by colonisation and persistent infection by a wide range of pathogens, most commonly *Pseudomonas aeruginosa* (PA). One of the factors that facilitates establishment of chronic lung infection is formation of biofilms which are structures resistant to antimicrobial drugs and immune attack. Biofilms are embedded within extracellular polymeric substance (EPS) that maintains the structure of biofilms. PA produces two important polysaccharides Pel and Psl, which have been implicated in promoting biofilm development and biofilm maintenance, respectively, as well as cell aggregation . To the best of our knowledge, there has not been any study showing the presence of specific immune receptors for the recognition of PA biofilms. C-type lectin receptors (CLRs) are pathogen-recognition receptors that contribute to the recognition of infectious agents through the detection of carbohydrates moieties representing a subset of pathogen-associated molecular patterns (PAMPs).

We hypothesized that CLRs such as DC-SIGN (CD209) and mannose receptor (MR) (CD206) could play a crucial role in the immune recognition of PA biofilms through the binding to different carbohydrate-containing components. Investigating the CLR-PA cross-talk and the response of immune cells expressing CLRs to different PA components could lead to a novel strategy to eradicate infections. The main aim of this thesis is to determine the capability of CLRs, particularly MR and DC-SIGN, to interact with PA biofilms.

We have shown that CTLD4-7, a region of MR, and DC-SIGN bind to PA biofilms with DC-SIGN binding significantly better than MR. Both lectins also recognised several independent preparations of EPS lacking Pel. Surprisingly, we found that DC-SIGN also binds to planktonic PA in the absence of Psl and Pel which indicates that DC-SIGN could recognize non-EPS carbohydrate-containing ligands in the bacteria. Further investigation unveiled that DC-SIGN requires the presence of the common polysaccharide antigen (CPA) which is shared among all PA serotypes to bind planktonic cells. These results indicate that CPA is a candidate ligand for DC-SIGN in PA. To determine the significance of these findings, assays incubating human dendritic cells with purified EPS and planktonic PA were performed but no definitive conclusion could be drawn.

These findings shed light on the potential impact of PA Psl and CPA-LPS on the recognition of PA by immune cells expressing CLRs and might open new avenues for therapeutic approaches.

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List of Abbreviations

AECs	Airway epithelia cells
AHL	N-acylhomoserine lactones
AP	Alkaline phosphatase
AQ	2-alkyl-4 quinolones
ASL	Airway surface liquid
BIR	Baculovirus inhibitor of apoptosis protein repeat
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3',5'-monophosphate
c-di-GMP	Cyclic dimeric guanosine monophosphate
CF	Cystic fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator gene
CLRs	C-type lectin receptors
CTLDs	C-type lectin-like domains
DCs	Dendritic cells
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
eDNA	Extracellular DNA
EF-2	Elongate factor
ENaC	Epithelial Na^+ channel
EPS	Extracellular polymeric substance
ETA	Exotoxin A
F1gE	Flagellar hook protein
FAD	Flavin adenine dinucleotide
GAC	Global activator of antibiotic and cyanide
HAA	Hydroxyalkanoyloxy alkanoic acids
HHA	Hippeastrum hybrid Amaryllis
HHQ	2-Heptyl-4-quinolone
HHQNO	2-Heptyl-4-quinolone N-oxide
HIV	Human immunodeficiency virus
HOCL	Hypochlorous acid
HXA3	Hepoxilin A3
IATS	International Antigenic Typing Scheme

IRF	Interferon regulatory transcription factor
ITAM	Immunoreceptor tyrosine-based activation motif
LB	Luria-Bertani
LPS	Lipopolysaccharide
LRRs	Leucine-rich repeats
LTB4	Leukotriene B4
MAPK	Mitogen-activated protein kinases
MBL	Mannan binding lectin
mBTL	Meta-bromo-thiolactone
MDRPA	Multidrug-resistant *P. aeruginosa*
MPO	Myeloperoxidase
MR	Mannose receptor
MRSA	Methicillin-resistant *Staphylococcus aureus*
MyD88	Myeloid differentiation 88
NETs	Neutrophil extracellular traps
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NHQ	2-Nonyl-4-quinolone
NLRP	Nucleotide-binding oligomerization domain, Leucine-rich Repeat and Pyrin domain
NLRs	Nucleotide-binding oligomerization domain (NOD)-like receptors
NOD	Nucleotide-binding oligomerization domain
OM	Outer membrane
OMVs	Outer membrane vesicles
PA	*P. aeruginosa*
PAMPs	Pathogen associated molecular patterns
PGN	Peptidoglycan
PLA2	Phospholipase A2
PLC	Phospholipase C
PMNs	Polymorphonuclear leukocytes
PQS	2-Heptyl-3-hydroxy-4-quinolone
PRR	Pattern recognition receptors
Psl	Polysaccharide synthesis locus
PYD	Pyrin domain
QS	Quorum sensing
GM-CSF	Granulocyte macrophage colony–stimulating factor

RLRs	Retinoic acid-inducible gene (RIG)-I-like receptors
ROS	Reactive oxygen species
Sky	Spleen tyrosine kinase
T3SS	Type III secretion system
TCA	Tri-chloro-acetic acid
TIR	Toll/IL-1 receptor
TIRAP	TIR-associated protein
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor- α
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein-inducing IFN-β

Chapter 1: General introduction

1.1 *P. aeruginosa* (PA)

1.1.1 PA Infections

The term ESKAPE (***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa,* and ***E****nterobacter* spp) is an acronym that refers to a group of bacterial pathogens of concern to global health systems due to their association with antimicrobial resistance (Santajit and Indrawattana, 2016). ESKAPE pathogens have been found to be responsible for major nosocomial infections (Rice, 2010) and have the ability to escape the biological actions of antimicrobial agents and antibiotics (Pogue et al., 2015), and represent a paradigm shifts in disease pathogenesis and resistance (Pendleton et al., 2013). Nosocomial infections are hospital acquired infections and can occur within two days of admission to a hospital, less than five days after discharge, or a month after an operation (Revelas, 2012). PA is a leading cause of nosocomial infections and accounts for 10.1% of nosocomial infections in United States hospitals (Sadikot et al., 2005b). The increased rate of infections with multidrug-resistant PA (MDRPA) is a concern because of limited availability of effective antimicrobial options (Obritsch et al., 2005). ESKAPE pathogens employ several mechanisms of drug resistance including, destruction of a drug's activity, biofilm formation, modification of drug binding sites, and changing the cell's permeability which, in turn, reduces intracellular drug accumulation (Santajit and Indrawattana, 2016). For instance, the most common mechanism of imipenem resistance in PA is a combination of drug inactivation and reduced intracellular drug accumulation, with AmpC and porin changes being

involved, respectively (Poole, 2005, Cai et al., 2009). AmpC β-lactamases are important enzymes encoded on the chromosomes of many pathogens including PA. They have the ability to mediate resistance to cephalothin, penicillins, and inhibitors of β-lactamase (Jacoby, 2009). The outer membrane of PA contains porins, the proteins that allow passage of hydrophilic substances such as antibiotics (Delcour, 2009). A reduction in the amount of PA porins decreases drug inflow into the cells (Fukuoka et al., 1993).

PA has the ability to exhibit different phenotypes allowing it to cause either acute or chronic infection (Balasubramanian et al., 2015). In order to initiate infection, PA planktonic cells express several acute virulence factors such as flagella, pili, and type III secretion system (T3SS). PA also expresses multiple virulence factors regulated by quorum-sensing systems (QS) such as proteases, cyanide, elastase, rhamnolipid, and phenazines (Balasubramanian et al., 2012a). Bacterial motility is also an important factor at this stage (Furukawa et al., 2006). During acute infection, PA cells are antibiotic sensitive unless the infection was initiated by an antibiotic-resistant strain (Hogardt and Heesemann, 2011). Upon switch from acute to chronic infection, PA forms biofilm which is considered a critical factor allowing PA to persist and accumulate multiple mutations (López-Causapé et al., 2013). This is associated with a conversion to mucoid phenotype and gain of resistance to antibiotics (Høiby, 2011). The global activator of antibiotic and cyanide (GAC) within the PA genome plays a role in the transition from acute to chronic infection. The GAC system consists of two transmembrane sensors, GacS and GacA (Jimenez et al., 2012, Heeb and Haas, 2001). Both sensors activate expression of small regulatory RNAs, rgRsmY and rgRsmZ, which in turn deactivate the negative regulator RNA-binding protein RsmA (Brencic et al., 2009), a posttranscriptional regulatory protein that controls the expression of many of the genes involved in

virulence during both acute and chronic infection (Mulcahy et al., 2008b). AmpR is a transcriptional factor involved in regulation of antibiotic resistance, proteases, and QS (Kong et al., 2005). Lack of AmpR downregulates T3SS system, reduces production of acute virulence factors, increase antibiotic resistance, and enhances formation of biofilm (Kumari et al., 2014, Balasubramanian et al., 2012b). Hence, both the GAC system and AmpR, among others, control biofilm, antibiotic resistance, and QS regulation, and aid PA to establish either acute or chronic infection.

PA is a major medical problem to the healthcare system (Mendiratta et al., 2005). It is an opportunistic pathogen because it takes advantage of a weakened immune system or breached barriers to cause severe illness (Goldberg, 2010). PA is considered an important pathogen causing serious infections such as pulmonary CF disease, ventilator-associated pneumonia, and burn-wound infection (Mesaros et al., 2007).

1.1.1.1 Burn-wound infection

PA is a key pathogen in chronic burn-wound infections (Gonzalez et al., 2018). Damaged skin and host defences deficiency contribute to the infection complexity (Lyczak et al., 2000b). The burn-wound infection starts with colonization of the wound by bacterial cells and then subsequently biofilm formation on the surface of the epithelial layer (Bielecki et al., 2008). Many virulance factors can mediate the dissemination of PA through the injured tissues. For example, neutrophils elastase has the ability to degrade the host fibronectin, an important component of the extracellular matrix during wound healing (Grinnell and Zhu, 1996). Using a burn-wound mouse model, mutations in *lasR* and *lasA,* components of PA QS, have

shown reduction in both virulence and spreading of bacterial cells on the burned skin. However, the most significant reduction was observed with the double mutants *lasR* and *lasA* compared to WT PAO1, *ΔlasR* PAO1, or *ΔlasA* PAO1, suggesting that QS plays a role in burn-wound infection by promoting biofilm formation, which makes the burn-wound healing difficult (Rumbaugh et al., 1999).

1.1.1.2 Ventilator-associated pneumonia (VAP)

PA has been shown to be responsible for most of VAP cases (Park, 2005). Long term intubated patients might acquire VAP causing pathogens from different sources such as respiratory equipment, sinks, and health workers (Vincent, 2012). Mechanical ventilation has been demonstrated to be associated with VAP (Kalanuria et al., 2014). VAP is the most common hospital acquired infection in critical care facilities and is associated with up to 40% mortality (Gunasekera and Gratrix, 2016). In order to establish VAP, pathogens normally access the lower respiratory tract and then adhere to it, causing persistent infection (Safdar et al., 2005). It has been shown that the endotracheal tube serves as a reservoir for VAP pathogens. A previous study found that the adherent pathogens produced biofilms, leading to poor prognosis (Koerner, 1997). 70% of VAP patients had the same pathogens isolated from both tracheal secretions and endotracheal tube biofilms (Adair et al., 1999). A study conducted on a group of VAP patients showed that patients with PA expressing T3SS showed worse clinical outcome (Hauser et al., 2002).

1.1.1.3 PA infection in CF patients

CF is a life-threatening autosomal recessive disease (Hobbs et al., 2013a) caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator gene (CFTR) (Rogers et al., 2008). CF involves multiple organs such as the pancreas, lung, liver, and intestine (Abu-El-Haija et al., 2011). CFTR regulates the movement of water and chloride and sodium ions across the epithelium (Gaspar et al., 2013), therefore, any defect in CFTR leads to viscous secretions, diminished muco-cilliary clearance and pulmonary infections (Bonfield et al., 2012). The CFTR protein is found on the apical membrane of epithelial cells (Denning et al., 1992), and it functions as an ATP-dependent membrane transporter which is activated by cyclic adenosine 3',5'-monophosphate (cAMP)-dependent phosphorylation (Gadsby et al., 2006). The homeostasis of chloride, sodium and other ions is maintained by CFTR (Schwiebert et al., 1999).

Since 1989, when the gene encoding CFTR was first identified (Sheppard and Welsh, 1999), more than 1500 mutations have been described (Sebro et al., 2012). The most common mutation is ΔF508, which accounts for 70% of CF cases (Davis, 2006). Less common CFTR mutations are G551D, N1303K, and G542X (Bobadilla et al., 2002). Different mutations have different effects on CFTR. Mutations are classified into six groups based on their molecular mechanisms. Table 1 summarizes the different mutations which result in either altered or loss of CFTR function (Lubamba et al., 2012b, Zielenski, 2000).

Table 1.1. Classification of CFTR mutations

Class	Mutation prototype	Consequences
I	▪ G542X ▪ W1282X ▪ R553X	▪ Lack or defect in CFTR biosynthesis ▪ Abnormal proteins cleared efficiently from the cell ▪ Most severe mutations
II	▪ F508Ddel ▪ N1303K	▪ Immature CFTR is synthesised ▪ No CFTR at the apical membrane
III	▪ G551D	▪ Mature CFTR is synthesized ▪ Inactive non-functional protein due to defective regulation
IV	▪ R334W ▪ G314E ▪ D1152H	▪ Normal amount of CFTR at the apical membrane ▪ Impaired chloride conductance
V	▪ A455E	▪ Defect in CFTR synthesis ▪ The amount of functional CFTR is reduced
VI	▪ Q1412X	▪ Intact CFTR is produced ▪ Unstable protein is found at the apical membrane

In addition to acting as a cAMP-regulated chloride channel (Lubamba et al., 2012a), it has been shown that CFTR is expressed in endosomes which suggests that it may be involved in regulating endosomal pH (Lukacs et al., 1992).

Some gene-based therapeutic agents have been approved to act on CFTR. Ivacaftor, a CFTR potentiator, is used to treat CF patients with the G551D mutation. It mainly increases the opening time of CFTR allowing chloride ions to flow via CFTR proteins channels at the epithelial cell surface. Ivacaftor has been shown to successfully improve lung function (Condren and Bradshaw, 2013). Lumacaftor (VX-809) is used to treat patients with the ΔF508 mutation. It can facilitate the CFTR trafficking within the cells allowing the protein to reach the cell membrane (Boyle et al., 2014). Another agent is VX-661 which has shown improvement when

administered with Ivacaftor to treat CF patients with the ΔF508 mutation (Donaldson et al., 2013).

Airway dehydration due to chloride depletion and sodium increase is a hallmark of CF disease (George et al., 2009). The epithelial Na^+ channel (ENaC) is responsible for absorbing sodium in airway epithelia, while chloride is secreted by CFTR. This maintains electrolytes balance leading to sufficient hydration of the airway surface liquid (ASL) (Mall et al., 2004). Defective CFTR results in reduced chloride level, hyperactivity of ENaC, which in turn increases sodium absorption (Hobbs et al., 2013b). All these factors contribute to mucus dehydration leading to: 1) insufficient mucus clearance and 2) decreased ASL, resulting in impairment of pathogen clearance and, subsequently, establishment of chronic infection (Gaspar et al., 2013). PA infection is a well-established cause of morbidity in CF (Mowat et al., 2011), although other pathogens have also been found in the CF lung. Lung function failure in CF has also been associated with Methicillin-resistant *Staphylococcus aureus* (MRSA) (Dasenbrook et al., 2008). In addition, *Haemophilus influenze* frequently colonizes the CF lungs (Cardines et al., 2012), and *Aspergillus fumigatus* has been isolated from sputum of CF patients (De Vrankrijker et al., 2011). CF patients show high levels of *Burkholderia* spp in the salivary fluid (Coutinho et al., 2008). Also, *Streptococcus pneumoniae*, and *Klebsiella pneumoniae* have been found in CF patients' sputum (Jones et al., 2018). PA chronic infection is prevalent in about 80% of adult CF patients (Pressler et al., 2011). Also, CF adult patients might suffer pulmonary exacerbations due to PA infection (Aaron et al., 2004).

Biofilms have been identified in the CF lung (Kovach et al., 2017). Biofilm growth in CF is associated with several mutations, adaptation of the bacteria to the lung conditions and resistance to antibiotics (Høiby et al., 2010). PA CF isolates display

altered expression of virulence factors which affects motility, antimicrobial resistance, lipopolysaccharide (LPS) structure, and production of some secreted products such as pyocyanin (Hoboth et al., 2009). PA pyocyanin can inhibit the function of antioxidants and block chloride transport in human bronchoepithelial cells (Hassett et al., 1992, Cormet-Boyaka et al., 2016). During chronic infection PA undergoes a phenotypic change which involves production of biofilm matrix, called extracellular polymeric substance (EPS), an important player in biofilm formation (Bragonzi et al., 2005). PA can also adopt a mucoid phenotype in the CF lung (Troxler et al., 2012). Once PA becomes mucoid and forms biofilm, the eradication of infection is very difficult. This leads to immune response disturbance, impaired pulmonary function, and persistence of chronic disease (Hartl et al., 2012b).

1.1.2 Characteristics of PA

PA is a Gram-negative, rod-shaped, and motile, opportunistic pathogen commonly found ubiquitously in the environment due to its ability to utilize numerous energy sources (Gellatly and Hancock, 2013). PA versatility is underpinned by its large genome (6.3 million base pairs) that endows the bacterium with metabolic adaptability and tightly regulated gene expression programmes. This flexibility allows PA to grow vigorously in diverse ecological niches (Sadikot et al., 2005a, Rossolini and Mantengoli, 2005). PA utilizes many exoproducts such as elastase and exotoxin A to evade the immune response allowing the persistence of PA infections (Sadikot et al., 2005a, Mariencheck et al., 2003b). Chronic lung infection in CF, bacteraemia in burn-wound patients, and acute ulcerative keratitis are the most common human diseases caused by PA (Lyczak et al., 2000a). In CF, the presence of PA infection is associated with poor lung function and increased

morbidity and mortality (Lavoie et al., 2011a). A previous report showed that 80% of CF patients have PA infection (Crull et al., 2016). PA has the ability to resist multiple classes of antibiotics and antimicrobial agents such as ciprofloxacin and levofloxacin (Lister et al., 2009). In order to establish a chronic infection, PA produces a variety of virulence factors and surface structures which contribute to its pathogenicity and play a role in motility and adhesion (Murray et al., 2010). Biofilm formation is one of the strategies used by PA to establish and maintain chronic infections (Høiby et al., 2010).

1.1.3 Virulence factors of PA

PA express many virulence determinants that enable PA to abrogate the immune response, establish chronic infection and survive in extremely aggressive environments (Gellatly and Hancock, 2013) (Figure 1.1). As mentioned above, isolates from acute infections express a wide range of virulence factors but some of these virulence determinants such as flagella and pili are missing in isolates from chronic CF lung and chronic burn-wound infections (Hogardt and Heesemann, 2010). The contribution of key virulence factors to PA infection is described below.

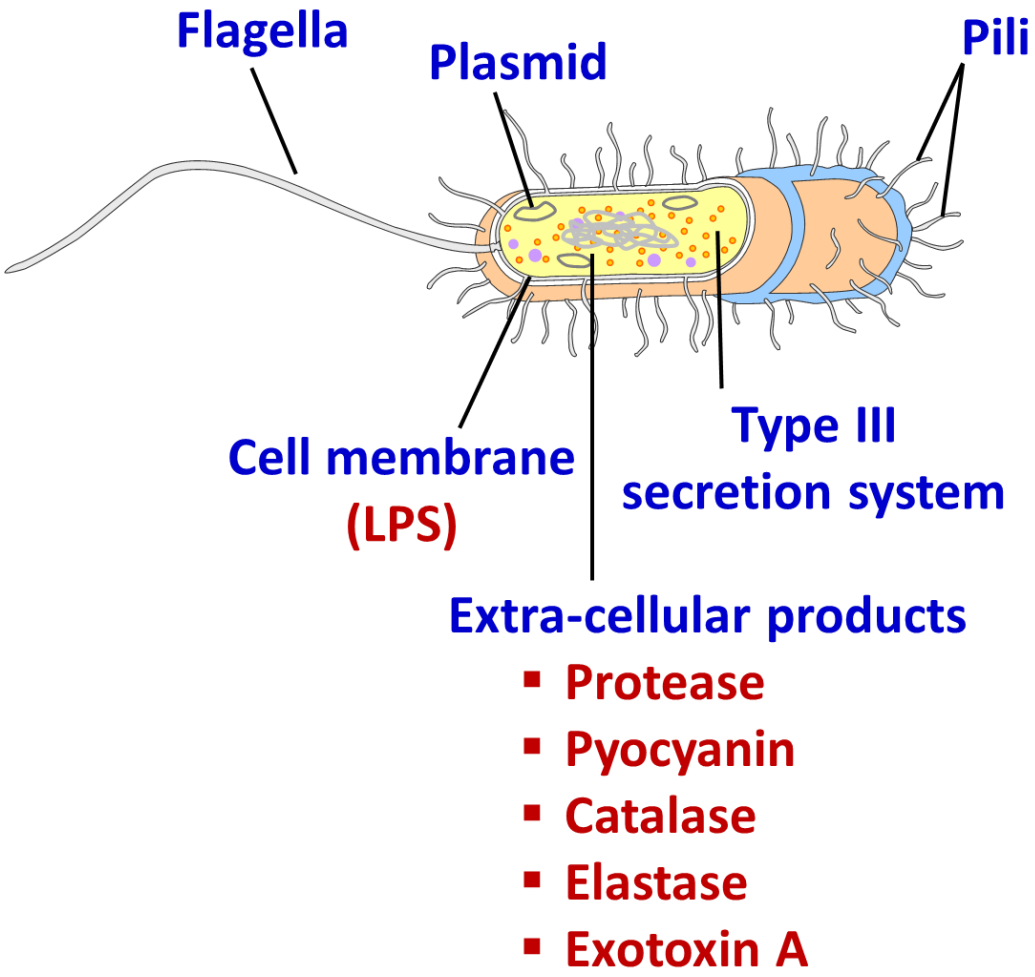


Figure 1.1: Virulence factors in PA.

This image illustrates the surface virulence factors such as flagellum, LPS, pilus, and secreted virulence factors that include products such as pyocyanin, catalase, proteases and exotoxin A, and T3SS apparatus that is considered a major determinant of PA cytotoxicity. Image adapted from (Sadikot et al., 2005b).

1.1.3.1 Flagella and pili

The single polar flagellum is required for PA motility, biofilm initiation and adhesion to host cells (Bucior et al., 2012). Despite the critical role that flagella play in acute infection, it has been demonstrated that some strains isolated from the CF lung do

not express this surface structure (Tart et al., 2005). The mechanism underlying this observation has been elucidated. During infection, flagellin, the structural protein of flagellum, is recognized by TLR-5, which is used by the immune system to detect PA and induce cytokine production (Rumbo et al., 2006, Zhang et al., 2005). In order to evade the host immune system, PA represses or downregulates flagellin synthesis (Wolfgang et al., 2004); this can occur when neutrophil elastase degrades the flagellar hook protein (F1gE). Reduced levels of F1gE induces accumulation of the anti-sigma factor (F1gM) within the bacterium inhibiting *fliC*, the gene encoding flagellin (Jyot et al., 2007).

Pili are composed of minor pilin units which assemble to form fine filamentous surface structures (Nguyen et al., 2015). Pili play an important role in twitching motility and facilitating biofilm formation. Pili also promote the initial colonization of epithelial cells by binding to asialoGM1 at the epithelial cell membrane (Alarcon et al., 2009, Comolli et al., 1999). This binding leads to activation of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mucin production (Du et al., 2006, Li et al., 2003). Piliated PA strains were shown to promote more severe pneumonia compared to non-piliated mutants in a mouse lung infection model (Tang et al., 1995).

1.1.3.2 Type III secretion system (T3SS)

T3SS is the mechanism by which PA injects toxins into the host cells (Bleves et al., 2010). This system is regulated by 43 genes that encode the secretion apparatus (Veesenmeyer et al., 2009). Four effector toxins or exoenzymes have been shown to be injected by T3SS: ExoU, ExoS, ExoT, and ExoY. T3SS is organised in a sequential manner to transport T3SS exoenzymes into host cells

(Hauser, 2009). Most clinical strains express ExoU and ExoS, which are the major toxins, while ExoT and ExoY, the minor toxins, are expressed by all strains (Gellatly and Hancock, 2013). ExoS and ExoT are bifunctional toxins that possess both ADP-ribosyltransferase (ADPRT) and Rho GTPase activating protein activity (Hauser, 2009). ExoY is a soluble adenylate cyclase (Ochoa et al., 2012). ExoU, the most cytotoxic of T3SS effectors, lyses the intoxicated mammalian cells through its phospholipase A2 (PLA2) activity (Sawa et al., 2016, Lee et al., 2007), whose activity leads to loss of plasma membrane and rapid cell death (Sato and Frank, 2014).

The effect of T3SS exoenzymes is as follows: ExoS alters cell function by inhibiting phagocytosis, endocytosis, and host cell DNA synthesis (Rangel et al., 2015, Frank, 1997). ExoT inhibits the process of epithelial cell wound repair and hinders host cell division (Wood et al., 2015). As mentioned above ExoY has adenylate cyclase activity. A recent study showed that ExoY increased the level of cAMP in endothelial cells leading to increased vascular permeability. In order to respond to infection, epithelial cells produce the neutrophil chemo-attractant hepoxilin A3 (HXA3) which initiates neutrophil migration. Recruited neutrophils produce leukotriene B4 (LTB4) to amplify the recruitment process. It has been demonstrated that cytosolic phospholipase A2α (cPLA2α) regulates the activity of both HXA3 and LTB4. ExoU displays the ability to augment cPLA2α levels leading to exacerbated neutrophil recruitment (Pazos et al., 2017).

Expression of T3SS is less predominant during chronic infection such as during infection of the CF lung (Sousa and Pereira, 2014). These effectors are expressed at some point during infection but eventually PA loses the ability to produce T3SS

over time in CF airways (Chen et al., 2016, Yahr and Wolfgang, 2006). Mutation within the *mucA* gene leads to overproduction of alginate during chronic infection; it has been shown that overproduction of alginate supresses T3SS activity during PA chronic infection (Wu et al., 2004). The mechanism is not clear yet, but it is thought that algiante overproduction might cause metabolic stress affecting T3SS function (Yahr and Wolfgang, 2006) .

1.1.3.3 Lectins

PA produces two types of lectins LecA and LecB that bind to galactose and fucose, respectively, in a calcium dependant manner (Blanchard et al., 2008, Cioci et al., 2003). LecA and LecB were initially identified in the cytoplasm of PA; however, large amounts have been detected on the outer membrane suggesting that they might be important for adhesion (Tielker et al., 2005, Glick and Garber, 1983). It has been shown that LecA decreases the growth of epithelial cells resulting in respiratory epithelial cell injury. Additionally, LecA has been found to promote the permeability of intestinal epithelium. This increases the absorption of exotoxin A, an essential virulance factor (Bajolet-Laudinat et al., 1994, Laughlin et al., 2000). Protease IV, can digest important proteins such as immunoglobulins, complement components, and fibrinogen. A *lecB* mutant has shown defective protease IV function, indicating that *lecB* might play a role in the regulation of protease IV virulence activity (Engel et al., 1998). Additionally, both lectins can help PA to inhibit ciliary beating. In addition, LecA and LecB facilitate bacterial aggregation and play a role in biofilm formation (Tielker et al., 2005, Diggle et al., 2006a). It has been shown that a *lecA* mutant was unable to form biofilm compared to WT (Diggle et al., 2006b).

1.1.3.4 Lipopolysacharide (LPS)

LPS assembles the outer layer of the outer membrane in Gram negative bacteria. It plays an important role in antigenicity, the inflammatory response, and in promoting interactions with antibiotics (King et al., 2009b). PA LPS domain consists of three regions :1) A membrane-anchored lipid A, 2) core region, 3) O-specific polysaccharide (O-antigen) (Kocincova and Lam, 2011). Numerous investigators have studied PA LPS due its importance to both the bacterium and host response. LPS structure and pathogenicity will be discussed in detail in chapter 5.

1.1.3.5 Secreted virulence factors

Exotoxin A (ETA) is an important cytotoxic virulence factor. *ToxA* is the structural gene of ETA (Ochsner et al., 1996) and *ToxA* transcription is regulated by *toxR* (Walker et al., 1994). ETA recognizes cells through binding to α2 macroglobulin receptor/low density lipoprotein receptor (CD91) (Kounnas et al., 1992). Structurally, ETA is a 66KD single polypeptide and consists of three functional domains:1) domain Ia which is responsible for cell recognition, 2) domain II plays a role in toxin transportation through cellular membranes, and 3) domain III has the ability to catalyse the ADP-ribosylation of elongate factor (EF-2) which leads to blocking biosynthesis of protein and ultimately cell death (Morlon-Guyot et al., 2009, Wolf and Elsässer-Beile, 2009).

Elastase (33 kD) is encoded by PA *lasB* gene (Casilag et al., 2016, Rust et al., 1996). Elastase hydrolyses extracellular matrix components and breaches

epithelial barriers by disrupting tight junctions. It can affect the immune system by degrading numerous components such as TNF-α, IFN-γ, and IL-8 (Kuang et al., 2011). It also has been demonstrated to affect host cells by cleaving immunoglobulins, surfactant protein A and D, and complement, consistent with its contribution to PA pathogenesis (Mariencheck et al., 2003a).

Phospholipase C (PLC) is a soluble protein which has a catalytic activity. It is mainly found in the cytosol and can be translocated to the plasma membrane (Fukami, 2002). PLC can hydrolyse phospholipids such phosphatidylcholine and sphingomyelin resulting in tissue damage (Terada et al., 1999). PLC can also promote bacterial invasion and cause fatal injuries to host cells (Sadikot et al., 2005b). It also contributes to suppression of the neutrophil respiratory burst activity which promotes PA persistent chronic infection (Terada et al., 1999).

Rhamnolipids are mixtures of glycolipids and rhamnose moieties linked to 3-hydroxyalkanoyloxy alkanoic fatty acids (HAA). Rhamnolipids have an essential role during chronic PA infection. They have the ability to dissolve various insoluble substances, reduce surface tension, and increase swarming activity (Deziel et al., 2003). Dynein is responsible for cilia movement (Lodish et al., 2000). Rhamnolipids have been shown to remove dynein resulting in impaired ciliary function. (Bloodgood, 2013).

1.2 Quorum sensing (QS)

QS is a cell density-dependent gene regulation system that coordinates gene expression, virulence production, and biofilm formation (Rutherford and Bassler,

2012). PA expresses three main QS systems: las, rhl and pqs. The las system consists of the transcriptional regulator (lasr) and the synthase protein (lasI). The rhl system consists of rhlI and rhlr proteins (Smith and Iglewski, 2003, Rampioni et al., 2016). For las and rhl, QS PA uses N-acylhomoserine lactone (AHL) molecules or autoinducers to establish signalling networks and coordinate the production of virulence factors (Davis et al., 2010, Zhu et al., 2002). Two AHLs have been identified in PA, [N-(3-oxododecanoyl)-l-homoserine lactone (3O-C12-HSL)], produced by the las system, and [N-butyryl-L-homoserine lactone (C4-HSL)], produced by the rhl system (Nakagami et al., 2011). It has been shown that these AHL molecules cause apoptosis of neutrophils and macrophages (Tateda et al., 2003). The las system regulates secretion of some virulence factors such as elastase, alkaline protease, exotoxin A, and biofilm formation (Rasamiravaka et al., 2015). The rhl system activates the production of rhamnolipids, pyocyanin, and the cytotoxic lectins LecA and LecB (Grishin et al., 2015, Aendekerk et al., 2005). It has been shown that the las system plays a crucial role in biofilm development and maturation (Passos da Silva et al., 2017). A *lasI* mutant formed flat undifferentiated biofilms compared to controls. Also, in the same experiment, when both biofilms were treated with a detergent, the *lasI* mutant biofilm was dispersed and diminished, while the WT control biofilm did not show detectable effect (Heydorn et al., 2002). The rhl system controls the expression of several PA products including the carbohydrate Pel, which is involved in the generation of EPS which, as mentioned above, comprises the biofilm matrix. *lasI* and *rhlI* mutants exhibited reduction in the transcription of the *pel* operon (Sakuragi and Kolter, 2007). PA produces extracellular DNA (eDNA) which is important component of the PA biofilm. It has been shown that eDNA generation is dependent on the QS system suggesting that QS mutants might affect biofilm formation compared to WT (Allesen-Holm et al., 2006). Meta-bromo-thiolactone (mBTL) has been reported to inhibit lasr and rhlr. O'Loughlin and colleagues have demonstrated that mBTL

partially inhibited both lasr and lhlr, which in turn inhibited biofilm formation (O'Loughlin et al., 2013). All these studies might indicate that there is a link between biofilm and the QS system.

2-alkyl-4 quinolones (AQs) are involved in the *pqs* QS system as signalling molecules (Rampioni et al., 2016). Genes coding for pyocyanin, exoenzymes, hydrogen cyanide, lectins, rhamnolipids, pyochelin and pyoverdine as well as biofilm development require AQs (Heeb et al., 2011). PA produces more than fifty AQs (Lahiri and Ghosh, 2017), including 2-heptyl-3-hydroxy-4-quinolone (PQS), 2-heptyl-4-quinolone (HHQ), 2-nonyl-4-quinolone (NHQ) and 2-heptyl-4-quinolone N-oxide (HHQNO) (Tarighi et al., 2008). A five-gene,operon the *pqsABCDE* operon, is responsible for AQs synthesis in PA (Dubern and Diggle, 2008). PQS binds to PqsR, which leads to *pqsABCDE* expression (Brouwer et al., 2014). The *pqsABCD* genes are involved HHQ synthesis, the PQS precursor (Ha et al., 2011). *PqsH* encodes a flavin adenine dinucleotide (FAD)-dependent monooxygenase, which aids conversion of HHQ into PQS (Wells et al., 2017). *PqsE* function is not fully understood, but it has been reported that this gene acts independently of PQS and *PqsR* (Farrow et al., 2008). Rampioni *et al* showed that a *pqsE* mutation has no effect on the synthesis of AQ; however, the overexpression of *PqsE* abrogates PqsA activity, which consequently inhibits AQ synthesis (Rampioni et al., 2010).

Many studies have shown the important role of PQS in biofilm formation. Treatment of PAO1 cultures with PQS enhanced the attachment of bacterial cells to the substrate (Diggle et al., 2003). Possible mechanisms for this observation could be the induction of LecA, the galactophilic lectin, by PQS (Diggle et al., 2006b) or PQS-mediated eDNAs release (Allesen-Holm et al., 2006). D'Argenio *et*

al has demonstrated that PQS can lyse a population of cells leading to eDNAs release (D'Argenio et al., 2002). A *pqsA* mutant formed biofilm that contains low eDNAs compared to WT (Chiang et al., 2013). It also has been shown that PA strains that exhibit high autolysis activity have increased PQS levels, which correlates with enhanced biofilm formation (Häussler and Becker, 2008). AQs have been detected in sputum and urine of CF patients (Rampioni et al., 2016). PA strains isolated from two year old CF patients have shown increased AQs levels compared to a WT PAO1 lab strain (Dubern and Diggle, 2008). Mutations in *pqsA* and *pqsC* affected AQ synthesis, resulting in poor biofilm formation (Tettmann et al., 2016). It also has been demonstrated that mutations in *pqsB*, *pqsC* and/or *pqsD* completely obliterate AQ production (Rampioni et al., 2016). All these studies support the crucial role of AQ in biofilm formation.

1.3 PA biofilms

Biofilms are highly organized communities of bacteria (Ma et al., 2009a). Biofilms are widely distributed; microbial biofilms have been reported at high temperature (about 50^{o}C) (Jones et al., 2010) and have been found in a glacier samples (Yang et al., 2009). Biofilms also can present in drinking water pipes making them potential biohazards (Hallam et al., 2001). Medical devices such as contact lenses, mechanical heart valves, and urinary catheters have been reported as biofilm substrates (Wu et al., 2015). All ESAKPE pathogens exhibit the ability to form biofilms to endure harsh environmental conditions (Bales et al., 2013). These communities are enclosed in an EPS which is composed of polysaccharides, nucleic acids, and proteins (Das et al., 2013). EPS maintains the integrity of biofilms protecting bacterial cells from harsh environmental conditions (Stoodley et al., 2002b). Once biofilms are established, they become resistant to antimicrobial

agents and protected from the immune system. This facilitates chronic infections making the eradication of infection extremely difficult (Lewis, 2007, Mah et al., 2003). The persistent lung chronic infection in CF is attributed to the presence of biofilms (Hauser et al., 2011b).

The PA biofilms development process is considered to consist of five-stages (Figure 1.2): (1) reversible cell attachment to the substrate,(2) irreversible cells attachment and cell proliferation, (3) loss of flagellar motility and EPS production, (4) biofilm maturation, and (5) detachment and dispersion of planktonic cells to either colonise a new surface or continue planktonic life style (Stoodley et al., 2002b). Chua *et al* demonstrated that dispersed cells display high expression levels of genes involved in motility and T3SS making them more virulent (Chua et al., 2014). In the same experiment, Chua and colleges showed that dispersed cells exhibit low levels of c-di-GMP, allowing more motility and fast growth. Based on the dependency on polysaccharide synthesis locus (Psl) (1.3.3.2) and/or Pel (1.3.3.3) for biofilm formation, Colvin *et al*. proposed four classes of PA strains. Class 1: strains that rely on Pel, such as PA14. Class 2: strains that have a Psl-dominant matrix, such as PAO1. Class 3: strains that can form a biofilm using either Pel or Psl and only have impaired ability to form biofilm if both pathways are mutated (EPS-redundant matrix). Class 4: matrix overproducers, which are those strains that overproduce Pel and Psl to form a significant amount of biofilm; in case of *ps*l deletion, *pel* could compensate for the loss and vice versa. This reduces the impact of deletion on biofilm formation (Colvin et al., 2012b). Understanding biofilm structure and major players in biofilm formation might lead to the development of novel therapeutic approaches to eradicate biofilms.

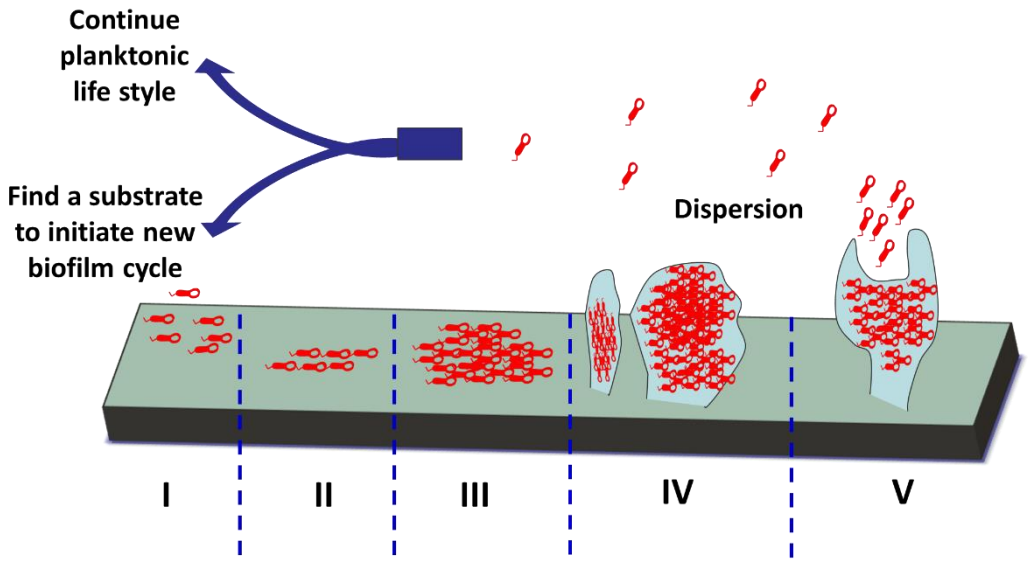


Figure 1.2: Establishment of PA biofilms.

The sequential process of biofilm formation is initiated by reversible cell attachment to the substrate (stage 1). Irreversible cell attachment and cell proliferation (stage 2), followed by stage 3 in which there is loss of flagella and cell motility and EPS is produced. The next stage is biofilm maturation, stage 4, followed by stage 5 when single cells disperse from the biofilm. Diagram adapted from (Stoodley et al., 2002b).

1.3.1 Role of proteins in biofilm

Biofilm proteins play a crucial role in facilitating surface adherence, matrix molecule interaction, and matrix stabilization (Mann and Wozniak, 2012). The proteins implicated in PA biofilms include LecA, LecB, and CdrA (Fong and Yildiz, 2015).The two-partner secretion (TPS) system (TPS) facilitates the translocation of large proteins across the outer membrane (Guérin et al., 2017). The cyclic diguanylate-regulated TPS partner A (CdrA) locus includes the TPS system with high adhesion and transportation activities. Western analysis shows that *CdrA* encodes a 150 kDa secreted protein (Borlee et al., 2010b). CdrA displays a rod-shape with a β-helical structure (Mann and Wozniak, 2012) and contains several

adhesion domains including a carbohydrate-dependent hemagglutination activity domain, a glycine-rich sugar-binding domain (Borlee et al., 2010b). PA CdrA has the ability to bind carbohydrates, promoting the interaction between matrix molecules (Vozza et al., 2016). CdrA was named because of its expression in response to high c-di-GMP (Cooley et al., 2016). It has been demonstrated that CdrA interacts with PA Psl, making it a key factor in biofilm formation (Kovach et al., 2017). In liquid cultures, CdrA overproduction increases cell auto-aggregation (Chua et al., 2015). A *CdrA* mutant showed a decreased biofilm biomass and structural integrity, suggesting that CdrA-Psl interaction could facilitates the cross-talk between Psl and biofilm components. (Borlee et al., 2010b).

1.3.2 Role of eDNA in biofilm

Many studies have demonstrated the importance of eDNA as a structural component of biofilms. For instance, a DNase enzyme isolated from *Bacillus licheniformis,* has been shown to act as an anti-biofilm agent in this marine bacteria (Nijland et al., 2010). It also has been reported that a DNase enzyme induced biofilm dispersal by several PA isolates from chronic rhinosinusitis patients (Shields et al., 2013). Biofilms that contain large amount of eDNA were not dispersed in response to DNase; however, the treatment weakened the biofilm structure (Grande et al., 2011, Lappann et al., 2010). Treatment of biofilms with DNase increased antimicrobial sensitivity (Martins et al., 2012, Tetz and Tetz, 2010). On the other hand, adding exogenous DNA to PA biofilms increased their resistance to antibiotics such as gentamicin and other aminoglycoside antibiotics (Lewenza, 2013, Mulcahy et al., 2008a). These studies support the critical role of eDNA in stabilizing bacterial biofilms. A recent study demonstrated that the PQS QS system modulates release of eDNA during biofilm formation (Allesen-Holm et

al., 2006). The origin of eDNA in the matrix can be genomic DNA released through a cell autolysis mechanism, QS system, or outer membrane vesicles (OMVs) (Wilton et al., 2016). Confocal microscopy showed that Pel cross-links eDNA in the biofilm through ionic interactions (Jennings et al., 2015). Gloag *el al* has shown that eDNA maintains efficient flow of bacterial cells facilitating cells migration and ensuring sufficient supply of cells (Gloag et al., 2013).

1.3.3 Role of carbohydrates in biofilm formation

EPS maintains biofilm integrity and provides protection against antibiotics and the host defence. EPS acts as a structural platform that facilitates cell-cell interaction and cell-surface attachment (Wingender et al., 1999). In addition to proteins and eDNA, three main polysaccharides produced by PA have been identified: alginate, Psl, and Pel (Ryder et al., 2007b).

1.3.3.1 Alginate

Alginate is a linear polymer of guluronic acid and mannuronic acid (Zhao et al., 2016). During chronic infection PA undergoes a phenotypic change due to the *mucA* mutation, which leads to overproduction of alginate, protecting PA from host immune cell attack and antimicrobial agents (Song et al., 2003). Mathee *et al* demonstrated that treating non-mucoid PA with low level of the oxygen radical hydrogen peroxide (H_2O_2), which is produced by polymorphonuclear leukocytes (PMNs), overproduces alginate. This suggests that the phenotypic conversion in response to H_2O_2 is one of the mechanisms that PA utilizes to evade immune response (Mathee et al., 1999). PA alginate also has been shown to reduce the

PMN chemotaxis and inhibit complement activation (Pedersen et al., 1990). Mucoid PA is characterized by alginate overproduction. Some mucoid PA strains rely on alginate to form biofilms and establish chronic lung infection (Hentzer et al., 2001b). However, a recent study showed that 50% of isolated mucoid bacteria rely on Psl to form biofilms (Jones and Wozniak, 2017). It has been demonstrated that overproduction of alginate helps PA to resist opsonic and non-opsonic phagocytosis (Rowe III, 2013). Patients with mucoid isolates have demonstrated increased humoral response and lower lung function (Martha et al., 2010). Alginate has been shown to inhibit the activity of some antimicrobial agents such as tobramycin (Nichols et al., 1988). The early stages of CF lung infection start with non-mucoid isolates which then become mucoid at later stages (Sousa and Pereira, 2014).

1.3.3.2 Psl

Psl is composed of mannose, glucose, and rhamnose in a 3:1:1 ratio (Ma et al., 2009b, Jones and Wozniak, 2017). The Psl locus consists of 15 genes that encode proteins involved in polysaccharide synthesis (Ma et al., 2006a) (Figure 1.4). Inactivation of the *pslA* and *pslB* genes in the laboratory PAO1 strain alters cell-cell and cell-surface interactions, which suggests that Psl is an important factor in maintaining biofilm integrity and cell aggregation (Ma et al., 2006b). PAO1 Psl appears to be helically distributed around the cell surface when labelled with lectins specific to mannose (Figure 1.3) (Ma et al., 2009a). LecB binds to Psl which stabilizes biofilm structure (Fong and Yildiz, 2015). It has been demonstrated that arabinose-induced Psl expressing bacteria deposit a trail of Psl. This facilitates the movement of the subsequent cells generating a positive feedback mechanism for cell-cell encounter and biofilm formation (Zhao et al., 2013). DiGiandomenico *et al* showed that anti-Psl antibodies reduced PAO1 attachment to A549 human

pulmonary epithelial cells. Also in the same report, using a mouse acute lethal pneumonia model, DiGiandomenico and colleges demonstrated that Psl antibodies deliver powerful protection against PAO1 (DiGiandomenico et al., 2012).

Many factors have been involved in *psl* regulation and biofilm overproduction. Recently, the universal bacterial second messenger cyclic (c)-di-GMP has been involved in many functions such as cell cycle regulation, biofilm formation, motility, and virulence factor excretion. C-di-GMP also positively regulates production of Pel and Psl polysaccharides (Hickman et al., 2005a). Moreover, it has also been shown that Psl elevates c-di-GMP levels, which leads to the generation of a positive feedback loop that results in continuous stimulation of biofilm formation (Irie et al., 2012a). In contrast, low levels of c-di-GMP promote a planktonic lifestyle (Borlee et al., 2010a). It also has been shown that biofilms with high levels of c-di-GMP gain greater resistance to antibiotics (Byrd et al., 2011) due to the ability of c-di-GMP to control efflux pump genes (Gupta et al., 2014). PA *wspF* gene is a member of the *wsp* operon, which consists of eight genes, *wspA-R*. The *wspF* gene encodes a methyl-esterase, which acts on the *wspR*. When wspR is activated, it produces c-di-GMP (Huangyutitham, 2013). The mutation of the *wspF* gene results in *wspR* activation which leads to elevation of c-di-GMP level. It has been shown that mutation of *wspF* upregulates *psl* and *pel* genes (Chung et al., 2008).

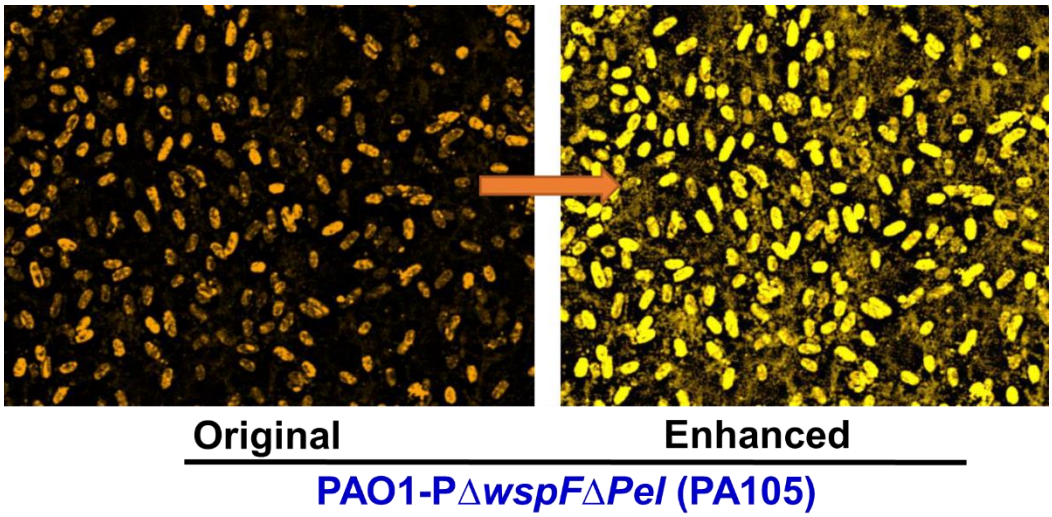


Figure 1.3: Super resolution images of extracellular matrix formation showing Psl anchored to PA surface.

Biofilm of PAO1-PΔ*wspF*Δ*Pel* (PA105) was generated on glass cover slips for 72 hrs. Psl was detected using 20µg/ml of hippeastrum hybrid Amaryllis (HHA) lectin. Images were generated by Tamanna Rahman, a former PhD student, in Dr Martinez-Pomares's lab.

Psl has been found to promote NF-κB activation in A549 cells (Byrd et al., 2010). Moreover, it has been shown that Psl could reduce neutrophil reactive oxygen species (ROS) production and phagocytosis. This is accomplished by reducing complement-mediated opsonisation (Mishra et al., 2012a). In addition, Psl promotes antibiotic resistance; activation of Psl by c-di-GMP leads to elevated antimicrobial tolerance in PA (Song et al., 2018). An in vitro study demonstrated that Psl monoclonal antibody improved PA killing by phagocytes and limited the attachment to lung cells (Ray et al., 2017, DiGiandomenico et al., 2012). Therefore, targeting Psl with a specific antibody might be a novel approach that provides protection against PA infection.

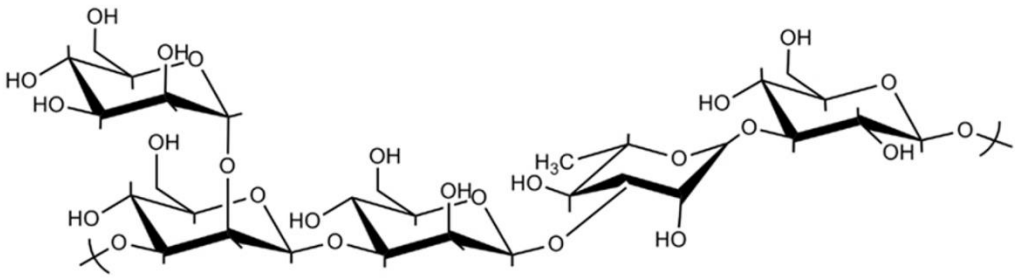


Figure 1.4: Structure of Psl polysaccharide.

Psl polysaccharide is composed of repeating pentameric units of D-mannose, L-rhamnose, and D-glucose residues. (Franklin et al., 2011).

1.3.3.3 Pel

Pel is the polysaccharide that the highly virulent laboratory strain PA14 relies on to produce biofilms (Friedman and Kolter, 2004b, Friedman and Kolter, 2004a). The *pel* locus consists of seven genes and very little is known of their contribution to Pel production. It has been suggested that the Pel biosynthesis machinery utilises enzymes involved in the synthesis pathways of other carbohydrates to provide the essential sugar precursors of Pel (Franklin et al., 2011). Recent data showed that Pel is positively charged and composed of acetylated galactosamine and glucosamine sugars (Jennings et al., 2015). Pel binds eDNA in the biofilm through ionic interactions (Jennings et al., 2015). Previous studies suggest that Psl is the primary polysaccharide for biofilm matrix formation in PAO1, but when *psl* is deleted, *pel* is upregulated to compensate for the absence of Psl by cross linking eDNA (Jennings et al., 2015). Pel maintains cell-to-cell interaction in PA14 biofilm and increases resistance to some antibiotics such as aminoglycoside (Colvin et al., 2011b). Overproduction of Pel in PAO1 and PA14 increased tolerance to both gentamicin and tobramycin compared to parental strains, suggesting that Pel could enhance resistance to antibiotics (Colvin et al., 2011b). Therefore, it was

suggested that Pel is more important for providing biofilm stability and could play an important role in protecting the biofilm and maintaining its structural integrity.

1.4 Role of innate immune cells during PA infection

1.4.1 Airway epithelial cells (AECs)

AECs cover the airways and are considered as the first defence mechanism during the interaction between respiratory pathogens and the host. These cells perform several functions such as formation of a mechanical barrier, regulation of ion transport, secretion of mucus, expression of receptors involved in recognition of pathogen associated molecular patterns (PAMPs), as well as secretion of cytokines and chemokines, which play a crucial role in immune cell activation and recruitment (Bals et al., 1999, Koller et al., 2009). Based on their functional and biochemical features, epithelial cells are classified into three main groups:1) basal, 2) ciliated, and 3) secretory (goblet cells) (Ioannidis et al., 2012). The most predominant type is ciliated, which comprise more that 50% of AECs (Yaghi and Dolovich, 2016). Each ciliated cell displays approx. 300 cilia enabling movement of mucus from the lung to the throat (Tilley et al., 2015). Secretory cells are involved in mucin production (Adler et al., 2013). Mucin is a heavily glycosylated molecule that aids innate immune response by trapping foreign particles (Brockhausen et al., 2009). The airway dehydration due to chloride depletion and sodium increase caused by CFTR dysfunction is a hallmark of CF disease (Schmidt et al., 2016). Due to the dehydration conditions, the glycosylation of mucin is impaired preventing epithelial cells from trapping foreign particles, which leads to improper ciliary clearance (Ehre et al., 2014).

Epithelial cells produce antibacterial agents such as the collectins SP-A and SP-D that bind and opsonize microbial pathogens (Lavoie et al., 2011). In addition to that, epithelial cells contribute to lung defence by secreting complement proteins which are implicated in promoting phagocytosis (Gellatly and Hancock, 2013), Also it has been demonstrated that epithelial cells produce cytokines such as IL-1β, IL-6, TNFα, -IL-2, and MCP-1 upon TLR activation, which lead to recruitment and activation of other immune cells (Williams et al., 2010). Epithelial cells also play a role in immunity against pathogens by secreting some anti-microbial agents such as defensins, lysozyme, and lactoferrin (Leiva-Juárez et al., 2018).

CFTR mutations contribute to chronic infection (Hartl et al., 2012a). CFTR mutations may lead to: 1) depletion of ASL due to the imbalance between sodium and chloride (Zhou et al., 2011) , 2) accumulation of mucus which diminishes ciliary function (Boucher, 2004), 3) persistent neutrophil migration to the lung which increases the neutrophil elastase levels leading to aggressive lung damage (Hartl et al., 2012a).

A study used a mouse model that overexpress (βENaC) to mimic the high absorption of sodium in CF. In this study, depletion of ASL was observed due to the imbalance between sodium and chloride which indicates that ASL impairment is at least in part due to defects in epithelial ion transport (Zhou et al., 2011). CFTR influences the response of AECs. This role has been supported by a study that exposed CFTR–deficient AECs to PA and showed an increase in the neutrophil chemoattractant, IL-8. This might explain the hyper-inflammatory state in CF (Ratner and Mueller, 2012).

1.4.2 Macrophages

Macrophages are important phagocytic cells. They play a crucial role in host defence and tissue repair through their large capacity to engulf microbes and foreign particles. Hence, they contribute substantially to homeostasis and protection against infectious diseases (Gordon and Martinez-Pomares, 2017, Chazaud, 2014, Wynn et al., 2013, Aderem, 2003). Tissue resident macrophages (TRMs) are distributed throughout the body. There are different subsets of TRMs according to the organ in which they reside. For instance, alveolar macrophages (AMs) are located in the alveolar spaces of the lung, microglia are the macrophages in brain, Kupffer cells are the resident macrophages in liver, renal macrophages are located in kidneys, osteoclasts are the macrophages in bone, and dermal macrophages are located in the skin (Xu and Shinohara, 2017). Upon demand, circulating monocytes can be recruited to the injury/infection sites, and subsequently differentiate into macrophages (Smith et al., 2018). The contribution of circulating monocytes to resident macrophage populations is tissue-dependent. AMs originate from foetal embryonic monocytes. The differentiation of AMs requires GM-CSF (Gordon and Martinez-Pomares, 2017, McGovern et al., 2014). The numbers of AMs are maintained through recruitment (Aberdein et al., 2013). This contrasts to dermal macrophages which have short a half-life and are replenished through recruitment of monocytes (McGovern et al., 2014).

Two main macrophages activation pathways have been identified, classical and alternative. In the classical pathway, macrophages are activated with IFN-γ, the canonical Th1 cytokine, and/or PAMPs, while in the alternative pathway, macrophages are activated with IL-4, IL-10, or IL-13 (Gordon and Martinez, 2010). AMs are considered the first line defence against antigens in the lung. Therefore, they are key players during pulmonary immune responses (Seto et al., 2014).

Many studies have demonstrated the roles of macrophages in controlling PA infection through phagocytosis, ROS production, cytokine secretion, and iron sequestration (Ganz, 2012, Hespanhol and Mantovani, 2002, Forman and Torres, 2002). It has been shown that murine AMs were able to secrete chemokine (C-X-C motif) CXCL1 and tumour necrosis factor (TNF-α) in response to PA LPS and flagellin, respectively (Lavoie et al., 2011b). AMs produce IL-1β which induces epithelial cells to secrete neutrophil chemokines (Marriott et al., 2012). A previous mouse PA lung infection model showed that reduction of AMs was accompanied with a decreased level of macrophage Inflammatory protein-2 (MIP-2) and CXCL-1 affecting the neutrophils recruitment, which in turn impairs the infection clearance (Tumpey et al., 2002). In agreement with this observation, Singh *et al* demonstrated that macrophages (regardless of their activation) could not control PA growth (Singh et al., 2015). All these studies suggest that macrophages might not be able to control PA infection per se, however, they remain a substantial enhancer of neutrophil recruitment facilitating infection clearance.

There is growing evidence that CF macrophages have impaired ability to clear pathogens (Ratner and Mueller, 2012). Some studies have shown that CF macrophages lack killing ability which might be due to disrupted phagosome acidification caused by defective CFTR (Deriy et al., 2009, Di et al., 2006).

It has been demonstrated that CFTR inhibitors lead to elevated calcium level in macrophages which might inhibit macrophage activity (Shenoy et al., 2011). Also since CFTR is expressed in T cells (see below), it might be considered that defective CFTR in T cells could affect their ability to support macrophage activation resulting in persistent chronic infection (Ratner and Mueller, 2012). As

mentioned above macrophages become alternatively activated when exposed to Th2 cytokines IL- 4, and IL-13 (Gordon, 2003). It has been reported that CF patients show a high percentage of M2 and monocytes in an endotoxin tolerance state (Murphy et al., 2010b, del Fresno et al., 2009). This observation suggests that macrophages may play a role in CF lung destruction due to poor antigen presentation and impaired phagocytic activity.

1.4.3 Neutrophils

Neutrophils or polymorphonuclear cells possess potent phagocytic activity (Lee et al., 2003b). They constitute about 60 % of whole white blood cells in humans (Mestas and Hughes, 2004). These cells originate from myeloid precursor cells in the bone marrow, where they also fully develop. G-CSF stimulates neutrophil proliferation and differentiation in the bone marrow (Furze and Rankin, 2008). In order to respond to infection, epithelial cells and macrophages promote neutrophil recruitment, differentiation and activation by releasing cytokines such as G-CSF, GM-CSF, IL-8, and TNF-α. Th17 cells, which are a subset of T helper cells, play an important role in immune response against extracellular pathogens by facilitating neutrophil and macrophage recruitment (Mantovani et al., 2011). A hallmark of Th17 is production of cytokines such as IL17A and IL17F. It has been demonstrated that IL17A is involved in neutrophil recruitment. (Guglani and Khader, 2010). Additionally, neutrophils can further promote neutrophilic responses by releasing IL-17A. (Garraud et al., 2012).

Neutrophils have the ability to internalise microbes into phagosomes and destroy them (Winterbourn and Kettle, 2013). This is accomplished by producing many killing agents such as ROS, cathepsin G, elastase, lysozyme, and gelatinase

(Guentsch et al., 2009). The neutrophils killing capacity is higher than that of macrophages. They are equipped with a variety of microbicidal mechanisms. Macrophages lack or have lower levels of several important antimicrobial agents that neutrophils possess such has defensins, cathelicidins, and lactoferrin (Silva and Correia-Neves, 2012). Myeloperoxidase (MPO), a major component of neutrophil granules with effective killing activity, has been found in circulating monocytes but is not present in mature macrophages (Maródi et al., 1998). One of neutrophil killing mechanisms involves the release of neutrophil extracellular traps (NETs). In 1996, Takei *et al* has discovered NET as a cell death pathway (Takei et al., 1996). NET is a meshwork of DNA structures that consist of chromatin bound to antimicrobial peptides with bactericidal activity including elastase, histones, lactoferrin, MPO, cathepsin G, gelatinase, and proteinase 3 (Delgado-Rizo et al., 2017, Brinkmann and Zychlinsky, 2007). However, their sustained presence may lead to tissue damage. Many studies have demonstrated the role of neutrophils in controlling PA infection. Koh *et al* showed that neutrophil-deficient mice were more susceptible to PA infection compared to WT. In a mouse PA infection model, Hirche *et al* demonstrated that neutrophil elastase is required for PA killing. CF patients show persistent bacterial infection and massive neutrophil infiltration due to excessive release of IL-17A, which leads to pulmonary injury. Singh *et al* found that IL17 A production by circulating immune cells negatively correlates with lung function, i.e. lung function is better when Il-17A is less (Singh et al., 2015).

CF patients show persistent bacterial infection and massive neutrophil infiltration. Painter *et al* have been successful in localizing CFTR expression in neutrophils (Painter et al., 2006), which indicate that CF pathology can not only be attributed to CFTR deficiency in AEC but also to CFTR neutrophils. In order to investigate the contribution of neutrophils to lung damage in CF, CFTR $^{-/-}$ neutrophils were

transferred to healthy mice and this led to severe lung injury and inflammation (Su et al., 2011). This raises the question whether a defective CFTR could disrupt neutrophil function. Moreover, a study has shown that CF neutrophils displayed a reduced phagocytic activity, and the authors proposed that this defect is due to expression of mutant CFTR during neutrophil maturation (Ratner and Mueller, 2012).

Other groups have also studied the effect of hypochlorous acid (HOCL), a toxic agent generated by neutrophils whose formation requires chloride ion (Nauseef, 2007). They demonstrated that CFTR inhibitors reduced the transportation of chloride into the phagosomes in neutrophils resulting in impaired bacterial killing (Painter et al., 2010, Painter et al., 2008). In addition to that, increased degranulation of MPO (Koller et al., 1995), chemotaxis (IL-8) (Porro et al., 2001), and apoptosis (Watt et al., 2005) have been reported in CF. All these findings appear to support the role of CFTR in regulating neutrophil function. Certainly, further studies should be conducted to elucidate the particular contribution of CFTR expressed by neutrophils in modulating the immune response in CF disease.

1.4.4 Lymphocytes

Lymphocyte consists of three major subtypes: natural killer cells (NK), T cell, and B cell (Delves et al., 2006). NK cells are considered to be innate immune cells and are involved in killing virus-infected cells and controlling tumours (Vivier et al., 2011). B and T cells are essential components of the adaptive immune response. B cells mediate humoral immunity through the production of antibodies while T cells are responsible for cell-mediated immune responses. Several classes of T cells have been identified: T helper ($CD4^+$) and cytotoxic T cells (CD8+). T helper

cells are subdivided into Th1, Th2, Th17, and T regulatory cells (Zheng, 2013, Delves et al., 2006). Recently, the alterations of adaptive immune response in CF have begun to receive more attention. Allergic reactions, dermatitis, and hyper-inflammatory immune response have been observed in CF patients which may indicate that the adaptive immune response might play a role in disease (Ratner and Mueller, 2012). For example, elevated IL-4, IL-13, and IgE due to exposure to *A. fumigatus* have been reported in some CF patients and CF mice (Knutsen et al., 2004, Muller et al., 2006, Laufer et al., 1984). It has also been demonstrated that Th1 response correlates with better lung function in CF patients compared to Th2 response in chronic CF patients infected with PA (Moser et al., 2002). These findings support the notion that lymphocytes show a Th2 skewing in CF. Also it has been shown that CF lungs show high numbers of Th2 cells and high level of IL-4 and IL-13 which further support Th2 bias in CF (Hartl et al., 2006). Moss *et al* has reported that CF T cells produce lower level of IFN-γ and higher levels of IL-10 (Moss et al., 2000). Therefore, this dysregulation in Th1/Th2 might impair the immune response to CF associated pathogens. Recently, there is a growing body of evidence indicating that Th17 cells that produce IL-17 cytokines may contribute to CF pathology by promoting neutrophil recruitment to the lung (Tan et al., 2011, Dubin and Kolls, 2011). The IL-17 cells are activated by IL-23, IL-6, and transforming growth factor beta (TGF-β) (Veldhoen et al., 2006). Some human studies have shown that IL-17 and IL-23 were significantly elevated in the sputum of CF patients with PA infection (McAllister et al., 2005, Decraene et al., 2010). Also it has been demonstrated that CF T cells have an intrinsic ability to differentiate into Th17 cells (Kushwah et al., 2013).

1.5 Role of Pattern recognition receptors (PRR) in PA recognition

Pathogen-associated molecular patterns (PAMPs) are conserved structures associated with microbes (Mogensen, 2009). These molecules are invariant among pathogens and are essential for pathogen survival (Janssens and Beyaert, 2003). The immune system depends on PRR to recognize PAMPs. The interaction between PAMPs and PRRs triggers signalling pathways to orchestrate the early immune response to infection (Takeuchi and Akira, 2010) and instruct acquired immunity (Palm and Medzhitov, 2009). PRRs have been shown to instruct both B and T cells to induce different immune responses (Michallet et al., 2013). PRRs can be found either as membrane-anchored or soluble proteins (Parker and Prince, 2011). PRRs are expressed by many immune cells such as macrophages, fibroblasts, dendritic cells, monocytes, and neutrophils (Newton and Dixit, 2012). PRRs have been classified into four main groups:1) Toll-like receptors (TLRs), 2) nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), 3) retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and 4) C-type lectin receptors (CLRs) (Takeuchi and Akira, 2010).

1.5.1 TLRs

There are 10 human TLRs (TLR-1 to TLR10). TLRs are either localized at the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) or in endosomes (TLR3, TLR7, TLR8, and TLR9) (Botos et al., 2011). Each TLR contains an ectodomain consisting of leucine-rich repeats (LRRs), which mediate PAMP recognition in addition to a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain, which initiates intracellular signalling (Kawasaki and Kawai, 2014). TLRs recognize a variety of PAMPs such as lipids, lipoproteins, nucleic acids and proteins (Kawai and Akira, 2009). To elicit appropriate immune responses via

activation of NF-κB, interferon regulatory transcription factor (IRFs), or mitogen-activated protein kinases (MAPK) pathways (Takeda and Akira, 2005), signalling through TLRs requires adaptors such as myeloid differentiation 88 (MyD88), TIR domain-containing adaptor protein-inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), and TIR-associated protein (TIRAP), which is also known as MyD88 adaptor-like protein (Mal) (Piao et al., 2013) (Figure 1.5).

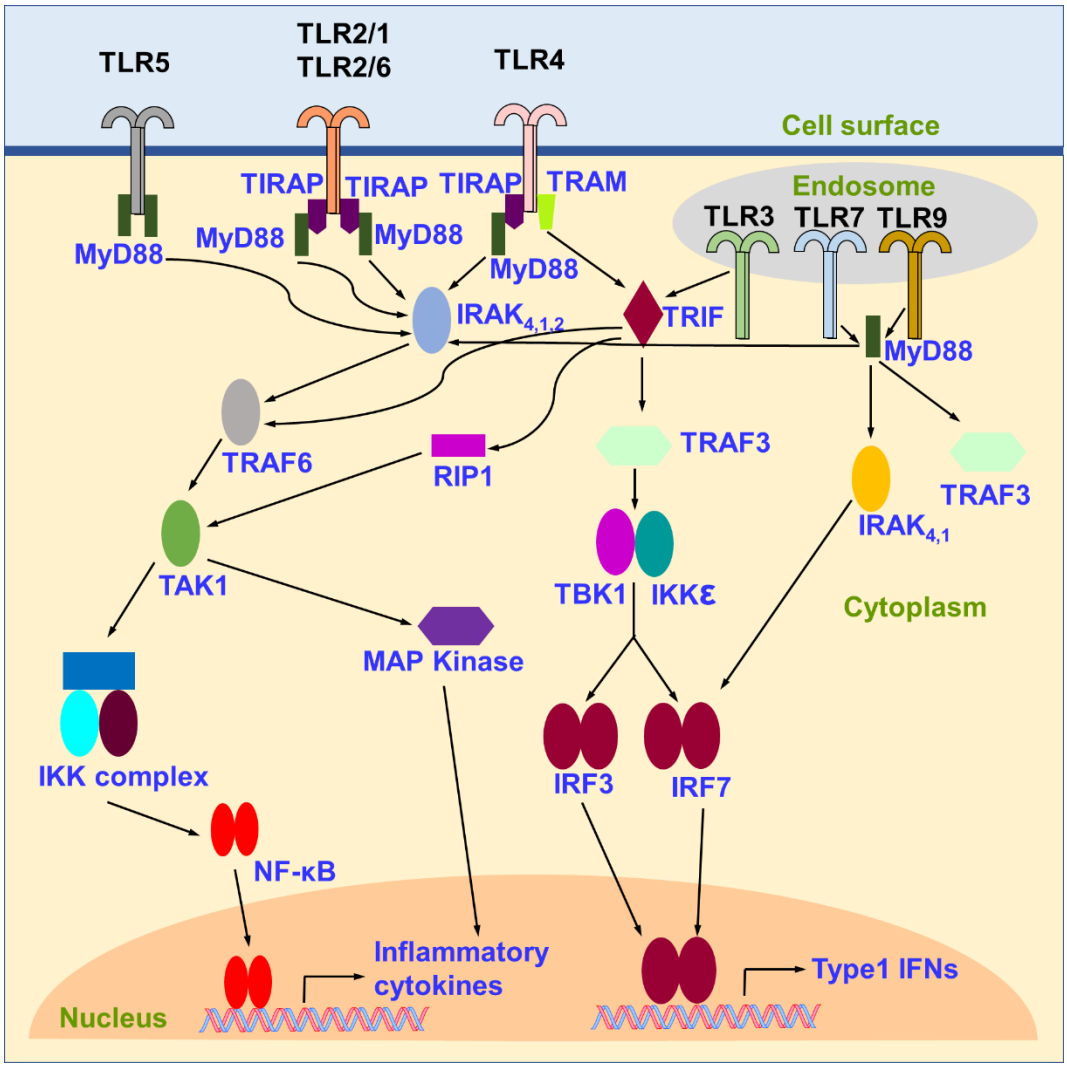


Figure 1.5: TLRs signalling pathways.

TLR1, TLR2, TLR4, TLR5, and TLR6 are cell surface receptors, while TLR3, TLR7, and TLR9 recognize their ligands in the endosome. All TLRs, except TLR3, rely on MyD88 for signalling. TLR2 and TLR4 require TIRAP as an adaptor to engage MyD88 through their TIR domain. TLR3 and TLR4 recruit TRIF. TLR4 requires the TRAM adaptor to signal through TRIF. Activation of MyD88-dependent pathways activates NFκB and MAPK pathways, whereas activation of TRIF-dependent pathway activates NFκB, MAPKs, IRF3, and IRF7. The activated NFκB subunits and IRF3,7 are transferred to the nucleus. NFκB and MAPK induce transcription of inflammatory cytokines whereas IRF3,7 initiate type I interferons transcription. The diagram was adapted from (Kumar et al., 2009)

All TLRs expect TLR3 rely on MyD88 to signal (Janssens and Beyaert, 2003). TLRs 2, 4 and 5 have an important role in PA recognition. TLR2 signalling requires TIRAP and MyD88 (O'Neill et al., 2003). The extracellular slime-glyco-lipoprotein (slime-GLP), alginate, and ExoS have been identified as TLR2 ligands (McIsaac et al., 2012). It has also been demonstrated that TLR2 recognizes PA through binding to pili which leads to epithelial cell activation (Sadikot et al., 2005b). TLR4 recognizes LPS, a major component of Gram-negative bacteria cell walls (Takeuchi et al., 1999). Both TRIF and MyD88 are involved the TLR4 signalling. TLR4 requires essential accessory co-receptors named CD14 and MD2 (see section 5.1 for details (Rajaiah et al., 2015). It has been shown that engagement of TLR4 is essential to clear PA lung infection (Faure et al., 2004). TLR5 recognises flagellin, the principal component of flagella. Binding of TLR5 to its ligand leads to the activation of MAPK (Zhang et al., 2007) and TLR5 also utilizes MyD88 to activate NF-κB (Tallant et al., 2004). This cascade appears to protect against PA infection through production of the chemokine IL-8 (Zhang et al., 2007).

1.5.2 Nucleotide-binding oligomerization domain (NOD)-like receptors (NLR)

Twenty members of NLR have been characterized (Zhong et al., 2013). NLR consists of a variable number of N-terminal effector domain, a Nucleotide-binding oligomerization domain (NOD), and a domain containing leucine rich repeats(LLRs) (Proell et al., 2008). Signalling by NLRs is initiated via homotypic protein-protein interactions through the effector domains which include caspase recruitment domain (CARD), pyrin domain (PYD) and baculovirus inhibitor of apoptosis protein repeat (BIR) (Lu et al., 2015). NOD1 and NOD2 are involved in recognition of peptidoglycan (PGN) fragments. NOD1 and NOD2 are activated by γ-d-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively, leading to NF-κB activation (Caruso et al., 2014). Nucleotide-binding oligomerization domain, Leucine-rich Repeat and Pyrin domain containing (NLRP) is a subgroup of the NLR family that can lead to the formation of Inflammasomes. Inflammasomes regulate caspase1 activity leading to processing and secretion of IL-1β and IL-18 (Schmidt and Lenz, 2012) and induction of a form of inflammatory cell death termed pyroptosis (Shen and Codogno, 2016). IL-1β secretion and pyroptosis requires processing of Gasdermin D by caspase-1 (Martín-Sánchez et al., 2017). Inflammasomes especially NLRP3 recognizes many agents including, *S. aureus*, and *Candida albicans* (Wu and Huang, 2017). Apoptosis-associated speck-like protein containing C-terminal caspase recruitment domain (ASC) is required for caspase-1 activation by the NLRP1 and NLRP3 inflammasomes. PA pili activate inflammasomes NLRP1 and NLRP3 in bone-marrow-derived murine macrophages (Lindestam Arlehamn and Evans, 2011). Several components of PA including T3SS system can activate the NLRC4 inflammasome (Vladimer et al., 2013). NLRC4, another member of the NLR family, has a CARD domain and could engage caspase 1 directly. Nevertheless, full caspase-1 activation in the case of NLRC4 still requires ASC. NLRC4 can activate caspase-1 during PA invasion.

However, the T3SS through the action of ExoU has been shown to inhibit caspase-1 activation (Taxman et al., 2010).

1.6 C type lectins receptors (CLRs)

The analysis of serum mannose-binding protein revealed the structure of C-type carbohydrate recognition domain (CRDs). CRDs formed of a hydrophobic core and disulfide bonds. There are additional conserved residues that form a calcium binding site. Many proteins can not bind to sugars due to the absence of the calcium binding site. This distinguishes between C-type CRDs that bind sugars and C-type lectin-like domains (CTLDs) which have a similar domain structure but lack lectin activity. The hallmark of all examined CRD–glycan complexes is the interaction of principal calcium with two monosaccharide vicinal hydroxyl groups. Mannose-type sugars such as mannose, N-acetyl-glucosamine and glucose contain adjacent equatorial 3-hydroxyl and 4-hydroxyl groups. Investigations have shown that some lectins contain in their primary calcium site glutamic acid-proline-asparagine (EPN) resides which determine their specifity for these sugars. Galactose-type sugars such as galactose and N-acetyl-galactosamine contain axial 4- hydroxyl group and lectin specific for these carbohydrates require glutamine-proline-aspartic acid (QPD) for binding. However, langerin, which is a specific lectin receptor of Langerhans cells, has been found to bind both sulphated galactose and mannose through the same binding site (Drickamer and Taylor, 2015). CLRs play crucial roles in regulating the immune response as PRRs, including cell adhesion and pathogen recognition. (Zelensky and Gready, 2005). CLRs are found either as secreted soluble proteins such as mannan binding lectin (MBL), surfactant protein-A and D (SP-A and D) or transmembrane proteins such as Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), mannose receptor (MR), dectin-1 and dectin-2 (Cambi et al., 2005).

Transmembrane proteins are divided into two groups based on the location of their amino N-terminus. Type I and type II membrane proteins have their N-terminus pointing outwards or into the cell cytoplasm, respectively. Type I CLRs such as the mannose receptor (MR, CD206) contain several C-type lectin-like domains (CTLDs), whereas type II CLRs such as dendritic cell-specific intercellular adhesion molecule-3-irabbing ion-integrin (DC-SIGN, CD209) have a single CTLD (Figdor et al., 2002). CLRs perform a variety of important biological functions, such as cellular activation, phagocytosis, cell adhesion, endocytosis, tissue remodelling and complement activation (Kerrigan and Brown, 2009). (Figure 1.6).

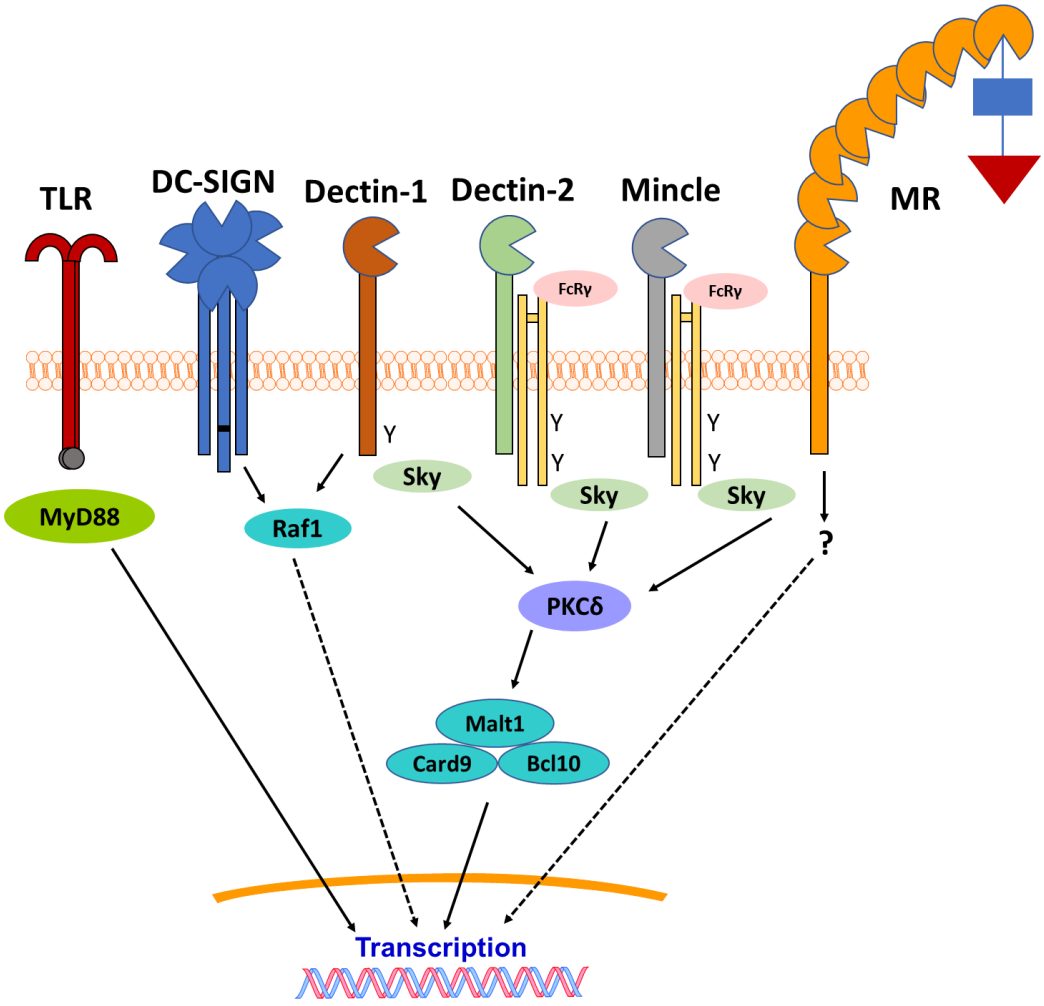


Figure 1.6: CLRs and their intracellular signaling pathways.

Dectin-1 activates spleen tyrosine kinase (Sky) directly via hemi-ITAMs, while mincle and dectin-2 require phosphorylation of immunoreceptor tyrosine-based

activation motif (ITAMs) of associated Fc gamma receptors (FcγR) chain. Activation of Sky activates the protein kinase C delta (PKCδ) pathway, which in turn activates the caspase-associated recruitment domain (Card9)- B-cell lymphoma (Bcl10)- mucosa-associated lymphoid tissue lymphoma translocation (Malt1) complex leading to transcription of inflammatory mediators. DC-SIGN and Dectin-1 can signal through the Raf-1 kinase pathway (dotted line) which can activate NF-κb. The mannose receptor has the ability to activate intracellular signalling, however, MR signalling mechanisms and pathways are poorly understood. Some CLRs have the ability to induce pathways that directly activates NF-κb, whereas other CLRs influence NF-κb activation by modulating TLR signalling. Adapted from (Hardison and Brown, 2012).

1.6.1 The mannose receptor

Mannose receptor (MR, CD206) is a 175 kDa type I CLR. It is expressed by selected populations of macrophages and dendritic cells (DCs), nonvascular endothelium, mesangial kidney cells, retinal pigment epithelium, and trachea smooth muscle cells (Martinez-Pomares, 2012). Less than 20% of MR is expressed on the cell surface while the rest is expressed intracellularly within early endosomes. MR is upregulated by IL-4, IL-10, and IL-13, and down-regulated by IFN-γ (Kerrigan and Brown, 2009). Many organisms can be recognized by MR, including *C. albicans, Leishmania, M. tuberculosis,* HIV, dengue virus*, K. pneumoniae,* and *Streptococcus pneumoniae* (Gazi and Martinez-Pomares, 2009). Structurally, MR consists of three extracellular regions: cysteine-rich domain (CR), fibronectin type II domain (FNII), and eight C-type lectin domain (CTLDs) and a short cytoplasmic tail. The CR domain is involved in binding to sulphated sugars, the FNII domain is capable of binding to types I, II, III, and IV collagen, and the CTLDs are responsible for recognizing sugars terminated in mannose, fucose, or N-acetyl glucosamine in a calcium-dependent manner.

It has been shown that the binding affinity of multiple CTLDs (4–8) is the same as for the whole receptor (Taylor et al., 2005a, Gazi and Martinez-Pomares, 2009). MR have numerous roles, which include the promotion of antigen presentation, clearance of host molecules, and cellular activation (Martinez-Pomares, 2012). However, the response of MR deficient mice to *C. albicans* was normal (Lee et al., 2003a). Several studies have established the role of MR to facilitate removal of lysosomal enzymes, neutrophil-derived myeloperoxidase, the pituitary hormones lutropin and thyrotropin as well as tissue plasminogen activator (Le Cabec et al., 2005). MR deficiency was associated with high levels of lysosomal hydrolases (Lee et al., 2002). This suggests that MR is a major player in homeostasis. It has been demonstrated that MR is involved in the phagocytosis of some pathogen such as *P. carinii*, yeast such as *Saccharomyces cerevisiae*, *leishmania, M. tuberculosis*, and *C. albicans* (Gorvel, 2004). Despite that fact that MR can recognize *Leishmania* cell surface via binding to mannose, there were no differences between MR-deficient and WT mice after infection (Akilov et al., 2007). However, Cos-I cells transfected with MR DNA were able to phagocytose *C. albicans*. (Gazi and Martinez-Pomares, 2009). This disagreement might indicate that MR is not essential for phagocytosis. However, MR might associate with FcRγ-chain and can modulate responses triggered by the FcRγ-chain and TLR4 (Rajaram et al., 2017b, Salazar et al., 2016a). MR has an endocytic activity to clear endogenous glycoproteins. It can recognize a wide range of allergens (Gazi and Martinez-Pomares, 2009, Martinez-Pomares, 2012). Also, Chavele and colleagues have shown that MR promotes crescentic glomerulonephritis (CGN) disease (Chavele et al., 2010). Despite the unknown signalling mechanisms of MR, it might be possible that MR signalling depends on the cellular activation and stimulus.

1.6.2 Dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN)

DC-SIGN, (CD209), belongs to the type II CLRs and is expressed by DCs and some macrophage populations (Kerrigan and Brown, 2009). DC-SIGN expression is induced by IL-4 and down-regulated by IFN-γ and TGF-β (Relloso et al., 2002). Originally, DC-SIGN was identified as a receptor that binds intercellular adhesion molecule 3 (ICAM3) to facilitate T-cell proliferation mediated by DCs. Recent studies revealed its role in detecting pathogens by binding to fucose and mannose, as well as blood group Lewis antigens in a calcium dependent manner (Ehlers, 2010, Geijtenbeek et al., 2000b). DC-SIGN has been found to recognise a wide range of pathogens, such as *C. albicans, M. tuberculosis, Schistosoma mansoni, Aspergillus fumigatus, Helicobacter pylori* and the human immunodeficiency virus (HIV) (Geijtenbeek et al., 2000b, Geijtenbeek et al., 2000a). DC-SIGN can recognize, HIV-1, HCV, Ebola, and as *M. tuberculosis* by binding to mannose (Gringhuis et al., 2009b, Pöhlmann et al., 2003, Lin et al., 2003), while binding to fucose enables DC-SIGN to detect *Schistosoma mansoni* and *H. pylori* (Gringhuis et al., 2014a). DC-SIGN consists of a CTLD, a stalk region that mediates multimerization, a single transmembrane region, and a cytoplasmic tail associated with a number of internalization motifs (Kerrigan and Brown, 2009). DC-SIGN undergoes tetrameric clustering, which increases the binding avidity (Mitchell et al., 2001).

Under normal homeostatic conditions, DC-SIGN is associated with the lymphocyte specific protein (LSP1). When DC-SIGN detects pathogens that contain mannose, Raf-1 is activated leading to NFκB activation. This promotes the transcription of genes encoding IL-1β, IL-6, IL-10, IL-12 and IL-23A, which in turn induce specific T cell responses. Whereas detection of fucose-containing pathogens by DC-SIGN leads to the detachment of Raf-1 from the DC-SIGN-LSP1 complex. This is

followed by activation of NFκB initiated by TLR4. This decreases the transcriptional genes of IL1B, IL-6, IL-12A, IL- 12B, and IL-23A and increases IL-10, skewing T cell differentiation to Th2 (Sprokholt et al., 2016, Gringhuis et al., 2014b). Intriguingly, accumulation of DCs expressing DC-SIGN positively correlates with the presence of biofilms in chronic rhinosinusitis with nasal polyps (CRSwNP) (Karosi et al., 2013).

1.6.3 Dectin-1

Dectin-1 is mainly expressed by macrophages, neutrophils and DCs (Herre et al., 2004). It possesses a single CTLD connected to a stalk region followed by a cytoplasmic tail, which consists of a motif similar to the immunoreceptor tyrosine-based activation motif (ITAM) termed by some authors hemITAM (Brown, 2006). β-1, 3-linked glucan has been characterized as the most important Dectin1 ligand and enables recognition of *M. tuberculosis*. *C. albicans*, *Aspergillus fumigatus*, and zymosan, *Saccharomyces cerevisiae* cell wall component (Brown et al., 2007, Dillon et al., 2006, Mikhalchik et al., 2000). Binding of Dectin-1 to its ligand is followed by activation of Syk-CARD9 signalling pathways. Dectin-1 induces expression of chemokines and cytokines including TNF-α, CXCL2, IL-2, IL-10 and IL-12 (Dostert and Tschopp, 2007). In addition to cytokine production, engagement of Dectin-1 promotes phagocytosis and the respiratory burst, i.e. ROS production (Goodridge et al., 2011). In a mouse model, lack of Dectin-1 increased the susceptibility to *C. albicans* infection due to impaired immune response including phagocytosis (Taylor et al., 2007). Dectin-1 plays an apparent role in adaptive immunity through promoting Th17 differentiation (Verma et al., 2015) . A previous study demonstrated that the susceptibility to *C. albicans* infection was correlated with reduced Th17 response in Dectin-1 deficiency (Carvalho et al., 2012).

Ferwerda *et al* has shown that humans deficient in dectin-1 are more susceptible to mucosal infection with *C. albicans* (Ferwerda et al., 2009).

1.7 Hypothesis and aims: C-type lectins as potential PRRs for PA

Tamanna Rahman, a former PhD student in Dr Martinez-Pomares' laboratory, showed that PA biofilms bind the CTLD4-7 region of MR and this binding is increased when Psl expression increases. Therefore, **we hypothesized that C-type lectins expressed by macrophages and DCs, such as MR and DC-SIGN, might contribute to the recognition of PA biofilms by immune cells through binding to EPS.**

Therefore, the overarching aims of this project are 1) to determine the capability of C-type lectins to interact with PA biofilms and purified EPS components, and 2) to investigate how EPS components affect the interaction of macrophages and DCs with PA. The specific aims for each results chapter are described below.

Specific aims for Chapter 3:

- Quantify biofilm formation by PA laboratory strains and clinical isolates.
- Determine the capability of MR and DC-SIGN to interact with PA biofilms.
- Examine whether the binding of MR and DC-SIGN is calcium- and carbohydrate-dependent.
- Evaluate and compare the binding of different DC-SIGN constructs to PA biofilms in different buffers.

Specific aims for Chapter 4:

- Establish a protocol to extract Psl from PA biofilms.
- Determine the capability of MR and DC-SIGN to interact with purified Psl.
- Evaluate and compare the binding of different DC-SIGN constructs to PA Psl using different techniques.
- Analyse how highly purified Psl preparations modulate the activation of human monocyte-derived DCs.

Specific aims for Chapter 5:

- Determine the capability of DC-SIGN to interact with planktonic PA (lab strains and clinical isolates).
- Investigate potential DC-SIGN ligand(s) on the outer membrane of PA.
- Examine the binding of DC-SIGN to PA LPS using LPS mutants, extracted PA LPS, and commercial purified PA LPS.
- Assess the binding of monocyte-derived huDCs to fixed planktonic PA using flow cytometry.

Chapter 2. Materials and Methods

2.1 Bacterial strains, culture conditions, and generation of growth curves

All the strains (Table 2.1), unless otherwise stated, were grown on Luria-Bertani (LB) agar plates from glycerol stocks stored at -80^{o}C and incubated overnight at 37°C. The following day, a single colony was selected and inoculated into 5 ml of X-vivo-15 (Lonza) media and incubated overnight at 37°C, at 200/220 rpm. The OD_{600nm} of cultures was adjusted to 0.01 in 20 ml of media in a 250 ml flask and incubated for 3 hrs at 37°C, shaking at 200/220 rpm prior to any experiment in order to reach mid log phase. For growth curves of Δ*wspF* mutants, after OD_{600nm} of overnight cultures was adjusted to 0.01 in 20 ml of X-vivo-15 media in a 250 ml flask, absorbance was measured at 600 nm hourly for 10 consecutive hrs in 1 ml samples using spectrophotometer (Thermo Scientific Biomate 3). PAO1-P strains were kindly provided by Dr. Yasuhiro Irie, University of Dayton, OH, USA).

Table 2.1. Strains used in the study

Strain	Features	Source
PAO1-P	WT PAO1 laboratory strain from Parsek's laboratory Normal cellular level of c-di GMP Expresses Psl and Pel	(Holloway et al., 1979)
PA176	PAO1-PΔ*wspF.* High cellular levels of c-di GMP Expresses Psl and Pel	(Hickman et al., 2005b)

PA105	PAO1-P∆*wspF*∆*Pel* High cellular levels of c-di GMP Overexpresses Psl Lacks Pel	(Irie et al., 2010)
PA106	PAO1-P∆*wspF*∆*Psl* High cellular levels of c-di GMP Overexpresses Pel Lacks Psl	
PA107	PAO1-P∆*wspF*∆*Pel*∆*Psl* High cellular levels of c-di GMP Lacks Psl and Pel Biofilm deficient	(Borlee et al., 2010a)
PAO1-N	WT PAO1 laboratory strain from Nottingham laboratory	Provided by Dr. Steve Diggle's lab, Nottingham University
PA wound isolates	Clinical samples isolated from wound, blood and bone	
PA-20	Mucoid PA CF isolate	Provided by Dr. Miguel Camara's lab, Nottingham University
PA-37	Mucoid PA CF isolate	
LESB58	non-mucoid CF isolate	
PA-PO13	mucoid CF isolate	
PA-A002	mucoid CF isolate	

PAO1-C	WT PAO1 laboratory strain from Khursigara's laboratory CPA^+/OSA^+	(Murphy et al., 2014a)
PAO1-C Δ*wbpM*	CPA^+/OSA^-	
PAO1-C Δ*rmd*	CPA^-/OSA^+	
PAO1-C Δ*wbpL*	CPA^-/OSA^-	(Murphy et al., 2014a)

2.2 Analysis of *wspF*, *Pel* and *Pel* mutations using polymerase chain reaction (PCR)

PCR was used to confirm Δ*wspF,* Δ*Psl,* and/or Δ*Pel* mutations in the strains PA176, PA105, PA106 and PA107. Primers designed to target the mutated gene(s) are shown in Table 2.2. PCR reactions were carried out in 25 µl reaction tubes containing 1 µl of overnight culture in LB broth, 0.1 µM each of reverse and forward primers, and 1x of GoTaq G2 green master mix (GoTaq PCR, Promega). The amplification process was as follows: a denaturation cycle at 96 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. This process was completed by an extra extension cycle at 72 °C for 5 min. PCR products (10 µL) were loaded into 1% (w/v) of agarose gel containing 0.01% (v/v) of Sybergreen safe in Tris-acetate-EDTA (TAE) buffer, that consist of [40 mM Tris (pH 7.6), 20 mM acetic acid and 1 mM EDTA], and electrophoresed for 30 min at 80 volts.

Table 2.2. Primers used to confirm specific mutations in the *WspF* mutants

***WspF* gene**	**Primer sequence**
Forward primer	AAGATTCAGAACGTCGGCTG
Reverse primer	GATGTGATTGTTGGTCCCCG
PslC* and *PslD	**Primer sequence**
Forward primer	GGTCTACCATTCCCACGATTAC
Reverse primer	TCAGCTCGTTGGCGATTT
PelA	**Primer sequence**
Forward primer	CCCTATCCGTTCCTCACCTC
Reverse primer	TACTGGGCCTCGAAGTTCTC

2.3 Biofilm quantification assays

2.3.1 Crystal violet assay

The OD of mid log phase cultures in X-vivo-15 was adjusted to 0.04 OD_{600nm} in X-vivo-15 media. After sterilizing the 96 well plate under UV light for 15 min, 100 µl of cultures were added into each well of a 96-well plate [Costar (9017, Corning) or Maxisorp (439454, Nunc immune-plate)]. The plate was incubated statically for 24 hrs at 37°C, 5%CO_2. After the incubation, the non-attached material was removed by washing the wells three times with 200 µl of HPLC water, which was added and sucked repeatedly from one edge of each well, to minimize biofilm interruption. The wells were then stained with 125 µl of 1% (w/v) crystal violet prepared in HPLC water and incubated for one hour at room temperature. After washing the wells three times, the stain was solubilized by adding 200 µl of 70% ethanol for 15 min; 125 µl was transferred into a clean 96 well Costar plate to measure the absorbance at 595 nm using Multiskan FC (Thermo Scientific).

2.3.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to quantify the biofilm formation of PA. Biofilms were developed on 96 well Maxisorp plates (439454, Nunc immune-plate) over 24 hrs as described in section 2.3.1 and then fixed with 50 µl of 2% (v/v) paraformaldehyde (15710-S, Electronic Microscopy Sciences, USA) prepared in PBS for 10 min at 4^{o}C. After washing the plate three times with PBS without calcium and magnesium (D8537, Sigma), a blocking step was carried out by adding 50 µl of 3% (w/v) bovine serum albumin (BSA) (80400-100, Alpha diagnostics) prepared in PBS to the wells. 50 µl of rabbit anti-*Pseudomonas* polyclonal antibody (ab68538, Abcam) diluted 1:1000 in PBS were added to the plate and incubated for 90 min at room temperature. After three washes in PBS, the plate was incubated with 50 µl of goat anti-rabbit IgG conjugated to alkaline phosphatase diluted 1:2000 (A3687, Sigma) in PBS for 1 hour at room temperature. After washing three times with AP buffer (100 mM Tris-HCl, 100 mM NaCl, 1 mM MgCl2, pH 9.5), 50 µl of p-Nitrophenyl phosphate substrate solution (1 mg/ml PNPP tablet and 0.2 M Tris buffer tablet, Sigma, in HPLC) were added to each well and incubated for 30-40 min at room temperature in the dark place. Absorbance was read at 405 nm using Multiskan FC (Thermo Scientific).

2.4 Preparation of MR (CD206) chimaeric proteins CTLD4–7-Fc and CR-FNII-CTLD1–3-Fc

2.4.1 Plasmid isolation

The *E. coli* strains containing plasmids encoding either CTLD4-7-Fc or CR-FNII-CTLD1–3-Fc (Martinez-Pomares et al., 2006) were grown on an LB agar plate at 37°C overnight. A single colony was inoculated in 5 ml of LB broth and incubated at 37°C overnight, shaking at 200 rpm. Following the incubation, the culture was

diluted 1:100 in 125 ml of LB broth and incubated at 37°C overnight, shaking at 200 rpm. Both the LB plate and the broth were supplemented with 50 µg/ml of ampicillin. Plasmids were purified using a Macherey Nagel Plasmid DNA Purification Kit.

2.4.2 Protein production

HEK293T were cultured in a T75 (Corning, USA) flask in pre-warmed Dulbecco's Modified Eagle's Media (D6421, Sigma) supplemented with 10% (v/v) foetal bovine serum (FBS) (F7524, Sigma), 2 mM L-glutamine (25030, Gibco), 100 units/ml penicillin, and 100 µg/ml streptomycin (P4333, Sigma) at 37°C in a humidified condition with 5%CO_2. The medium was changed every two days until the cells reached 90% confluency, at which point the cells were sub-cultured by removing the medium, washing with PBS without calcium or magnesium, and exposing the cells to 1X trypsin-EDTA solution in PBS (T4174, Sigma), which consists of 0.5mg/ml of trypsin and 0.2mg/ml of EDTA. The cells were then centrifuged at 350×g for 5 min at room temperature and transferred into two T175 (431080, Corning) flasks and maintained in the same conditions until they reached 90% confluency. Each T175 flask (431080, Corning) was passaged into three T175 flasks until we had collected 6 T175 flasks at 90% confluency. The medium was removed from each flask and replaced with 18 ml of fresh medium. To prepare the transfection mix, 108 ml of OPTIMEM (11058, Gibco) containing [2 mM L-glutamine (G7513, Sigma), 100 units/ml penicillin, 100 µg/ml streptomycin (P4333, Sigma), and 0.3% (v/v) of GeneJuice (Novagen)] was prepared and incubated for 10 min at room temperature. After that, 108 µg of plasmid were added to the mixture and incubated for 15 min at room temperature. After removing the medium from the flasks, the cells in the six T175 flasks were incubated with 18 ml of the prepared transfection mixture and maintained at 37°C in a humidified atmosphere

with 5% CO_2 overnight. The following day, the transfection mixture was removed and replaced with fresh OPTIMEM supplemented as above and incubated for five days. The supernatants were then collected into 250 ml centrifuge bottles (Corning, USA) and centrifuged at 2,100×g for 15 min at room temperature. The supernatants were then collected into a fresh 250 ml Corning bottle, supplemented with protease inhibitors (11836170001, cOmplete Mini, EDTA-free cocktail tablets, Roche) and stored at 4°C.

2.4.3 Protein purification

The protein was purified using a protein G-Sepharose column as follows; 75 µl of PBS without calcium or magnesium were added to the column (732-1010, Econo-Pac Chromatography Columns, BIORAD) and the position of the PBS was marked. After removing the PBS, protein G-Sepharose gel (17-0886-01, Gammabind plus sepharose, GE Healthcare) was added up to the mark. The column was washed with 25 ml of PBS and then the supernatant from transfected cells was passed through the column. The column was then washed with 50 ml of PBS and bound proteins were then eluted with 0.1M glycine (56-40-6, Fisher scientific) in endotoxin-free water (pH 2.8). Each 10 drops of the effluent were collected in clean 1.5 ml Eppendorf tubes containing 50 µl of 1M Tris-HCl (pH 8, Sigma). The eluted protein was detected using a Nanodrop (2000c, Thermo scientific) and only the fractions that contained protein were pooled and dialysed against Tris-saline Buffer in PBS (10 mM Tris HCL 7.4 PH, 10 mM $CaCl_2$, 154 mM NaCl). The protein was stored at -80 ˚C after measuring the protein concentration using the bicinchoninic acid (BCA) assay (23225, Pierce BCA Protein Assay Kit, Life Technologies).

2.5 Lipopolysaccharides (LPS) extraction

Extraction of LPS using the hot phenol-water method was carried out as described by (Rezania et al., 2011) as follows. Overnight bacterial cultures in X-vivo-15 medium were centrifuged at 10,000×g for 5 min at room temperature. The pellets were washed three time with PBS with calcium and magnesium (D1283, Sigma). Pellets were re-suspended in 10 ml PBS with calcium and magnesium and sonicated for 10 min on ice. 100 µg/ml of proteinase K (P2308, Sigma) was added to the suspension and incubated at 65°C for an hour to eliminate proteins. To eliminate the nucleic acids, the mixture was treated with 40 µg/ml of RNase (R6513, Sigma), 20 µg/ml of DNase (DN25, Sigma), 1 µl/ml of 20% $MgSO_4$ (M2643, Sigma), and 4 µl/ml of chloroform (67-66-3, Fisher scientific) and incubated at 37°C overnight. An equal volume of hot 90% (v/v) phenol (P4557, Sigma) was added to the mixture and kept at 70°C with vigorous shaking for 15 min. After cooling the mixture on ice, it was transferred into 1.5 ml polypropylene tubes and centrifuged at 8,500×g for 15 min. Supernatants were collected in a 15 ml falcon tube and 300 µl of distilled water were added in order to re-extract the phenol phase. In order to precipitate LPS, 0.5 M sodium acetate (CH_3COONa) (S2889, Sigma) final concentration and 10 volumes of 95% (v/v) ethanol were added to the extracts and kept at -20°C overnight. Samples were then centrifuged at 2000×g for 10 min at 4°C and the pellets were re-suspended in 1 ml of distilled water. The residual phenol was eliminated by dialysing the samples against distilled water. Finally, the extracted LPS products were lyophilized and stored at 4°C.

2.6 Preparation of fixed *P. aeruginosa*

Overnight PA cultures was centrifuged at 16,000×g for 5 min at 4°C. After washing twice with PBS without calcium and magnesium, the pellet was re-suspended in 4% (v/v) paraformaldehyde (15710-S, Electronic Microscopy Sciences, USA) in PBS for 30 min at 4°C for fixation. Then the culture was washed with PBS once; and adjusted to 0.5 OD_{600nm} in PBS.

2.7 Protein binding assays

Binding assays were used to test the binding of chimaeric proteins to fixed PA biofilms, fixed planktonic PA, purified Psl, extracted LPS, or commercial purified LPS. To test binding to biofilms, biofilms were developed on a Costar (9017, Corning) or Maxisorp (439454, Nunc immune-plate) plate over 24 hrs as described in 2.3.1 and then fixed with 50 µl of 2% (v/v) (15710-S, Electronic Microscopy Sciences, USA) prepared in PBS for 10 min at 4°C. For fixed PA, wells of Maxisorp plates (439454, Nunc immune-plate) were coated with 100 µl of fixed bacteria (see section 2.6 for preparation of fixed bacteria) in PBS and incubated at 4^{o}C overnight. Psl (prepared in house, see section 4.4), purified PAO1-serotype 10 LPS (L2262, Sigma), and extracted LPS (prepared as described in section 2.5) were added in 50 µl per well of different concentrations in 154 Mm NaCl to Maxisorp plates (439454, Nunc immune-plate) overnight at 37°C. In all instances, plates were washed three times with either TBS (10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 154 mM NaCl and 0.05% (v/v) Tween 20) or TSM binding buffer (20 mM Tris-HCl, pH 7.4,150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 1% (w/v) BSA). 50 µl of chimeric proteins CTLD4-7-Fc (prepared in house, see section2.4), Fc-DC-SIGN (R&D), biotinylated DC-SIGN (kindly provided by Dr. Daniel Mitchell, University of Warwick) or Fc-DC-SIGN-Ams (kindly provided by Dr. Juan Garcia Vallejo,

Amsterdam) were added to the plate in either (TBS or TSM binding buffer) or neat and incubated for 2 hrs at room temperature. After three washes in either TBS or TSM washing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.05% (v/v) Tween 20), the plate was incubated with 50 µl of anti-human Fc-conjugated to alkaline phosphatase (A9544, Sigma) or 50 µl of Streptavidin−alkaline phosphatase (S2890, Sigma) for 1 h at room temperature both diluted 1:1000 in TBS or TSM washing buffer. After washing three times with TBS or TSM washing buffer, the plate was washed twice with AP buffer (100 mM Tris-HCl, 100 mM NaCl, 1 mM $MgCl_2$, pH 9.5). Then 50 µl of p-Nitrophenyl phosphate substrate solution (1 mg/ml PNPP tablet and 0.2 M Tris buffer tablet, Sigma, in HPLC) were added to each well and incubated for 30-40 min at room temperature in the dark. Absorbance was read at 405 nm.

2.8 Carbohydrate inhibition assays

Inhibition assays were carried out in a TSB buffer with high salt (1M NaCl). CTLD4-7-Fc (see section 2.4) or Fc-DC-SIGN (R&D) were pre-incubated with different concentration of monosaccharides mannose (63579, Fluka), fucose (47870, Fluka), or galactose (4829, Fluka) in TSB buffer with high salt (1M NaCl) for 30 min at room temperature. After that pre-incubated protein was added to appropriate wells of Maxisorp plate (439454, Nunc immune-plate), coated with biofilms or polysaccharides polymers, and incubated for 2 hrs at room temperature. The binding was detected as described in section 2.7.

2.9 Generation of dendritic cells

Buffy coats (Blood Transfusion Service, Sheffield) were used to prepare human dendritic cells (DCs). After decontaminating the blood bags with 70% alcohol, the

blood (approximately 50 ml/bag) was transferred into a T75 flask and diluted with 90 ml of PBS without calcium and magnesium (total volume of the mixture must be 140 ml). 35 ml of the mixture were layered over 15 ml of Histopaque-1077 (H8889, Sigma) in 50 ml falcon tubes in order to isolate peripheral blood mononuclear cells (PBMCs) through density gradient centrifugation. Samples were centrifuged at 800×g for 30 min, low acceleration and deceleration, at room temperature using an Allegra X-15R centrifuge (Beckman Coulter). PBMCs were collected and washed three times with PBS. The monocytes were isolated using human CD14 MicroBeads (130-050-201, Miltenyi Biotec) following the manufacturer's protocol. After washing the purified monocytes twice with PBS, they were re-suspended in RPMI complete media which is composed of RPMI-1640 (R0883, Sigma), 10% (v/v) human AB serum (PAA Laboratories, UK), 2 mM L-glutamine (G7513, Sigma), 10 mM HEPES (15630056, Gibco), 800 IU/ml recombinant human granulocyte macrophage colony–stimulating factor (rhGM-CSF) (130-093-865, Miltenyi Biotec), and 500IU/ml recombinant human interleukin 4 (130-093-921, Miltenyi Biotec). Number of monocytes/ml and cell viability were determined using Trypan blue exclusion method. Using 24 well flat bottom tissue culture plates (Corning, USA), 500 µl of cell suspension containing 750,000 cells were seeded onto each well and incubated at 37^{o}C, 5%CO_2 for 7 days. On Day 3, 500 µl of fresh RPMI complete media containing growth factors were added to each well. On Day 7, the plate was placed on ice for 15 min and DCs harvested from the supernatants in a 15 ml flacon tube. The cells were pelleted by centrifugation at 350 x g, for 5 min, acceleration and deceleration 3, at 4^{o}C. After determining the cell viability and number, they were re-suspended in the appropriate media.

2.10 Analysis of cell surface markers using flow cytometry

DCs were re-suspended in X-vivo-15 media in a 15 ml falcon tube at a density of 1×10^6 cells /ml, centrifuged at 350 x g, for 5 min, acceleration and deceleration 3, at 4^{o}C and re-suspended in 1.5 ml of 5% (v/v) mouse serum (M5905, Sigma) in PBA solution [0.5% (w/v) BSA and 0.1% (w/v) sodium azide (S2002, Sigma) prepared in PBS], and incubated for 1 h at 4^{o}C. After washing the cells with cold PBA three times, the cell density was adjusted to 50,000 cells/45µl of PBA. Then the cells were incubated with mouse anti-human monoclonal antibodies or their matched isotype controls: CD206 (MMR)-Allophycocyanin (APC) (321109, Biolegend) or DC209 (DC-SIGN)-APC (330107, Biolegend) for 1 h at 4^{o}C (1:10 dilution). The cells were washed twice with cold PBA and re-suspended in fresh cold PBA and fixed with 0.5% (v/v) paraformaldehyde (15710-S, Electronic Microscopy Sciences, USA) in PBS at 4^{o}C. Cells were analysed using a FC500 Beckman Coulter flow cytometer; 10,000-20,000 events were collected. Data were analysed using Kaluza Software 1.5a.

2.11 Generation of biofilms on different substrates for confocal analysis

2.11.1 Biofilm formation

The OD of mid log phase cultures in X-vivo-15 was adjusted to 0.04 OD_{600nm} in X-vivo-15 media. 100 µl of cultures were added to Lab-Tek II chamber glass slides (154534, Thermofisher), Greiner cell star 96 well black plates (M0812, Sigma), or Cellview cell culture slide glass bottom (543079, Greiner bio-one). The slide was incubated statically for 24 hrs at 37°C and 5% CO_2. After the incubation, the non-attached materials were removed by washing the wells three times with HPLC water.

2.11.2 Incubation of biofilms with Fc chimeric proteins and fluorescent secondary antibody

Biofilms were fixed with 100 µl of 4% (v/v) cold paraformaldehyde (15710-S, Electronic Microscopy Sciences, USA) in PBS for 10 min at 4°C. Wells were washed three times with TSB buffer. In some instances, appropriate wells were stained with 100 µl of 2-10 µg/ml of FM 1-43 FX membrane dye (F35355, Thermofisher) in PBS for 30 min on ice. Following three washes with TSB buffer, 50 µl of 10 µg/ml CTLD4-7-Fc (see section2.4) or Fc-DC-SIGN (R&D) protein in TSB buffer were added to appropriate wells and incubated for 2 hrs at room temperature. After washing three times with TSB buffer, 100 µl of TSB Buffer containing 10 µg/ml goat anti-Human IgG conjugated to alexa fluor 647 (A21445, Invitrogen) and 3%(v/v) Donkey serum **(**D9663, Sigma**)** were added to wells and incubated for 1 h at room temperature. Following three washes with TSB, the DNA was stained with 100 µl of 2µg/ml HOECHST (33342, Sigma) in PBS for 15 min. The plate was washed with TSB and kept in 0.1% (w/v) sodium azide (S2002, Sigma) in TSB buffer at 4^{o}C in the dark.

2.11.3 Capturing Confocal images

Confocal images were generated using Zeiss LSM 710 under 60x magnification water immersion lens. The acquisition mode and fine focus were adjusted in order to scan an area of interest. Alexa fluor 647 was excited at 633 nm and images collected using the 641-645 nm filter. The FM 1-43 FX membrane dye (F35355, Thermofisher) was excited at 480 nm and images collected using the 410-470 filter. HOECHST was excited at 405 nm and images collected using the 510 nm filter. Images were processed using Zeiss ZEN lite.

2.12 Cell adhesion assay

Overnight cultures of PAO1-P (WT), PAO1-C (WT) (CPA^+/OSA^+), and PAO1-CΔ*rmd* (CPA^-/OSA^+) in X-vivo-15 media were washed three times in PBS at 10,000g for 10 min at 4ºC. Pellets were labelled with FM 1-43FX membrane dye (F35355, Thermofisher) in distilled water for 30 min on ice in the dark. After washing with cold PBS, bacteria were fixed with 4% (v/v) paraformaldehyde (15710-S, Electronic Microscopy Sciences, USA) in cold PBS at 4ºC. The bacteria were re-suspended in cold PBS and number of bacteria/ml was calculated based on OD_{600nm} following the relation: $3.5x10^8$ bacteria/ml=OD 4.23_{600nm}. After that, human DCs prepared as described in section 2.9 were washed with either X-vivo-15 media or TSM binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 1% (w/v) BSA) and kept on ice. 150,000 cells were transferred into 0.5 ml polypropylene Eppendorf tubes and the bacteria were added at the desired multiplicity of infection (MOI). In some instances, mannan (M3640, Sigma**)**, $Lewis^x$-PAA (0044-PA, Lectinity Holdings), Galactose-PAA (0023-PA, Lectinity Holdings), anti-DC-SIGN Ab (kindly provided by Dr. Juan Garcia Vallejo, Amsterdam), or purified Psl (see section 4.4) was added in order block the binding of the bacteria to DCs. Tubes were incubated at 37ºC or 4ºC for various times at 8 rpm. Following incubation, pellets were collected and re-suspended in either cold X-vivo15 media or cold TSM buffer. The samples then were transferred into pre-cold test tubes and analysed by flow cytometry using FC500 Beckman Coulter flow cytometer.

2.13 Analysis of modulation of DC phenotype by *P. aeruginosa* Psl preparations

2.13.1 Incubation of human DCs with Psl

96 well Maxisorp plate (439454, Nunc immune-plate) was coated with 50 µl/well of 100µg/ml of filtered Psl preparations , or mannan (M3640, Sigma) prepared in 154 mM Nacl and incubated overnight at 37^{o}C with 5%CO_2. Wells were washed three times with PBS without calcium and magnesium. 50 µl of 5×10^4 DC re-suspended in X-vivo-15 media were added into each well and incubated for 2 hrs at 37^{o}C with 5%CO_2. After checking the viability of the cells, 25 µl of 100 ng/ml of purified LPS PAO1-10 (L2262, Sigma) or 25 µl of fixed PAO1-P (WT) at MOI 50 were added to appropriate wells and incubated for 4 hrs at 37^{o}C with 5%CO_2. Supernatants were collected and stored at -80^{o}C for cytokines quantification.

2.13.2 Cytokine quantification

Magnetic luminex assay kit (LXSAHM-7, R&D) was used to measure the cytokines in supernatant. Color-coded magnetic micro-particles were coated with specific cytokines antibodies. After adding 50 µl of micro-particles to wells, 50 µl supernatant and standards were added to appropriate wells and incubated for 2 h at room temperature on a shaker at 800 rpm. Wells were washed three times with the provided washing buffer. Then 50 µl biotinylated antibody cocktail was added to each well and incubated for 1 h at room temperature on a shaker at 800 rpm. After washing the unbound biotinylated antibody three times, 50 µl of streptavidin conjugated to phycoerythrin (PE) were added and incubated for 30 min at room temperature on a shaker at 800 rpm. The wells were washed three times and the micro-particles were re-suspended in 100 µl of the washing buffer per well and

incubated for two min at room temperature on a shaker at 800 rpm. Samples and standard were read using Bio-Rad Bio-Plex 200 system

2.14 Statistical analysis

Statistical analysis was performed in GraphPad Prism v 7.02. Significance was calculated by either one-way ANOVA or Two-way ANOVA.

Chapter 3: Recognition of *Pseudomonas aeruginosa* biofilms by MR-CTLD4-7-Fc and Fc-DC-SIGN

3.1 Introduction

PA biofilms are highly organized bacterial communities that provide advantage to bacteria compared to the planktonic lifestyle (Ma et al., 2009b). Persistent chronic lung infection in CF is attributed to the presence of biofilms (Hauser et al., 2011a). During infection, the mechanism of resistance of PA biofilms against phagocytes is not completely understood but likely depends on extracellular polymeric substance (EPS) which is composed of polysaccharides, nucleic acids, proteins, and extracellular DNAs (eDNAs) (Borlee et al., 2010a, Irie et al., 2012a). EPS maintains the integrity of biofilms protecting bacterial cells from harsh environmental conditions and plays a crucial role in the adherence stage during the transition of cells from the planktonic to biofilm lifestyle (Stoodley et al., 2002a, Romling et al., 2013). The formation of biofilms has been reported in several human pathogens, such as *Staphylococcus aureus, Vibrio cholerae, Candida albicans, Legionella pneumophila, Klebsiella pneumoniae, Enterobacter, Listeria, Campylobacter* spp*., Escherichia coli,* and *Salmonella typhimurium* (Donlan, 2002, Stoodley et al., 2002a, Romling et al., 2013). Biofilms are widely distributed and found on different surfaces including medical devices, such as urinary catheters, water system piping, contact lenses as well as CF mucus plugs (Donlan, 2002, Ma et al., 2009b).

A crucial component of EPS is extracellular polysaccharides. These polysaccharides perform a variety of functions, such as promoting the attachment of cells to surfaces, maintaining the biofilm structure, and protecting from host

defence and harsh environmental agents. Three main polysaccharides have been identified in PA biofilms: alginate, Psl, and Pel (Ryder et al., 2007a). These polysaccharides varied in chemical structure and also in their biosynthetic mechanisms. Alginate facilitates the persistence of biofilms in CF patients and is commonly found in mucoid PA strains (Wozniak et al., 2003). Alginate is not essential in the formation of biofilms (Nivens et al., 2001) but enhances their antimicrobial resistance (Hentzer et al., 2001a). Psl and Pel are utilized by non-mucoid PA strains such as PAO1 and PA14 to form biofilms (Wozniak et al., 2003). Pel is positively charged and interact with eDNA through the ionic interaction. Pel is composed of 1-4 linked partially acetylated galactosamine and glucosamine sugars (Jennings et al., 2015). Pel serves as a protective factor and initiates cell-cell interaction that lead to maintaining the biofilm matrix (Colvin et al., 2011a). Psl is galactose and mannose rich (Ma et al., 2007a). Psl has a crucial role in initiation of biofilm formation (Jackson et al., 2004a), and is required for bacterial cell adherence to a surface and maintenance of biofilm structure (Ma et al., 2006a). Jackson *et al*. showed that a PA strain that overexpresses Psl produces more biofilms (Colvin et al., 2012c).

Many factors have been involved in *psl* regulation and biofilm overproduction. Recently, the universal bacterial second messenger cyclic (c)-di-GMP has been implicated in a number of functions, including cell cycle regulation, biofilm formation, motility, and virulence factor production. CF patients' airways have been found to contain rugose small-colony variant (RSCV) mucoid PA strains associated with high levels of c-di-GMP (Romling et al., 2013). C-di-GMP also positively regulates production of Pel and Psl polysaccharides (Hickman et al., 2005a). Moreover, it has also been shown that Psl elevates c-di-GMP levels, which leads to the generation of a positive feedback loop that results in the continuous

stimulation of biofilm formation (Irie et al., 2012a). In contrast, low levels of c-di-GMP promote a planktonic lifestyle (Borlee et al., 2010a). It also has been shown that biofilms with high levels of c-di-GMP gain greater resistance to antibiotics (Byrd et al., 2011) due to the ability of c-di-GMP to control efflux pump genes (Gupta et al., 2014). PA adherence and cell aggregation are controlled by a number of genes. The *wspF* mutation activates the *wspR* gene, which results in elevated levels of the c-di-GMP and RSCV phenotype (Hickman et al., 2005b). In addition, the *wspF* mutation has the ability to convert smooth wild-type colonies to RSCV and vice versa (Starkey et al., 2009b).

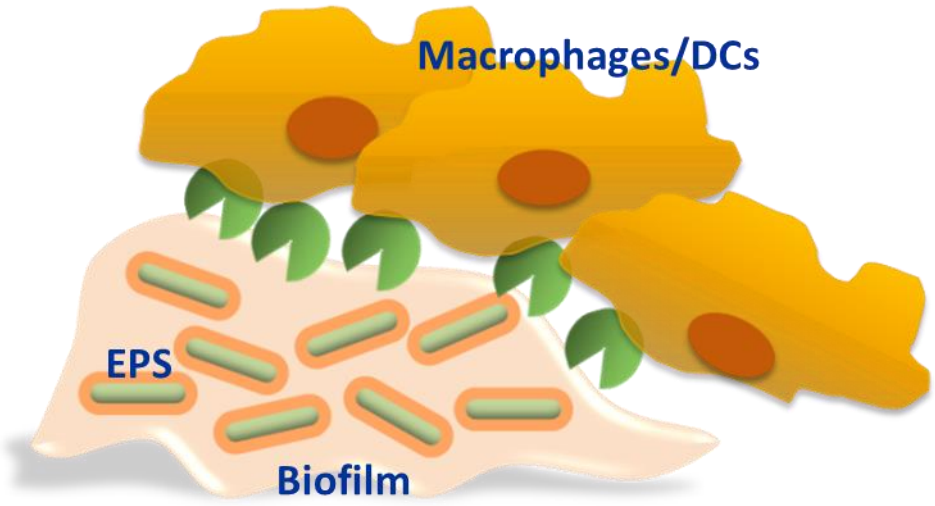
Few studies have been performed addressing the innate immune response to biofilm-associated infections. C-type lectin receptors (CLRs) play a crucial role in regulating the immune response as pattern recognition receptors (PRRs). CLRs have the ability to bind pathogen-associated molecular patterns (PAMPs), which lead to the expression of cytokines that determine the appropriate immune response (Geijtenbeek and Gringhuis, 2009). CLRs are found either as secreted soluble proteins or transmembrane proteins (Cambi et al., 2005). The latter can be divided into two groups based on the location of their amino N-terminus. Type I and type II have their termini pointing outward or into the cell cytoplasm, respectively. Type I CLRs contain several C-type lectin-like domains (CTLDs), whereas type II CLRs have a single CTLD (Figdor et al., 2002). CLRs perform a variety of important biological functions, such as phagocytosis, cell adhesion and complement activation (Kerrigan and Brown, 2009). Recognition of mannose, glucan, and fucose carbohydrates enables detection of most human pathogens. In particular, mannose is present on viruses, fungi, and mycobacteria. The last two can also be recognized through glucan. Fucose allows the recognition of helminths and some bacteria (van Kooyk and Rabinovich, 2008, Rothfuchs et al., 2007).

Dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN, CD209) belongs to the type II CLRs and is expressed by DCs and some macrophage populations (Kerrigan and Brown, 2009). DC-SIGN expression is induced by IL-4 and down-regulated by IFN-γ and TGF-β (Relloso et al., 2002). DC-SIGN consists of a CTLD, a stalk region that mediates multimerisation, a single transmembrane region, and a cytoplasmic tail associated with a number of internalization motifs (Kerrigan and Brown, 2009). DC-SIGN undergoes tetrameric clustering, which increases the binding avidity (Mitchell et al., 2001). It has the ability to bind to ligands that contain mannose and/or fucose (Geijtenbeek and Gringhuis, 2009). Originally, DC-SIGN was identified as a receptor that binds intercellular adhesion molecule 3 (ICAM3) to facilitate T-cell proliferation mediated by DCs. DC-SIGN has been found to detect a wide range of pathogens, such as *C. albicans, Mycobacterium tuberculosis, Schistosoma mansoni, Aspergillus fumigatus, Helicobacter pylori* and the human immunodeficiency virus (HIV) (Geijtenbeek et al., 2000b, Geijtenbeek et al., 2000a). Interaction of DC-SIGN with mannose containing ligands results in the phosphorylation of Raf-1, which in turn enhances the phosphorylation and acetylation of the nuclear factor (NF)-kB subunit p65, leading to an overall increase of the transcription gene levels such as IL12 (Gringhuis et al., 2009a). Intriguingly, accumulation of DCs expressing DC-SIGN positively correlates with presence of biofilms in chronic rhinosinusitis with nasal polyps (CRSwNP) (Karosi et al., 2013).

Mannose receptor (MR) is a type I CLR. It is expressed by selected populations of DCs and macrophages, and nonvascular endothelium among others (Martinez-Pomares, 2012). MR is upregulated by IL-4, IL-10, and IL-13, and down-regulated by IFN-γ (Kerrigan and Brown, 2009). Many organisms can be recognized by MR, such as *C. albicans, Leishmania, M. tuberculosis,* HIV, dengue virus*, K.*

pneumoniae, and *Streptococcus pneumoniae* (Gazi and Martinez-Pomares, 2009). MR has several roles, which include the promotion of antigen presentation, clearance of molecules, and cellular activation (Martinez-Pomares, 2012). Structurally, MR consists of three extracellular regions: cysteine-rich (CR) domain, fibronectin type II domain (FNII), and eight CTLDs and a short cytoplasmic tail. The CR domain is involved in binding to sulphated sugars, the FNII domain is capable of binding to types I, II, III, and IV collagen, and the CTLDs are responsible for recognizing sugars terminated in mannose, fucose, or N-acetyl glucosamine in a calcium-dependent manner. It has been shown that the binding affinity of multiple CTLDs (4–8) is the same as for a the whole receptor (Taylor et al., 2005a, Gazi and Martinez-Pomares, 2009).

3.2 Hypothesis



Tamanna Rahman, a former PhD student in Dr Martinez-Pomares's lab, has shown that PA biofilms bind the CTLD4-7 region of MR and this binding is increased when Psl expression increases. In view of the ability of CLRs to recognise pathogens and influence immune activation, we hypothesized that C-

type lectins such as MR and DC-SIGN, might contribute to recognition of PA polysaccharides within EPS.

3.3 Aims

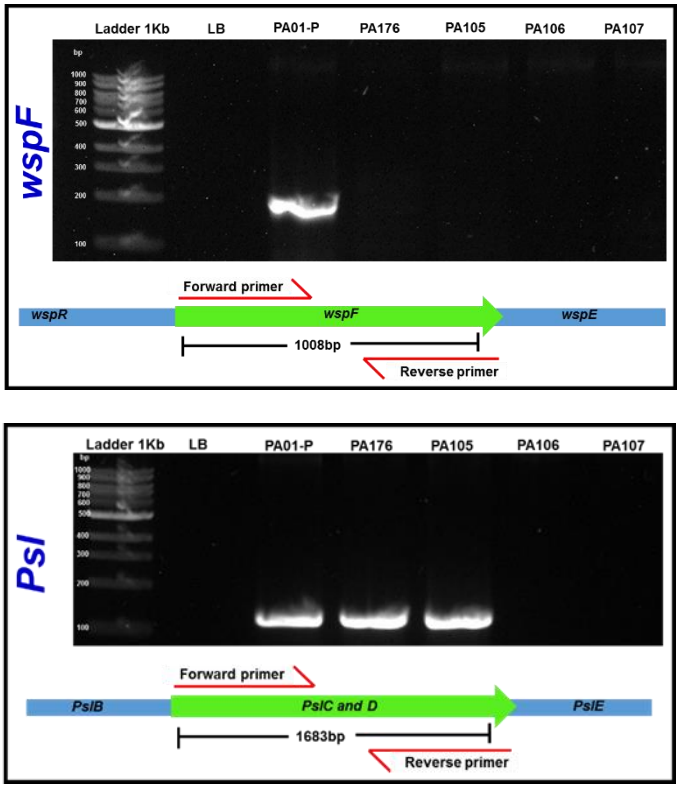
- Quantify biofilm formation of PA laboratory strains and clinical isolates.
- Determine the capability of MR and DC-SIGN to interact with PA biofilms.
- Examine whether the binding of MR and DC-SIGN is calcium and carbohydrate dependant.
- Evaluate and compare the binding of different DC-SIGN constructs to PA biofilms in different buffers.

3.4 Results

3.4.1 PCR analysis confirms mutations in PAO1-P strains with the *ΔwspF* background

In order to test if CLRs bind PA biofilms and display selective binding towards particular EPS components, different PA strains that express either Psl and/or Pel were investigated. The *pel* and *psl* operons, involved in the production of EPS, are upregulated by an increased level of c-di-GMP. It has been shown that the loss of the *wspF* gene is one of the factors that results in an increased level of c-di-GMP (Hickman et al., 2005a). Therefore, it was decided that the use of strains with the Δ*wspF* background would be beneficial for this study (Table 2.1). The generation of the deletion of the *wspF* gene in the PAO1-Parsek (P) strain was described by (Hickman et al., 2005a). The mutations of the *pel* and *psl* operons in the Δ*wspF* background were described by (Starkey et al., 2009b, Kirisits et al., 2005),

respectively. These mutations were confirmed by PCR using the primers and methodology described in section 2.2. Forward and reverse primers that anneal inside the *wspF* gene were designed and the presence of mutation was tested by assessing presence or absence of the corresponding PCR product. The Δ*wspF* mutation was present in all strains tested expect in PAO1-P. Then Δ*psl* mutation was confirmed using primers that target *pslC* and *pslD*. Primers that target *pelA* were used to check the Δ*pel* mutation. The *pel* mutation was only present in PAO1-P*ΔwspF*Δ*Pel* (PA105) and PAO1-P*ΔwspF*Δ*Pel*Δ*Psl* (PA107), while the *psl* mutation was present in PAO1-P*ΔwspF*Δ*Psl* (PA106) and PAO1-P*ΔwspF*Δ*Pel*Δ*Psl* (PA107) (Figure 3.1).



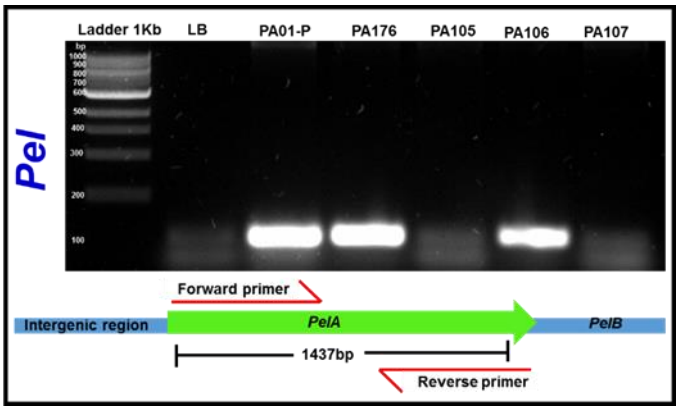


Figure 3.1: Confirmation of mutations in *wspF* and *psl* and/or *pel* operons using PCR in PA176, PA105, PA106 and PA107.

PCR reactions were carried out in 25 µl and contained 1µl of overnight culture, 0.1 µM of each of the reverse and forward primers, and 1X of GoTaq G2 green master mix in nuclease-free water. A 1 Kbp marker was used to determine size of PCR products.

3.4.2 Strains deficient in *wspF* that express Psl and/or Pel polysaccharides displayed altered growth in X-vivo-15 media

A commercial defined medium (X-vivo 15) was used to investigate the growth of the different PAO1-P-derived strains. Based on the growth curves, strains that overexpress Psl and/or Pel, such as PAO1-P*ΔwspF* (PA176), PAO1-P*ΔwspF*Δ*Pel* (PA105), and PAO1-P*ΔwspF*Δ*Psl* (PA106), grew slower than WT PAO1-P and PAO1-P*ΔwspF*Δ*Pel*Δ*Psl* (PA107), which either expressed normal Psl and Pel or was Psl/Pel deficient (Figure 3.2). This indicates that strains that express a high level of EPS due to the increased level of c-di-GMP might prefer to grow as biofilms instead of planktonic life style.

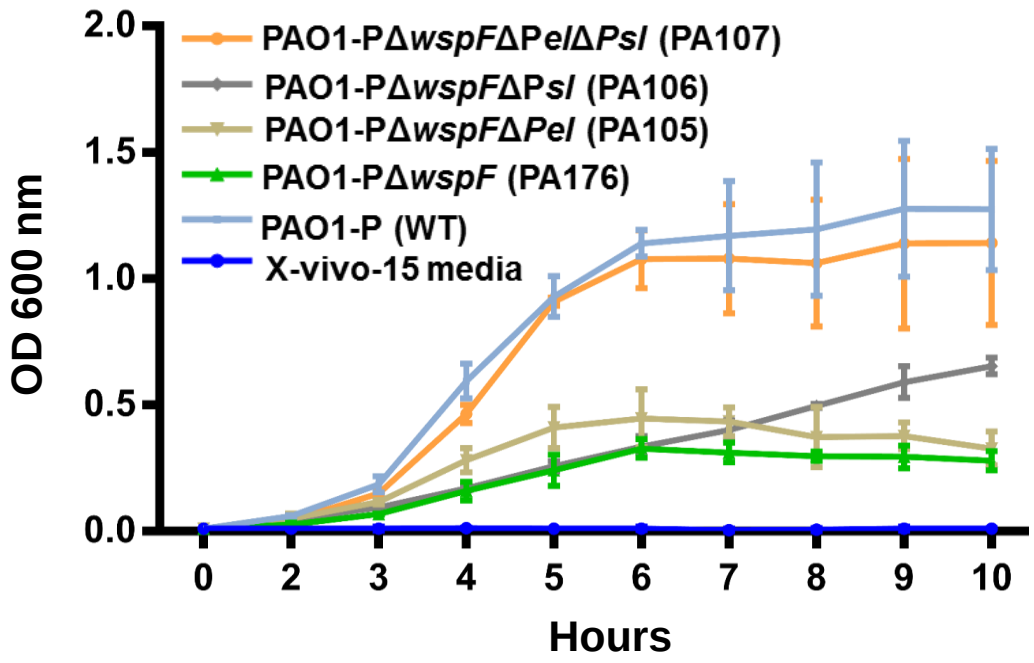


Figure 3.2: Growth curves of PAO1-P strains that express different combinations of EPS components in X-vivo-15 media.

The OD_{600nm} of overnight cultures were adjusted to 0.01 in 20 ml of pre-warmed X-vivo-15 media in a 250 ml conical flask and incubated at 37^{o}C with shaking at 200 rpm during the experiment. The absorbance was measured hourly at 600nm for 10 consecutive hours using a spectrophotometer. Error bars indicate the standard deviation (SD) of three independent repeats.

3.4.3 Lack of *wspF* alongside expression of Psl and/or Pel promotes biofilm formation

3.4.3.1 Quantification of biofilm formation by PAO1-P-derived mutant strains using crystal violet assay

After testing the growth of the PAO1-P-derived strains PA176, PA105, PA106 and PA107 in X-vivo 15, they were next tested for biofilm formation in the same medium using a crystal violet assay as described in section2.3.1. Data shows that all the strains displayed variable ability to form biofilm except PAO1-P*ΔwspF*Δ*PelΔPsl* (PA107) (Figure 3.3) which was confirmed as biofilm-deficient. The hyper-biofilm-

forming PAO1-P*ΔwspFΔPel* (PA105) strain produced two-fold more biofilm than PAO1-P*ΔwspF* (PA176), which had the same Δ*wspF* background. PAO1-P*ΔwspFΔPsl* (PA106) produced less biofilm than PA105. To conclude, the Δ*wspF* Δ*pel* strain which overexpresses Psl, formed more biofilm in comparison to the Δ*wspF*, Δ*wspF*Δ*psl*, and Δ*wspF*Δ*pel*Δ*psl* strains. This supports the role of Psl as a important component of the PA matrix. The results also indicate that the differences in growth between different strains appear to relate to their capacity to form biofilm.

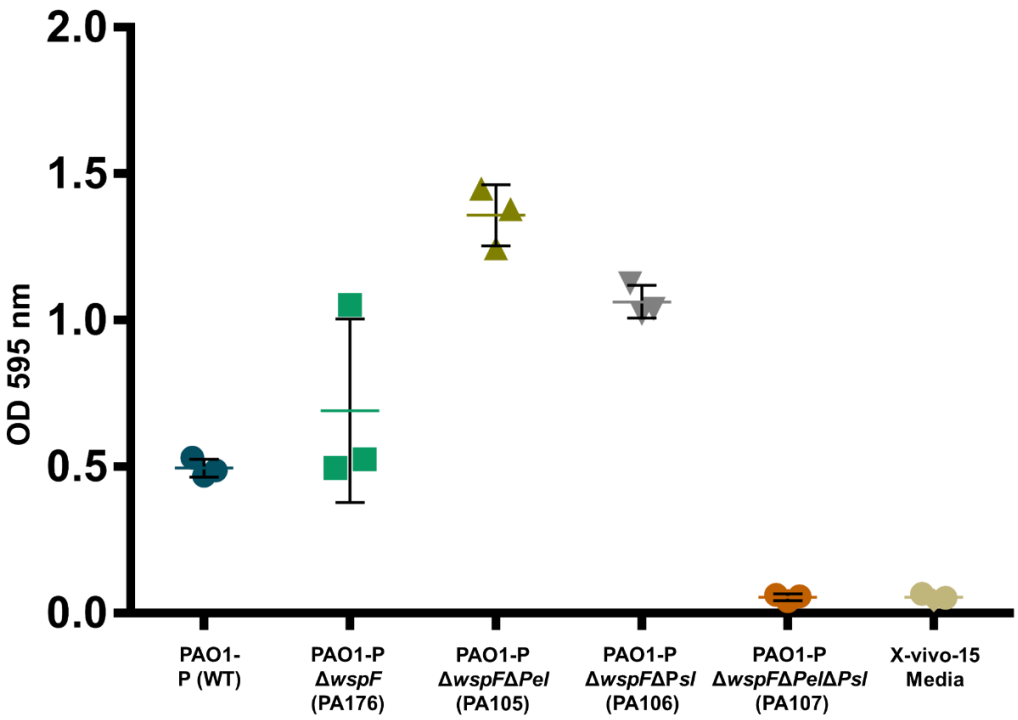


Figure 3.3: Assessment of biofilm formation of PAO1-P WT and Δ*wspF* strains in X-vivo-15 media using the crystal violet method.

Cultures in X-vivo-15 with 0.04 $OD_{600\ nm}$ were incubated statically on Costar plate for 24 h at 37°C with 5%CO_2. After washing the non-adherent material with HPLC water, the amount of biofilm was quantified by staining with 1 %(w/v) crystal violet and measuring the absorbance at 595 nm. Error bars indicate the standard deviation (SD) of three independent repeats.

3.4.3.2 Quantification of biofilm formation by PAO1-P-derived mutant strains using ELISA

Another approach was used to quantify biofilms formed by strains that express different combination of EPS polysaccharides. Biofilms grown in X-vivo-15 media were tested for PA biomass by analysing the binding of polyclonal anti-*Pseudomonas* antibody as described in section 2.3.2. We found that all the strains formed different amounts of biofilms depending on the types of EPS present. As above PAO1-P*ΔwspF*Δ*Pel*Δ*Psl* (PA107) failed to show biofilm formation. PAO1-P*ΔwspF*Δ*Pel* (PA105) and PAO1-P*ΔwspF*Δ*Psl* (PA106) formed more biofilm than PAO1-P*ΔwspF* (PA176) (Figure 3.4). This supports the crucial role of EPS carbohydrates (Psl or Pel) in biofilm formation. Using this assay differences in biofilm among strains PAO1-P, PA176, PA105 and PA106 were less obvious with PA106 biofilms presenting the highest PA biomass.

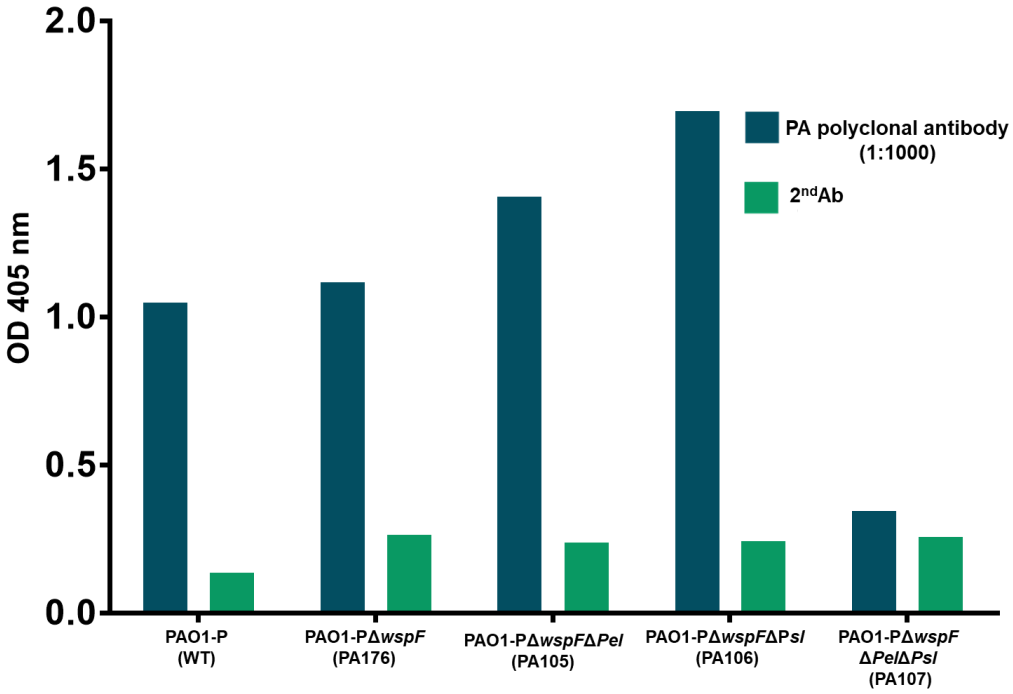


Figure 3.4: Quantification of biofilm formation by PAO1-P WT and Δ*wspF* strains in X-vivo-15 by ELISA.

The OD of mid log phase cultures were adjusted to 0.04 $OD_{600\ nm}$ in X-vivo-15 and incubated statically on a Maxisorp plate for 24 h at 37°C with 5%CO_2. After fixing the biofilm with 2% (v/v) formalin, it was incubated with rabbit anti-*Pseudomonas* polyclonal antibody (1:1000 dilution) for 90 min at room temperature. The binding was detected using anti-rabbit IgG Fc-specific antibody conjugated to AP (1:2000 dilution). Absorbance was read at 405 nm. N=2.

3.4.4 Biofilm is recognized by the CTLD4-7 region of MR and the extracellular region of DC-SIGN

After having established the methodology to generate consistent biofilms, we assessed whether chimeric proteins (CTLD4-7-Fc and Fc-DC-SIGN, containing the CTLD4-7- region of mouse MR or the extracellular region of DC-SIGN fused to the Fc portion of human IgG) were able to bind to biofilms. We wanted to explore the role of C-type lectins, particularly MR and DC-SIGN, in the recognition of PA biofilms. Thus, we used a combination of PA strains which have the same level of c-di-GMP but express different levels of EPS. Results show that CTLD4-7-Fc and Fc-DC-SIGN bind to PA biofilms in a dose-dependent manner and that DC-SIGN-Fc binds significantly better than CTLD4-7-Fc to all strains tested regardless of the different amounts and types of EPS they produce (Figure 3.5). Both CTLD4-7-Fc and Fc-DC-SIGN strongly recognised the positive control Mannose-PAA indicating that the reduced binding of CTLD4-7-Fc to biofilms compared to Fc-DC-SIGN is not due to reduced activity of the recombinant protein produced in house. In another attempt, in order to increase the binding affinity, CTLD4-7-Fc was pre-incubated with anti-human IgG Fc-specific antibody at room temperature for 30 mins, then the binding assay was conducted. Unfortunately, this didn't enhance the binding of CTLD4-7-Fc to biofilms (data not shown).

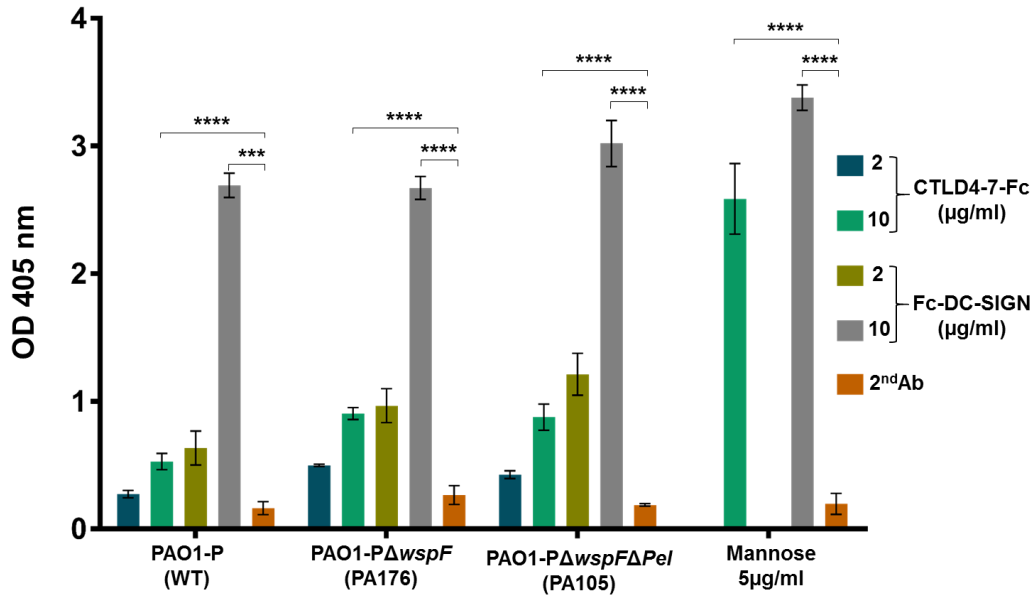


Figure 3.5: Binding of CTLD4-7-Fc and Fc-DC-SIGN to PAO1-P biofilms.

Biofilms from different strains, developed over 24 hours using Costar plates, were incubated with 2 and 10µg/ml of CTLD4-7-Fc and Fc-DC-SIGN chimeric proteins for 2 hours at room temperature. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000 dilution). Absorbance was read at 405 nm. Error bars indicate the standard deviation (SD) of three independent experimental repeats. Statistical significance was calculated by two-way ANOVA test. P values are represented as follows: *** = $p \leq 0.001$ and **** = $p \leq 0.0001$.

3.4.5 Dectin-1 shows very weak binding to PAO1-P biofilms

After we found different PAO1-P-derived strains recognized by both CTLD4-7-Fc and Fc-DC-SIGN chimeric proteins, we wanted to explore the ability of another important CLR, Dectin-1, (see section 11.6.3 for more details on Dectin-1) to bind PA biofilms. We used a combination of PA strains which have the same level of c-di-GMP but express different levels of EPS. Results show that Dectin-1 weakly binds to all strains but, surprisingly binding was negatively impacted by increased protein concentration (Figure 3.6).

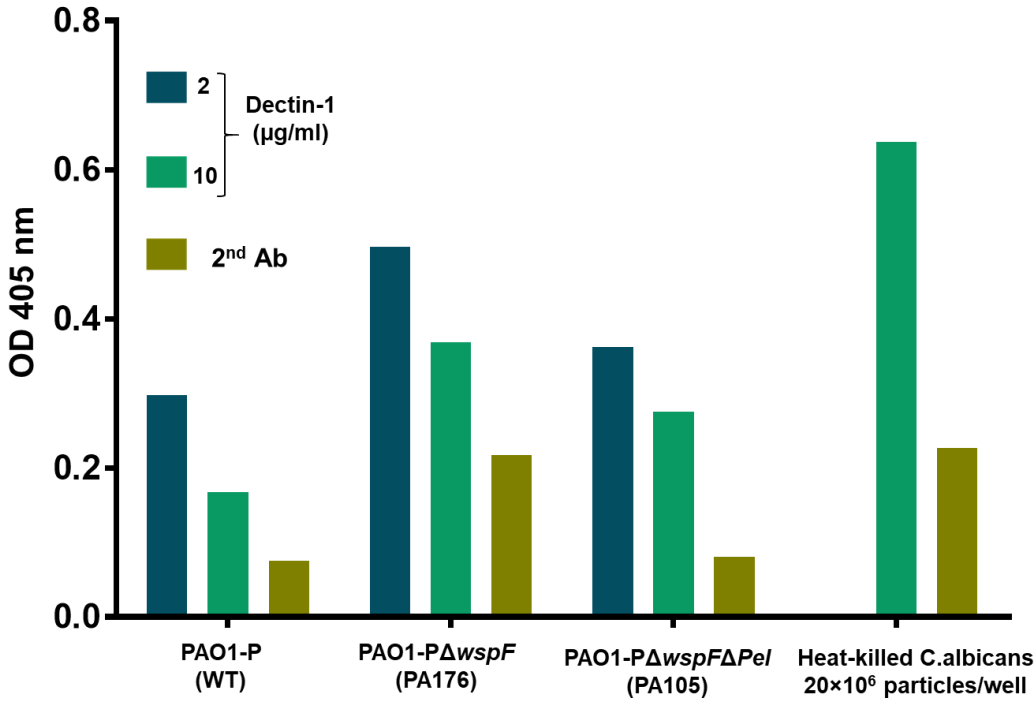


Figure 3.6: Binding of Dectin-1 to PAO1-P biofilms

Biofilms from different strains, developed over 24 hours using Costar plates, were incubated with 2 and 10µg/ml of Dectin-1 chimeric protein for 2 hours at room temperature. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000 dilution). Absorbance was read at 405 nm. N=2

3.4.6 The binding of the CTLD4-7-Fc and Fc-DC-SIGN to PAO1-P biofilms is calcium dependent

MaxiSorp plates have high protein-binding capacity, and researchers extensively use them for binding assays. We used both Costar and Maxisprp plates to compare the biofilms levels formed by different PAO1-P-derived strains. Data suggests that

there is no difference between plates; thus, it was decided to use Maxisorp plates in the subsequent binding assays (Figure 3.7).

It has been shown that the binding of MR-CTLD (4–8) and DC-SIGN to their carbohydrate ligands is calcium dependent. In order to confirm that the binding of these lectins to PA biofilms or carbohydrate positive controls such as mannose and fucose is calcium dependent, the binding assay was carried out in either TSB buffer, which contains calcium, or calcium–deficient TSB buffer. We found that MR and DC-SIGN strongly bound to PA biofilm, mannose, and fucose in TSB buffer with calcium, and observed a complete elimination of lectin binding in the absence of calcium (Figure 3.8). Data suggest that calcium is necessary for the binding of MR and DC-SIGN to PA biofilms.

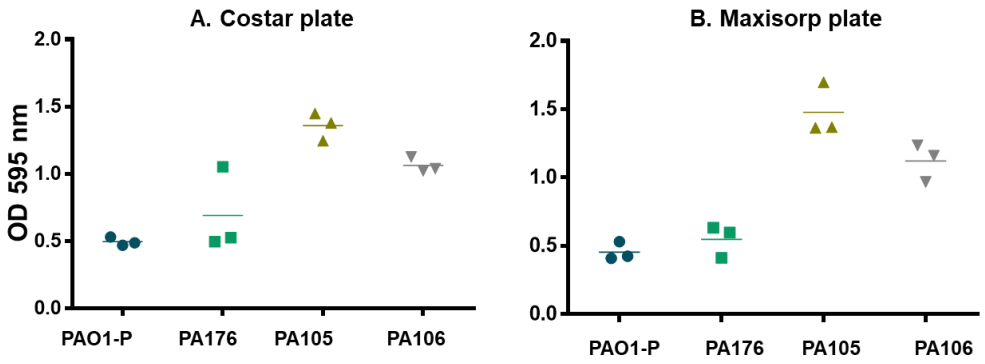
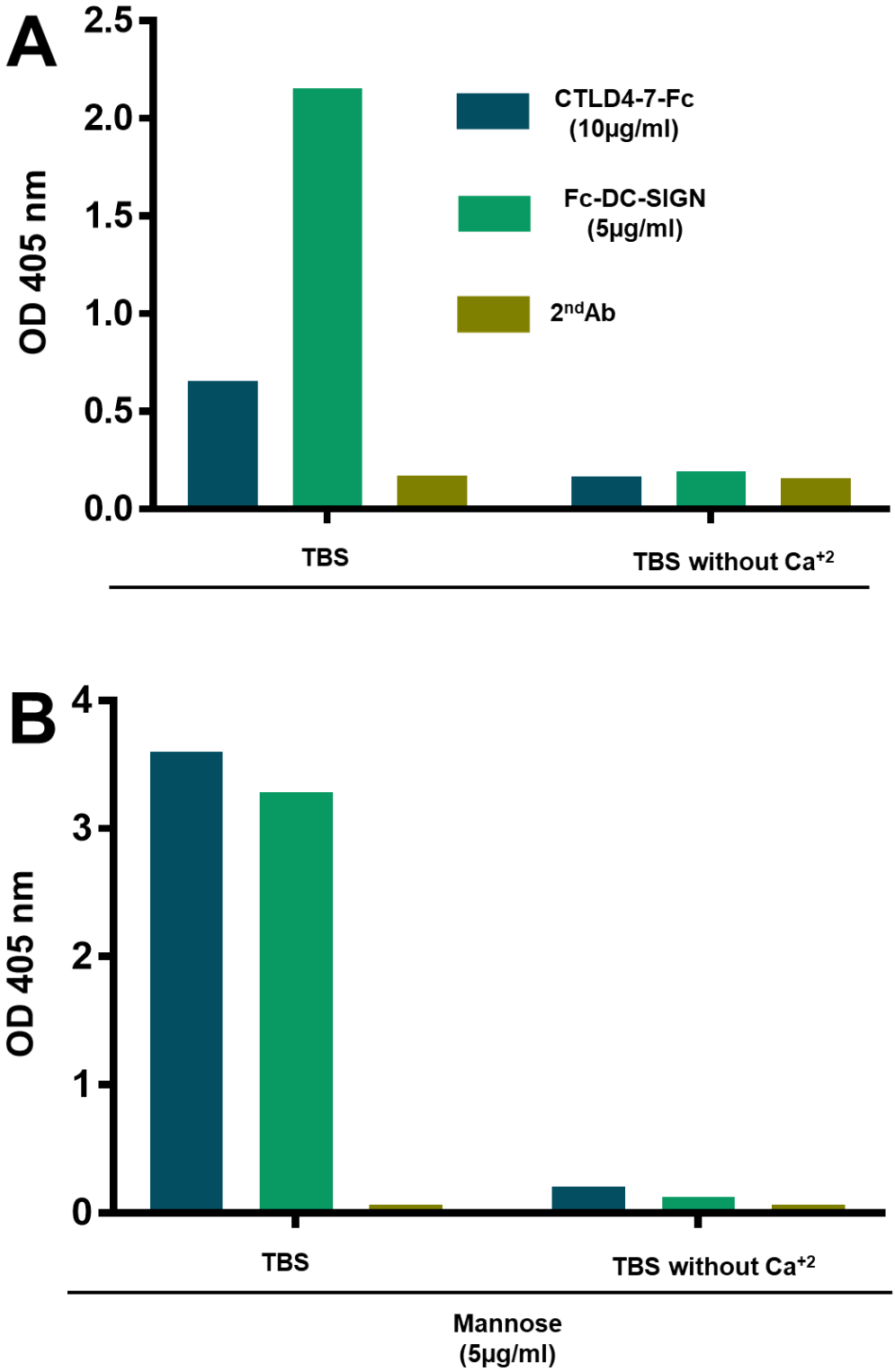


Figure 3.7: Comparison between the biofilms formed by different PAO1-P-derived strains using Costar plate (A) and Maxisorp plate (B).


A
2.5
2.0
1.5
1.0
0.5
0.0
OD 405 nm
CTLD4-7-Fc
(10µg/ml)
Fc-DC-SIGN
(5µg/ml)
2ndAb
TBS
TBS without Ca+2
B
4
3
2
1
0
OD 405 nm
TBS
TBS without Ca+2
Mannose
(5µg/ml)

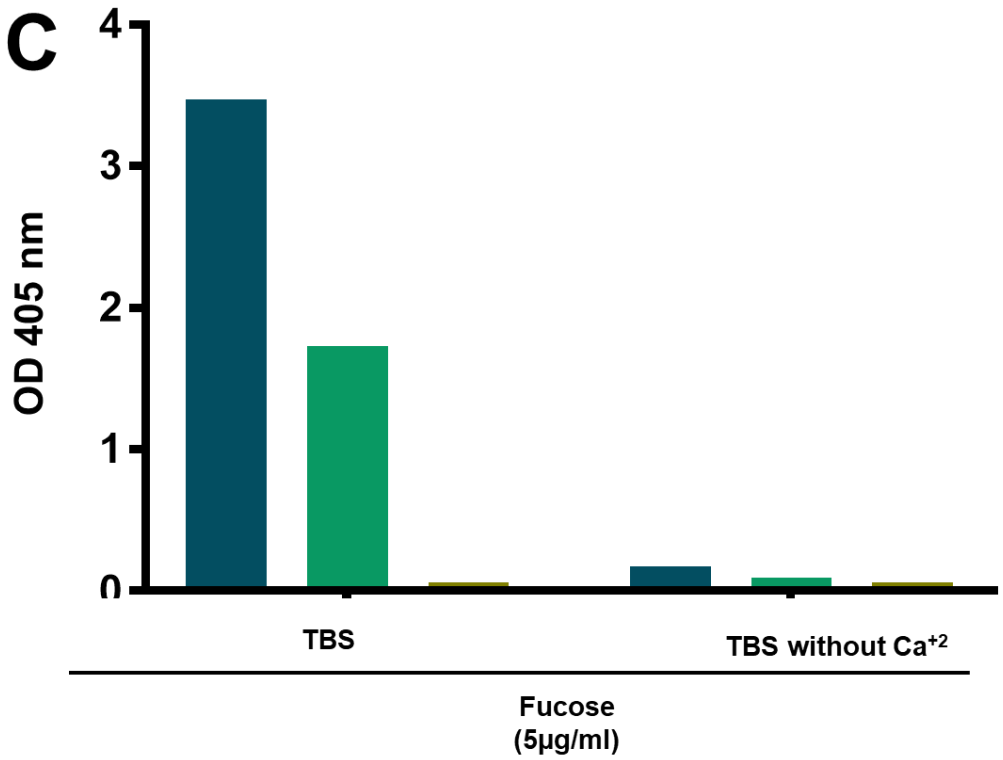


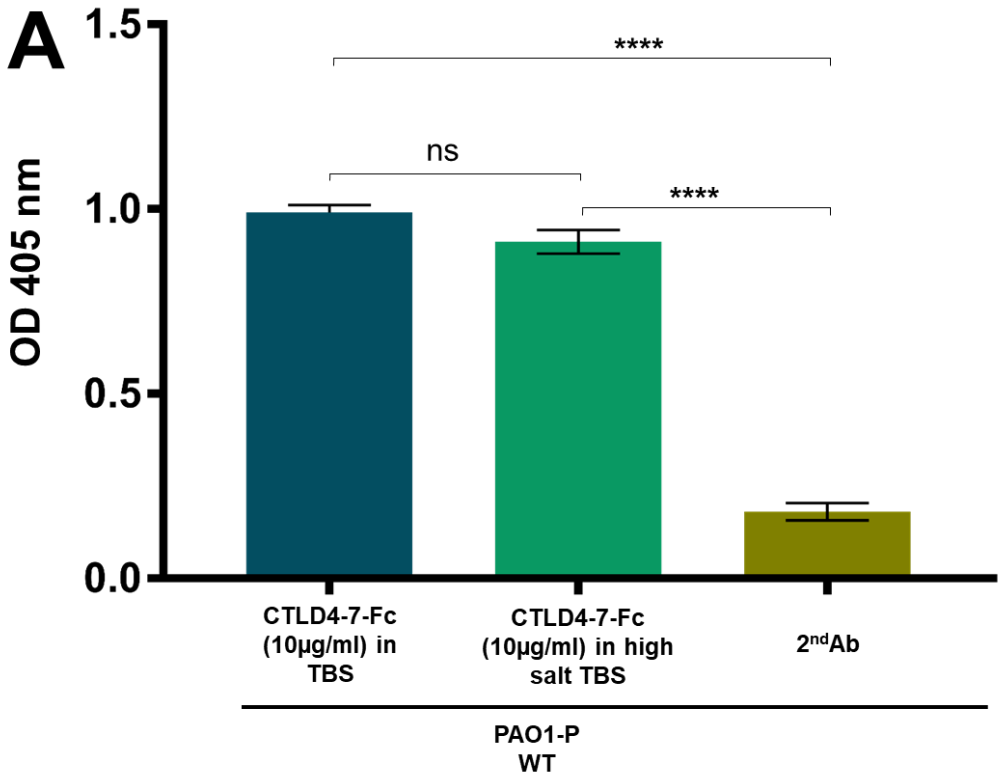
Figure 3.8: Binding of CTLD4-7-Fc and Fc-DC-SIGN to PAO1-P biofilms is calcium dependant.

PAO1-P WT biofilms developed over 24 hours on Maxisorp plate (A), mannose-PAA (B), or fucose-PAA (C) coated wells of Maxisorp plate were incubated for 2 hours at room temperature with 10µg/ml of CTLD4-7-Fc and 5µg/ml Fc-DC-SIGN chimeric proteins prepared in TSB buffer with and without calcium. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000 dilution). Absorbance was read at 405 nm. N=2.

3.4.7 The binding of CTLD4-7-Fc and Fc-DC-SIGN to PAO1-P biofilms is carbohydrate dependent

3.4.7.1 Binding of CTLD4-7-Fc and FC-DC-SIGN is not affected by the use of high salt buffer

To further confirm that the binding of CTLD4-7-Fc and Fc-DC-SIGN to PA biofilms is specific, it was considered necessary to test if the interaction of both proteins with PA biofilms could be inhibited by carbohydrates. Towards this aim, we first tested the effect of high salt on the binding of both proteins as, in our hands, lectin binding should not be affected by salt and high salt conditions make the inhibition assay more robust. We assessed the binding of MR and Fc-DC-SIGN to PA biofilms in either TSB with normal salt (0.154 M) or in TSB with high salt concentration (1M). We found that there is no effect of the high salt buffer, and MR or DC-SIGN binds in both buffers although a slight decrease was observed in the high salt condition (Figure 3.9).



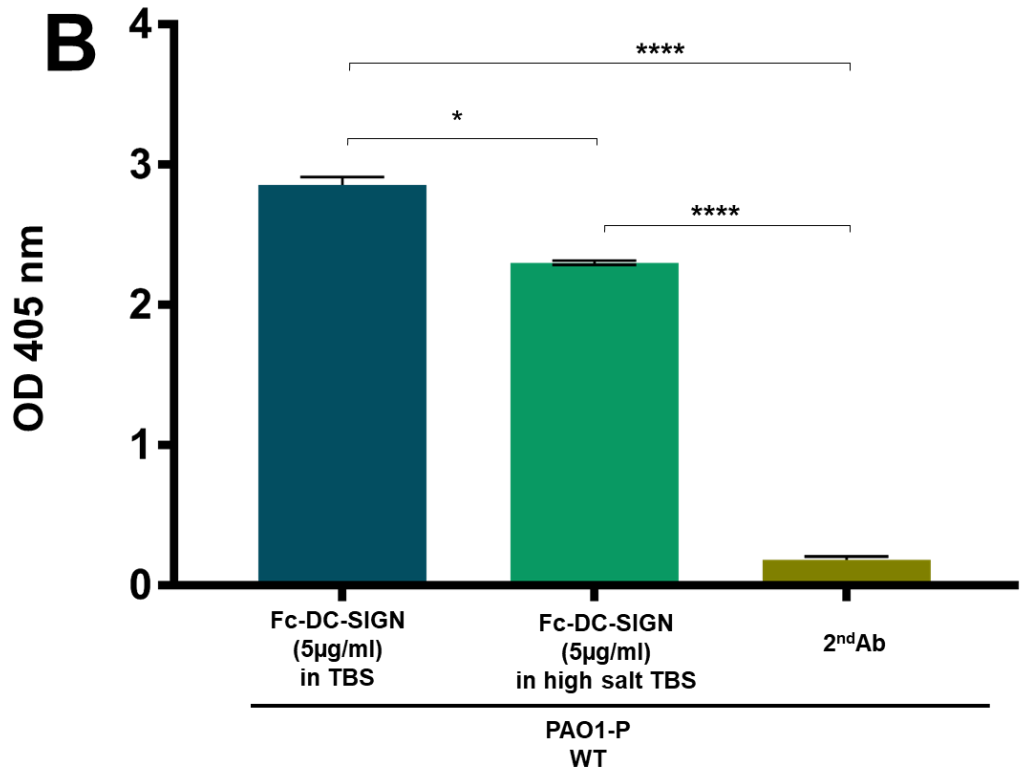


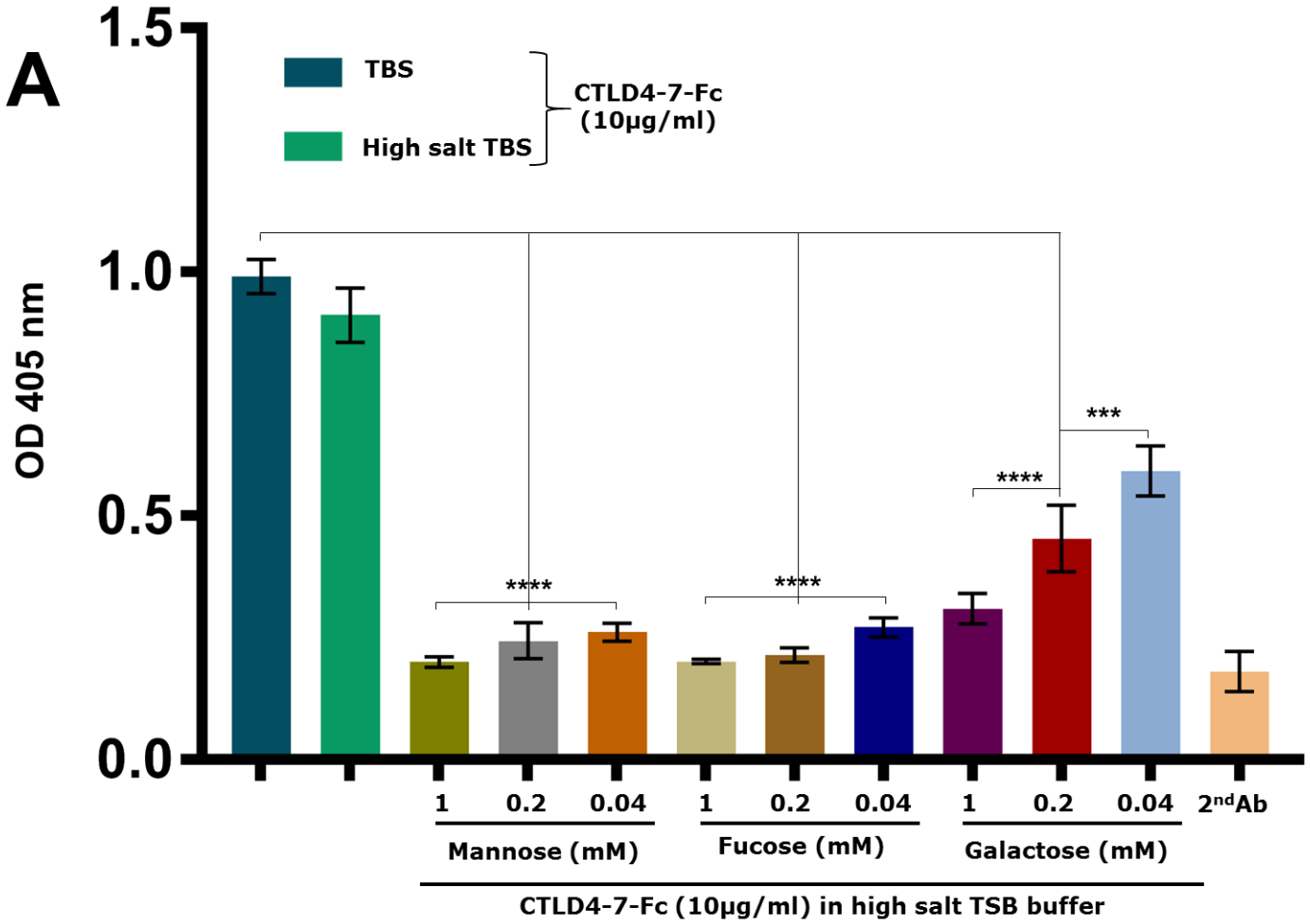
Figure 3.9: The binding of CTLD4-7-Fc and Fc-DC-SIGN is not affected by the use of high salt buffer.

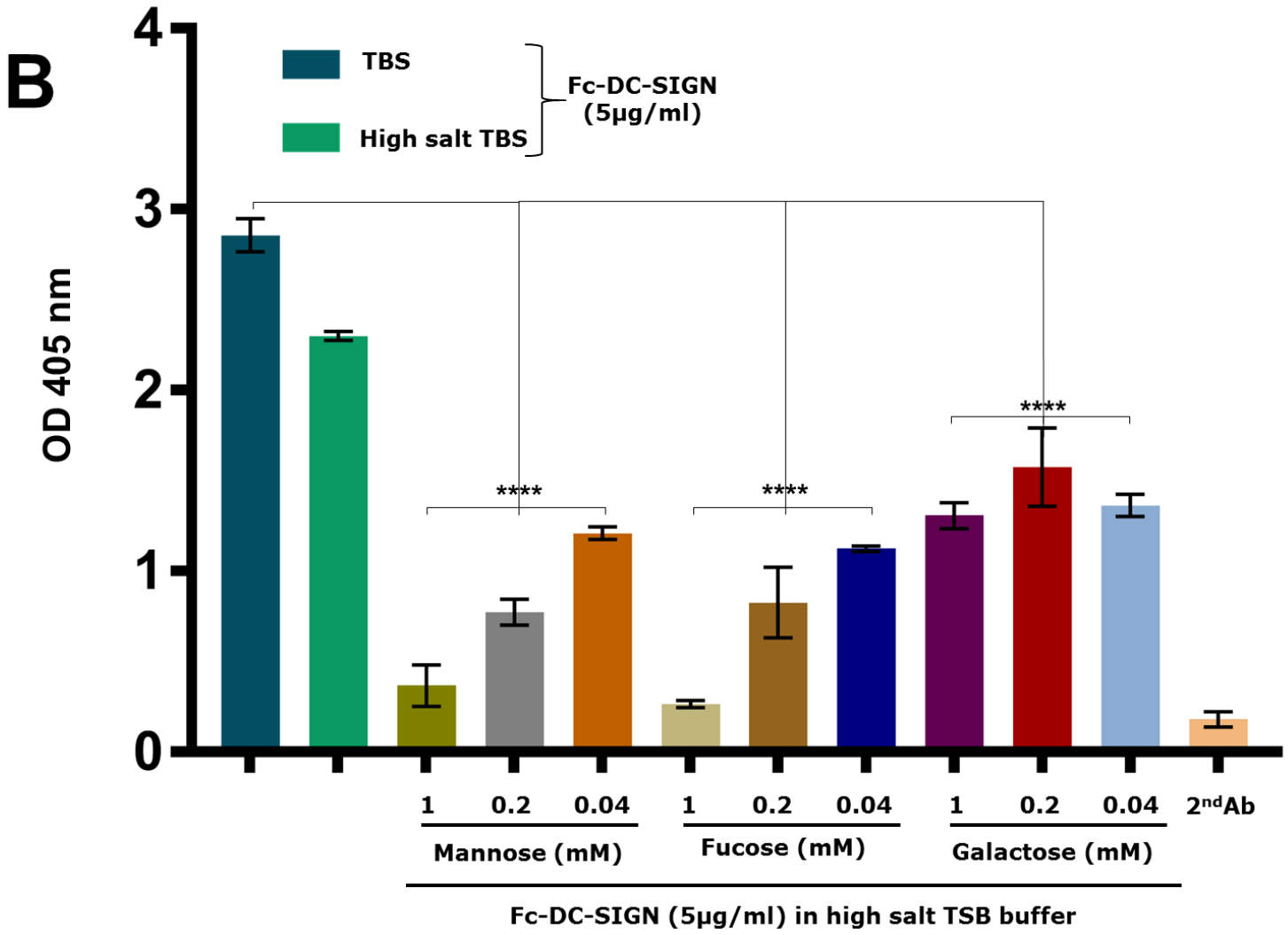
The binding of CTLD4-7-Fc (A) and Fc-DC-SIGN (B) to PAO1-P WT biofilms, developed over 24 hours, was assessed using TSB buffer with normal salt concentration (154 mM) or TSB buffer with high salt concentration (1 M). Error bars indicate the standard deviation (SD) of three independent repeats. Statistical significance was calculated by two-way ANOVA test. **** = $p \leq 0.0001$.

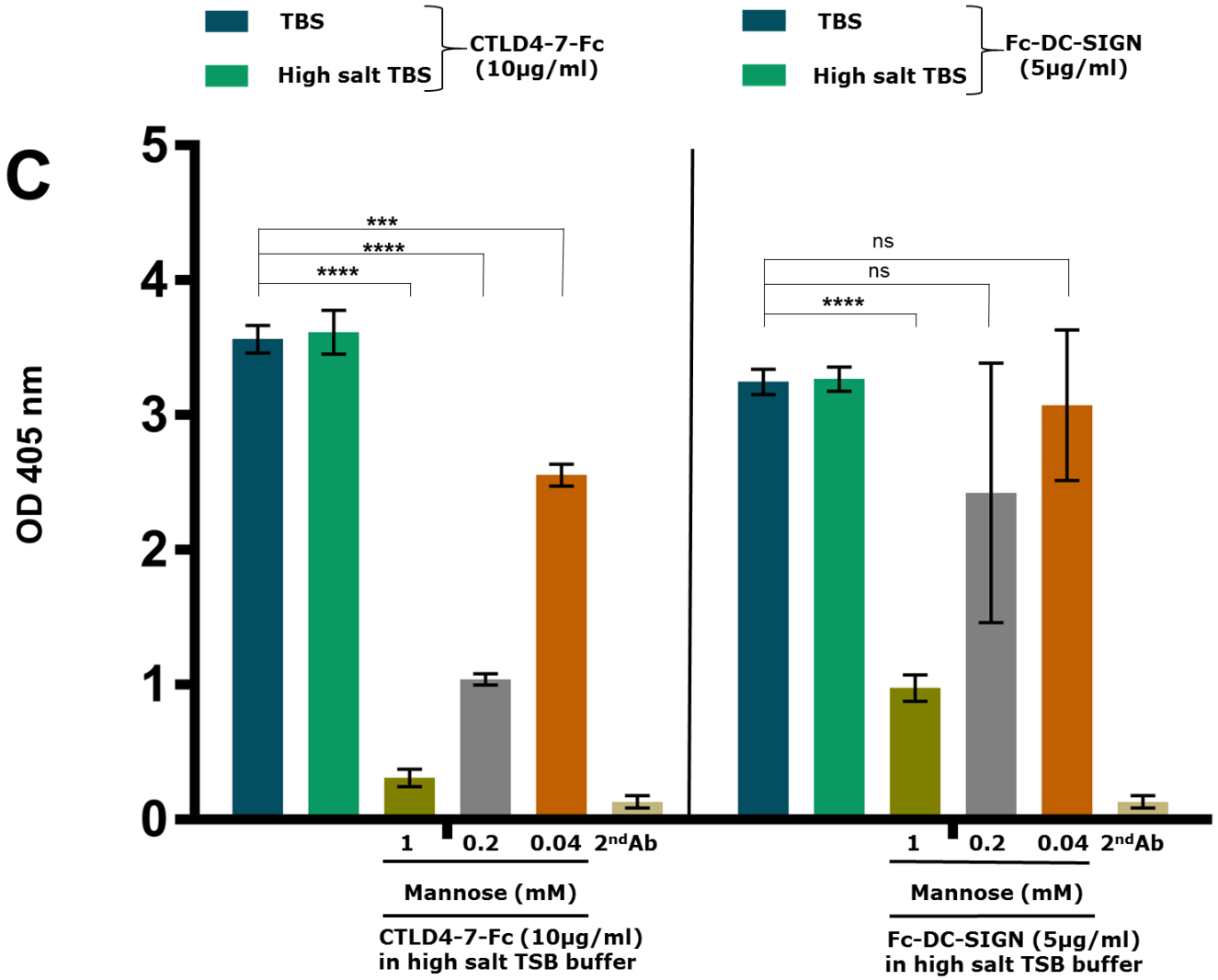
3.4.7.2 The inhibition of CTLD4-7-Fc and Fc-DC-SIGN binding

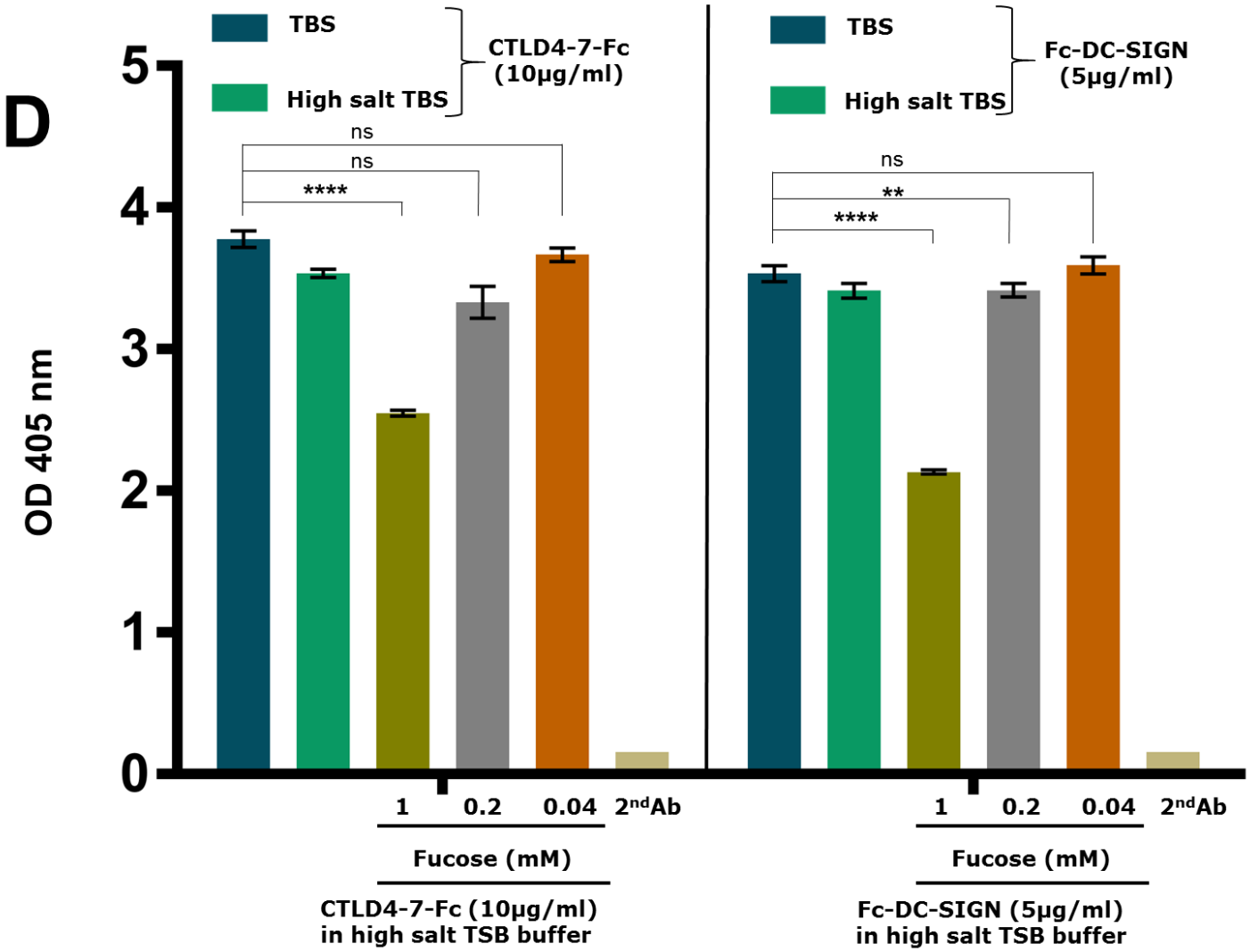
To determine whether the binding of MR and DC-SIGN to PAO1-P biofilms is carbohydrate dependent, the following monosaccharides (mannose, fucose, or galactose) were tested for their ability to inhibit the binding of MR and DC-SIGN to plates containing either PAO1-P biofilm or polysaccharides positive controls (mannose-PAA, fucose-PAA, or galactose-PAA). We used the monosaccharides at 1, 0.2, and 0.04 mM to determine whether inhibition was dose dependent.

Mannose and fucose were able to inhibit the binding of 10 µg/ml of MR-CTLDs (Figure 3.10-A) and 5 µg/ml of DC-SIGN (Figure 3.10-B) to the biofilms. The inhibition was dose-dependent and complete inhibition was reached at the concentration of 1 mM. Galactose was also able to inhibit the binding of both lectins to biofilms (Figure 3.10 [A and B]) but to a lesser extent compared to mannose and fucose. In the same experiments we tested whether these monosaccharides could inhibit the binding of MR and DC-SIGN to mannose-PAA, fucose-PAA, or galactose-PAA. The monosaccharides mannose and fucose were able to inhibit the binding of 10 µg/ml of MR-CTLDs and 5 µg/ml of DC-SIGN. The inhibition was dose-dependent in both cases although fucose was less able to inhibit binding to fucose-PAA than mannose to mannose-PAA (Figure 3.10 [C and D]). Intriguingly while CTLD4-7-Fc did not bind to galactose-PAA-coated wells, (Figure 3.10-E), Fc-DC-SIGN showed some binding to wells coated with galactose-PAA that could be readily inhibited by the presence of galactose (Figure 3.10-E).


A
1.5
1.0
0.5
0.0
OD 405 nm
TBS
High salt TBS
CTLD4-7-Fc (10µg/ml)
****
****
****
***
1
0.2
0.04
1
0.2
0.04
1
0.2
0.04
2ndAb
Mannose (mM)
Fucose (mM)
Galactose (mM)
CTLD4-7-Fc (10µg/ml) in high salt TSB buffer


B
TBS
High salt TBS
Fc-DC-SIGN
(5µg/ml)
OD 405 nm
4
3
2
1
0
****
****
****
1
0.2
0.04
1
0.2
0.04
1
0.2
0.04
2ndAb
Mannose (mM)
Fucose (mM)
Galactose (mM)
Fc-DC-SIGN (5µg/ml) in high salt TSB buffer


C
TBS
High salt TBS
CTLD4-7-Fc
(10µg/ml)
TBS
High salt TBS
Fc-DC-SIGN
(5µg/ml)
OD 405 nm
0
1
2
3
4
5
***
****
****
ns
ns
****
1
0.2
0.04
2ndAb
Mannose (mM)
CTLD4-7-Fc (10µg/ml)
in high salt TSB buffer
1
0.2
0.04
2ndAb
Mannose (mM)
Fc-DC-SIGN (5µg/ml)
in high salt TSB buffer


D
OD 405 nm
5
4
3
2
1
0
TBS
High salt TBS
CTLD4-7-Fc
(10µg/ml)
ns
ns
****
TBS
High salt TBS
Fc-DC-SIGN
(5µg/ml)
ns
**
****
1
0.2
0.04
2ndAb
Fucose (mM)
CTLD4-7-Fc (10µg/ml)
in high salt TSB buffer
1
0.2
0.04
2ndAb
Fucose (mM)
Fc-DC-SIGN (5µg/ml)
in high salt TSB buffer

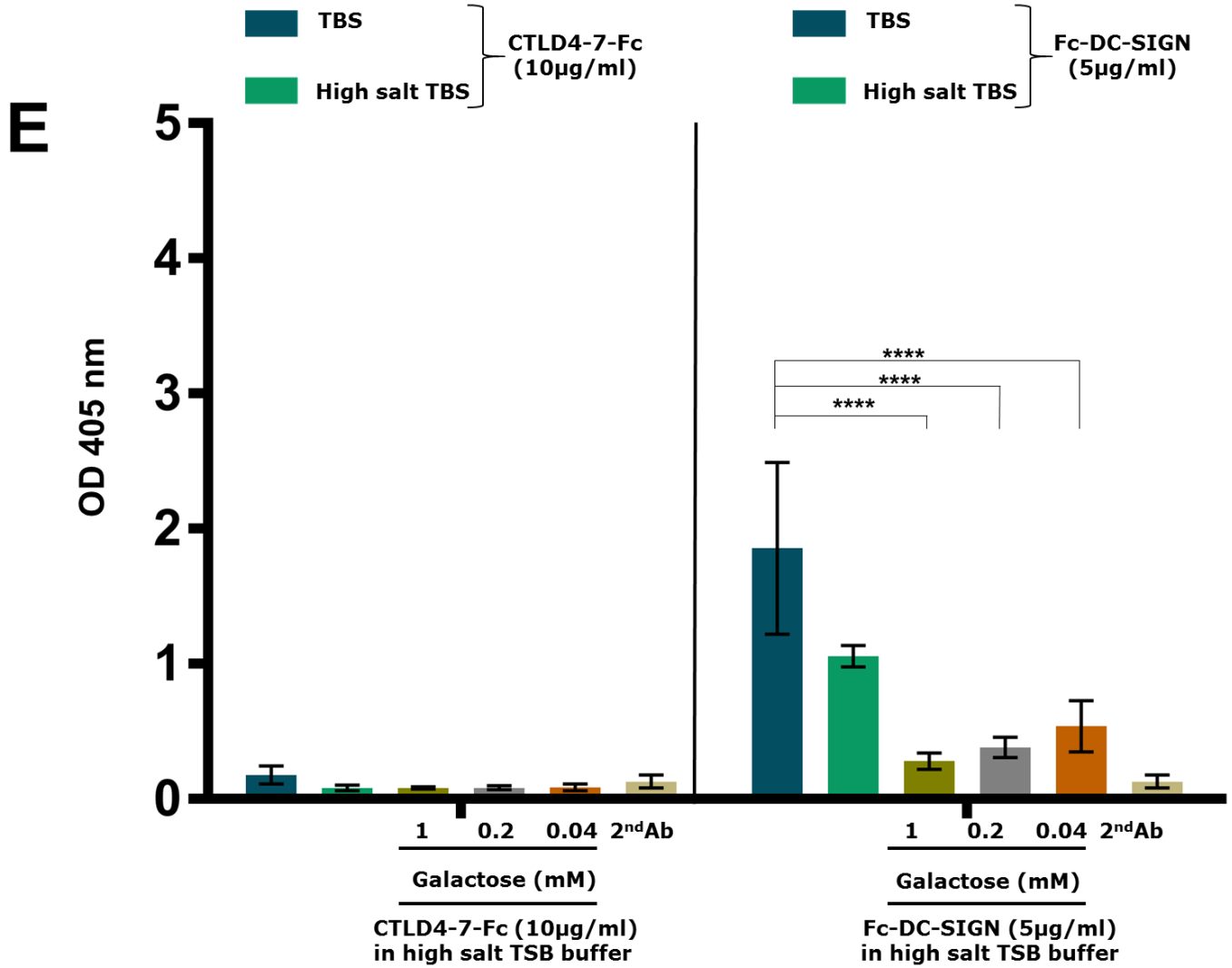

E
TBS
High salt TBS
CTLD4-7-Fc (10µg/ml)
TBS
High salt TBS
Fc-DC-SIGN (5µg/ml)
OD 405 nm
0
1
2
3
4
5
****
****
****
1
0.2
0.04
2ndAb
Galactose (mM)
CTLD4-7-Fc (10µg/ml) in high salt TSB buffer
1
0.2
0.04
2ndAb
Galactose (mM)
Fc-DC-SIGN (5µg/ml) in high salt TSB buffer

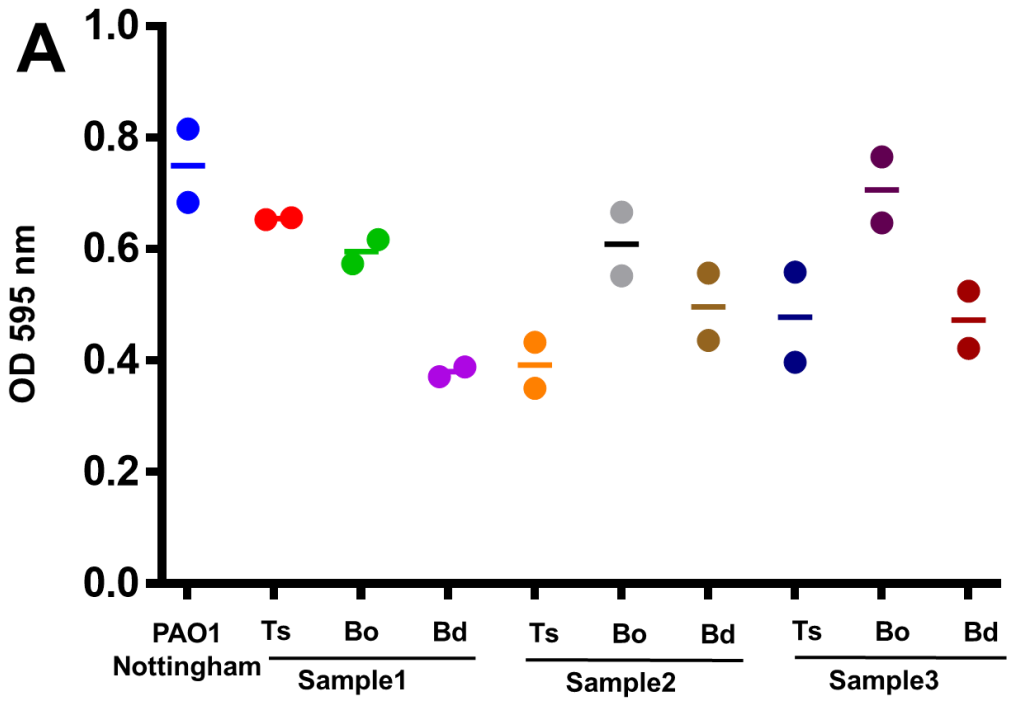
Figure 3.10: Inhibition of CTLD4-7-Fc and Fc-DC-SIGN binding to PA biofilms and control carbohydrates by monosaccharides in a high salt buffer.

CTLD4-7-Fc (10µg/ml) or Fc-DC-SIGN (5µg/ml) were pre-incubated with different concentrations of monosaccharides in TSB buffer with high salt (1M NaCl) for 30 min at room temperature and then added to Maxisorp plates containing 24 hrs old PAO1-P WT biofilms (A and B), mannose-PAA (C), fucose-PAA (D), or galactose-PAA (E) for 2 hours at room temperature. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000 dilution). Absorbance was read at 405 nm. Error bars indicate the standard deviation (SD) of three independent repeats. Statistical significance was calculated by two-way ANOVA test. P values are represented as follows: ns = $p > 0.05$, * $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, and **** = $p \leq 0.0001$.

3.4.8 Biofilms from PA wound isolates are recognized by the CTLD4-7 region of MR and the extracellular region of DC-SIGN

To confirm that the binding of CTLD4-7-Fc and Fc-DC-SIGN to biofilms occurred when using strains other than PAO1, biofilms generated by wound clinical PA isolates were also tested for CTLD4-7-Fc and Fc-DC-SIGN binding. Firstly, biofilm formation by PA wound isolates and the laboratory strain PAO1-Nottingham (Table 2.1) was tested using crystal violet assay. We used a combination of PA wound isolates collected from a single patient at different sites (bone, blood, or wound) and PAO1-Nottingham lab strain with a major deletion in the chromosome. We used the lab strain because the samples were collected from a patient admitted to a hospital in Nottingham city. Results showed that the clinical isolates tested generated biofilm (Figure 3.11-A). Next, we assessed whether CTLD4-7-Fc and Fc-DC-SIGN were able to bind to biofilms generated by these wound isolates. Results suggest that CTLD4-7-Fc and Fc-DC-SIGN bind to PA wounds biofilms

and that, as seen for PAO1, DC-SIGN-Fc binds significantly better than CTLD4-7-Fc to all samples (Figure 3.11).



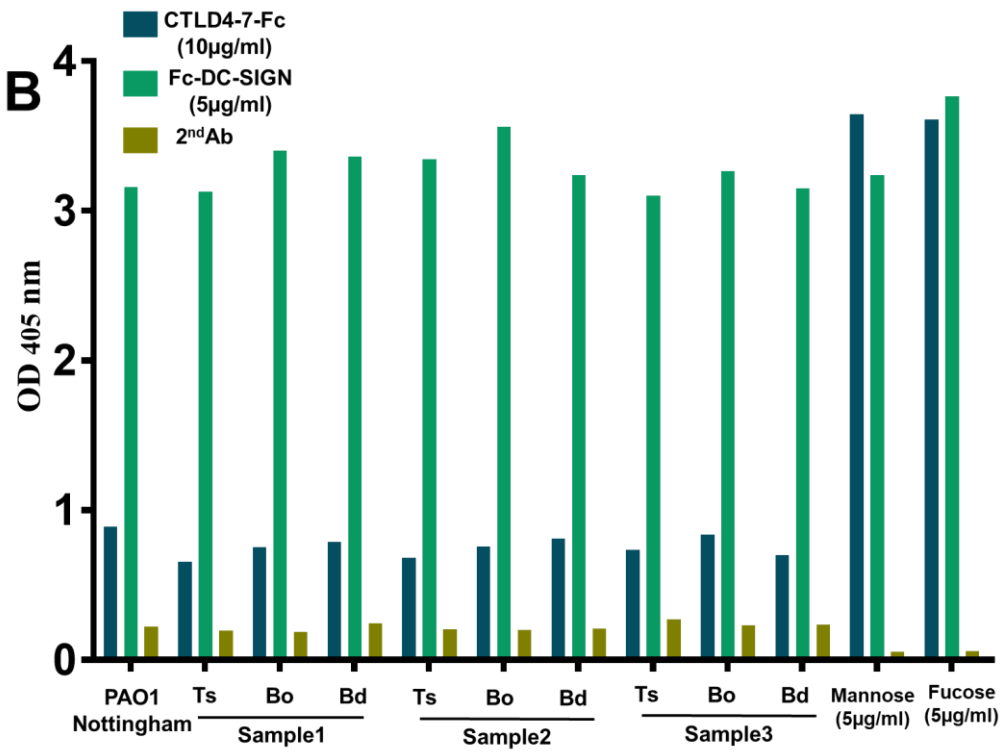
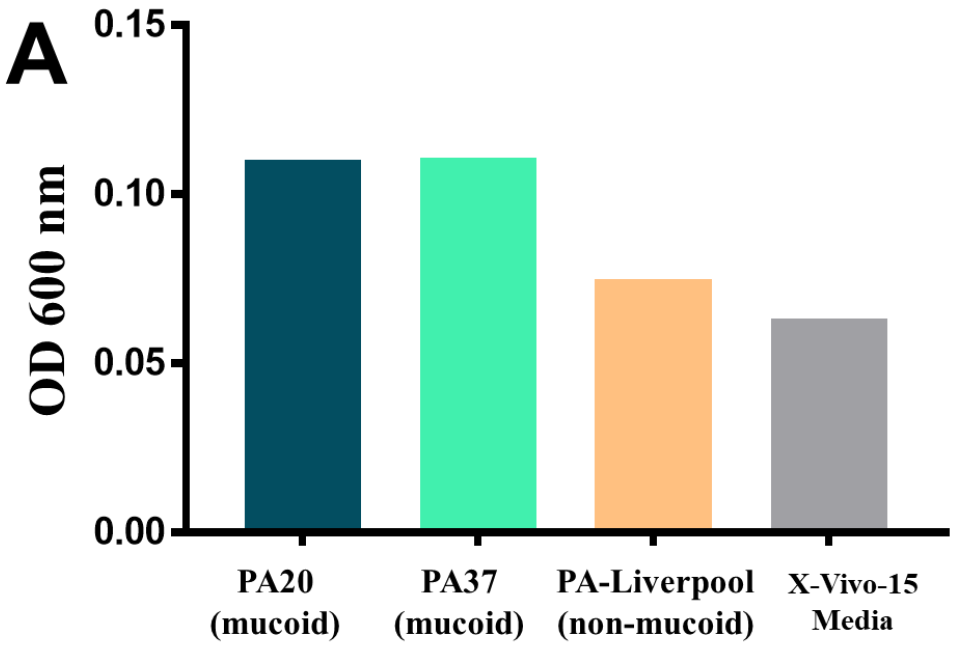


Figure 3.11: Assessment of biofilm formation by PA wound isolates (A) and binding of CTLD4-7-Fc and Fc-DC-SIGN to PA wound isolate biofilms (B).

Formation of biofilms by PA wound isolates and PAO1-Nottingham lab strain was assessed using the crystal violet method (A). Wound isolate biofilms and PAO1-Nottingham lab strain, developed over 24 hours using Maxisorp plates, were incubated with 10µg/ml of CTLD4-7-Fc and 5µg/ml Fc-DC-SIGN chimeric proteins for 2 hours at room temperature. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000 dilution). Absorbance was read at 405 nm (B). N=2.

3.4.9 Biofilms of PA CF isolates are recognized by the CTLD4-7 region of MR and the extracellular region of DC-SIGN

Biofilm formation of CF isolates (mucoid and non-mucoid) was tested using crystal violet assay. Data shows that both mucoid isolates formed very low amount of biofilm while PA-Liverpool (non-mucoid) failed to form biofilm (Figure 3.12-A). After that we wanted to explore the ability of MR and Fc-DC-SIGN to bind to CF isolates biofilms. We found that, in spite of the low level of biofilm formation, Fc-DC-SIGN binds to all the CF isolate biofilms regardless the pattern of growth (mucoid or non-mucoid) while MR shows only weak binding to the non-mucoid isolate (Figure 3.12-B).



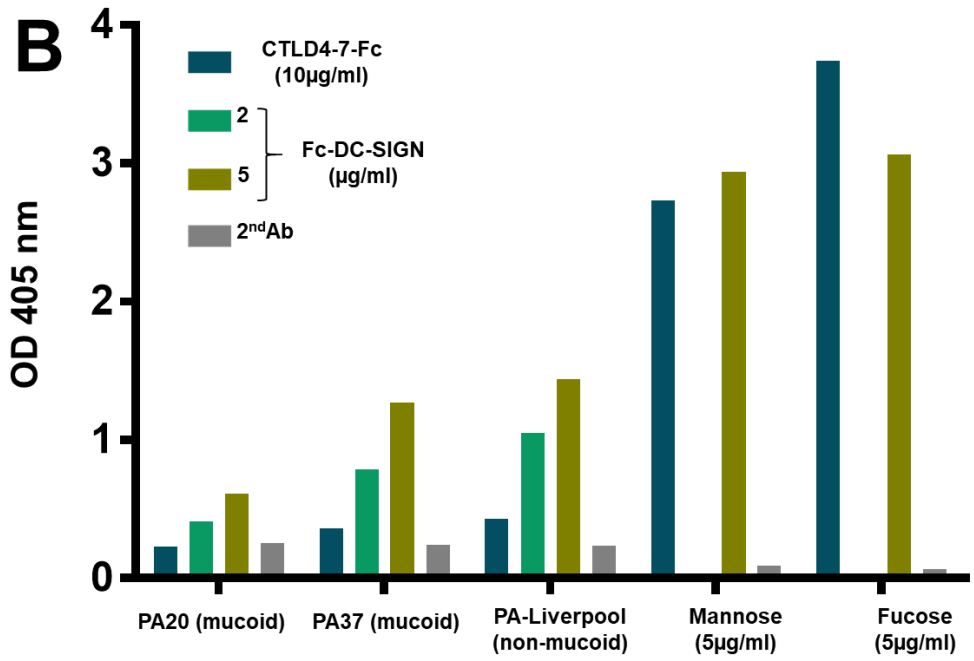


Figure 3.12: Binding of CTLD4-7-Fc and Fc-DC-SIGN to biofilms from CF isolates.

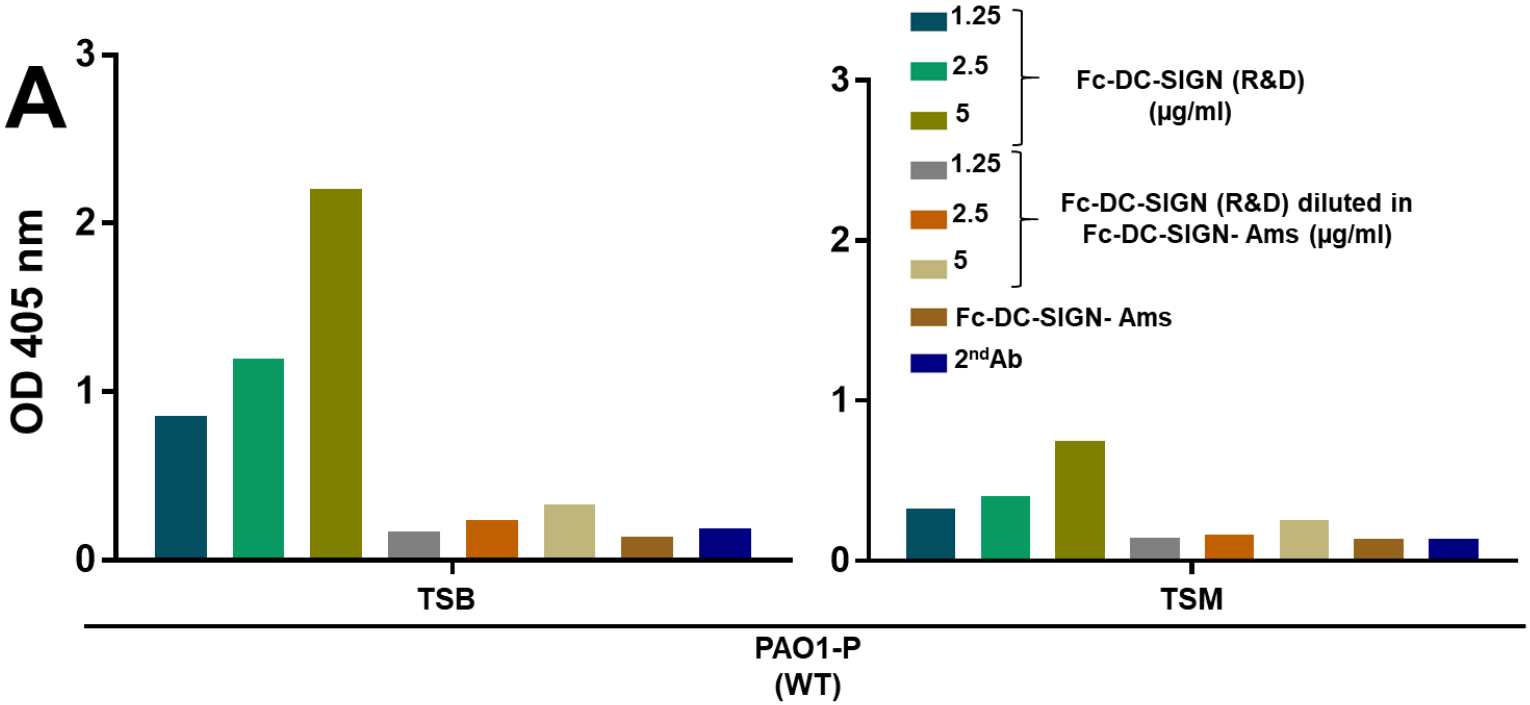
Formation of biofilms by PA CF isolates was assessed using the crystal violet method (A). Biofilms from CF isolates, developed over 24 hours using a Maxisorp plate, were incubated with 10µg/ml of CTLD4-7-Fc and 2 and 5µg/ml Fc-DC-SIGN chimeric proteins for 2 hours at room temperature. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000 dilution). Absorbance was read at 405 nm. N=2.

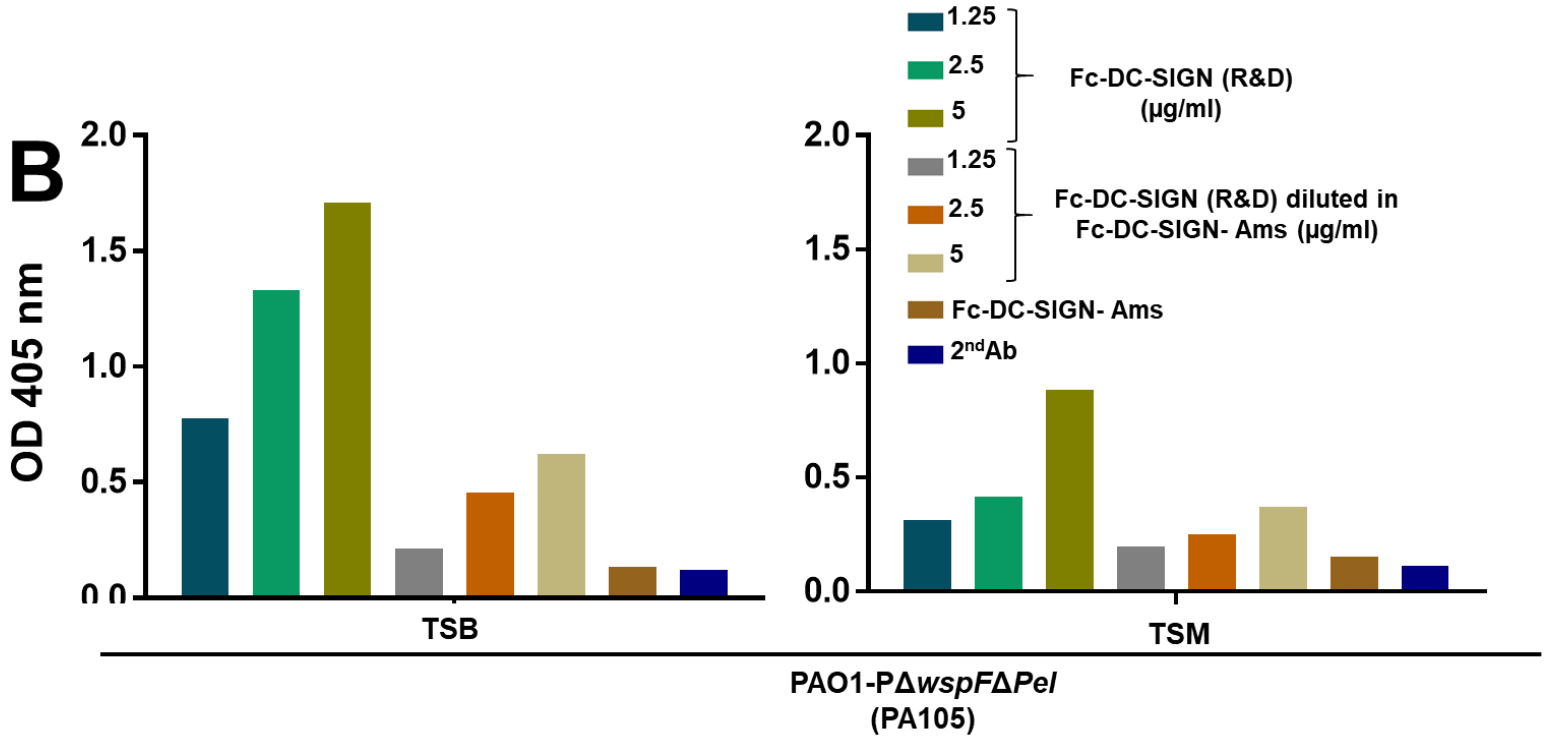
3.4.10 Comparison between the binding of Fc-DC-SIGN from R and D and Fc-DC-SIGN preparation obtained from Prof van Kooyk's laboratory.

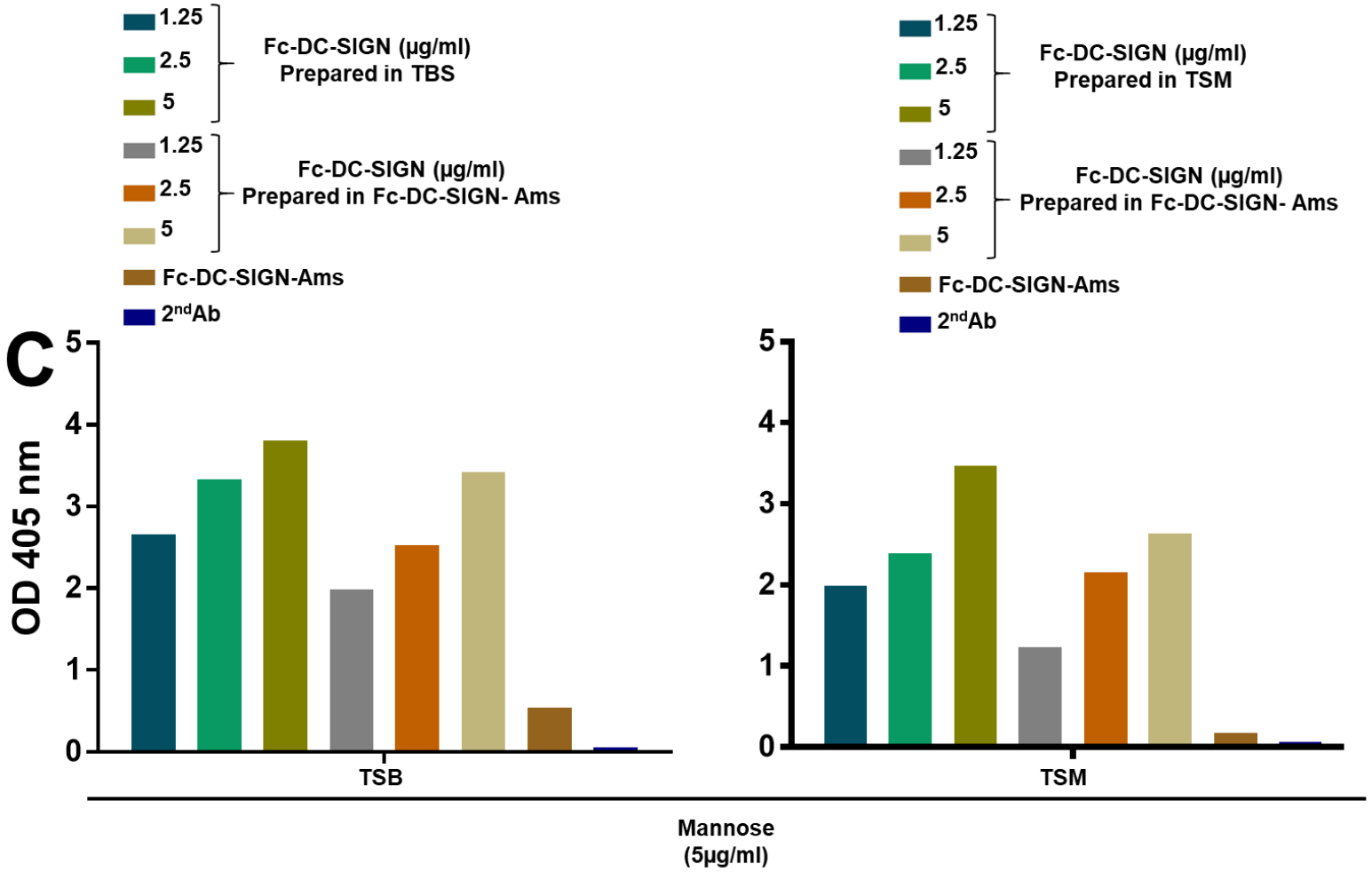
After we generated a substantial amount of data showing that Fc-DC-SIGN purchased from R&D strongly binds to PA biofilms in TSB buffer, we wanted to compare the binding of purified Fc-DC-SIGN (R&D) and Fc-DC-SIGN protein provided by our collaborator in Amsterdam (Fc-DC-SIGN-Ams). The Fc-DC-SIGN-

Ams preparation consisted of supernatants from eukaryotic cells transfected with the expression vectors for Fc-DC-SIGN and hence contains other proteins. Comparison between Fc-DC-SIGN (R&D) and Fc-DC-SIGN-Ams was performed using two different buffers TSB and TSM. We assessed three conditions: 1) Fc-DC-SIGN (R&D) diluted in either TSB or TSM, 2) Fc-DC-SIGN (R&D) diluted in DC-SIGN-Ams supernatant, and 3) DC-SIGN-Ams supernatant only. The binding to PAO1-P WT biofilms, PAO1-PΔ*wspF*Δ*Pel* (PA105), mannose-PAA, or galactose-PAA was examined in either TSB or TSM buffer using our standard binding assay.

Results suggest that (i) Fc-DC-SIGN (R&D) in TSM binds to PAO1-P and PAO1-PΔ*wspF*Δ*Pel* (PA105) biofilms less than in TSB; (ii) Fc-DC-SIGN (R&D) diluted in DC-SIGN-Ams supernatant binds only to biofilm when the assay was carried out in TSM buffer and binding was reduced compared to when the protein is diluted in TSB buffer; (iii) Fc-DC-SIGN-Ams protein preparation did not bind to PAO1-P biofilm neither in TSB nor in TSM (Figure 3.13 A and B). Fc-DC-SIGN (R&D) diluted in either TSB or TSM or Fc-DC-SIGN (R&D) diluted DC-SIGN-Ams supernatant bind to mannose-PAA, while DC-SIGN-Ams protein did not bind to mannose-PAA in either buffer (Figure 3.13-C). When we examined the binding of the three protein preparations to galactose-PAA, we found that Fc-DC-SIGN (R&D) diluted in either TSB or TSM binds to galactose-PAA (Figure 3.13-D) although binding to galactose TSM was substantially reduced compared to the TSB condition. Binding to galactose-PAA was less than to mannose-PAA.


A
OD 405 nm
3
2
1
0
TSB
TSM
PAO1-P
(WT)
1.25
2.5
5
Fc-DC-SIGN (R&D)
(µg/ml)
1.25
2.5
5
Fc-DC-SIGN (R&D) diluted in
Fc-DC-SIGN- Ams (µg/ml)
Fc-DC-SIGN- Ams
2ndAb


B
OD 405 nm
2.0
1.5
1.0
0.5
0.0
1.25
2.5
5
Fc-DC-SIGN (R&D)
(µg/ml)
1.25
2.5
5
Fc-DC-SIGN (R&D) diluted in
Fc-DC-SIGN- Ams (µg/ml)
Fc-DC-SIGN- Ams
2ndAb
TSB
TSM
PAO1-PΔwspFΔPel
(PA105)


C
1.25
2.5
5
Fc-DC-SIGN (µg/ml)
Prepared in TBS
1.25
2.5
5
Fc-DC-SIGN (µg/ml)
Prepared in Fc-DC-SIGN- Ams
Fc-DC-SIGN-Ams
2ndAb
OD 405 nm
0
1
2
3
4
5
TSB
1.25
2.5
5
Fc-DC-SIGN (µg/ml)
Prepared in TSM
1.25
2.5
5
Fc-DC-SIGN (µg/ml)
Prepared in Fc-DC-SIGN- Ams
Fc-DC-SIGN-Ams
2ndAb
0
1
2
3
4
5
TSM
Mannose
(5µg/ml)

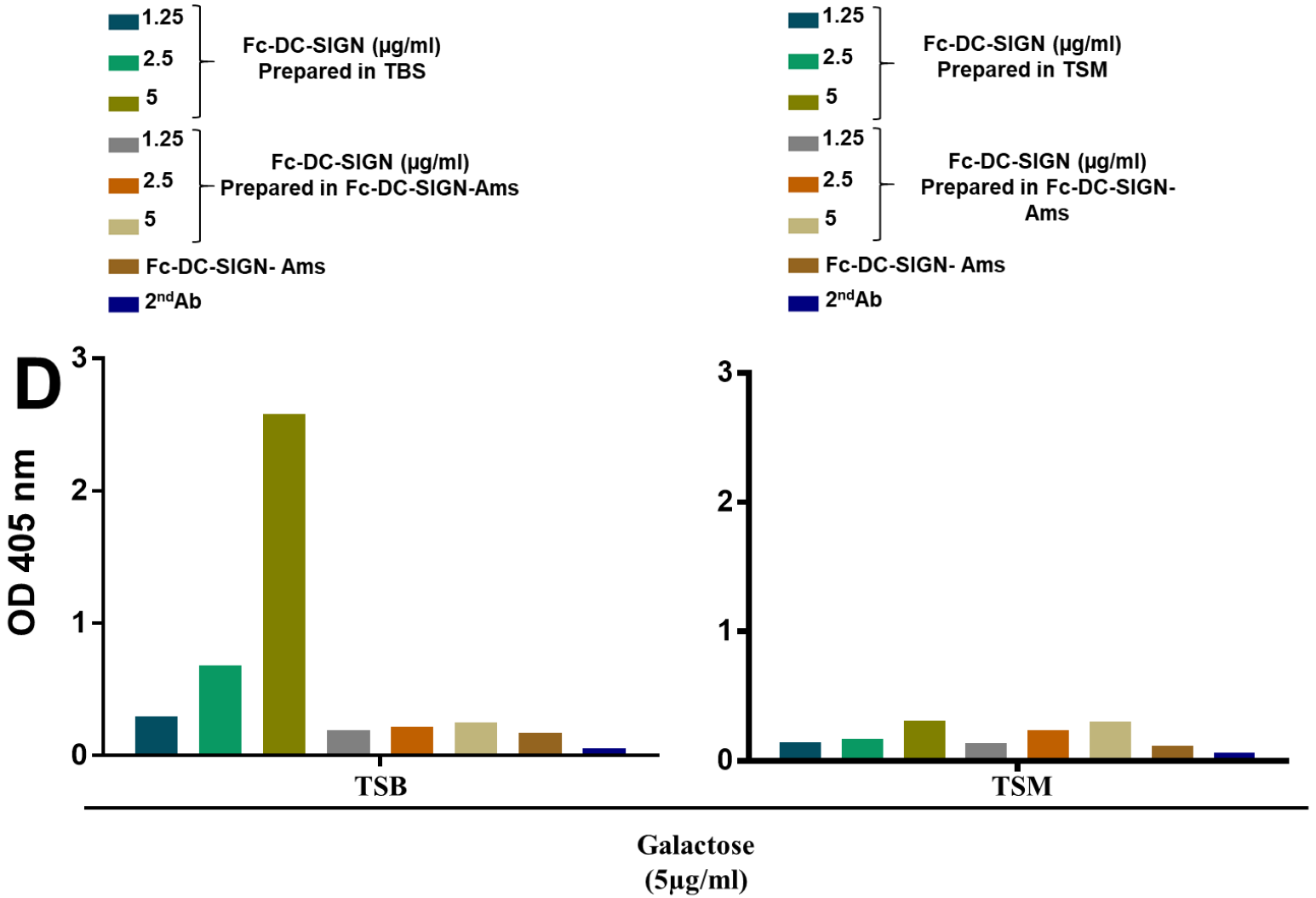

D
OD 405 nm
1.25
2.5
5
Fc-DC-SIGN (µg/ml)
Prepared in TBS
1.25
2.5
5
Fc-DC-SIGN (µg/ml)
Prepared in Fc-DC-SIGN-Ams
Fc-DC-SIGN- Ams
2nd Ab
1.25
2.5
5
Fc-DC-SIGN (µg/ml)
Prepared in TSM
1.25
2.5
5
Fc-DC-SIGN (µg/ml)
Prepared in Fc-DC-SIGN-Ams
Fc-DC-SIGN- Ams
2nd Ab
0
1
2
3
TSB
TSM
Galactose
(5µg/ml)

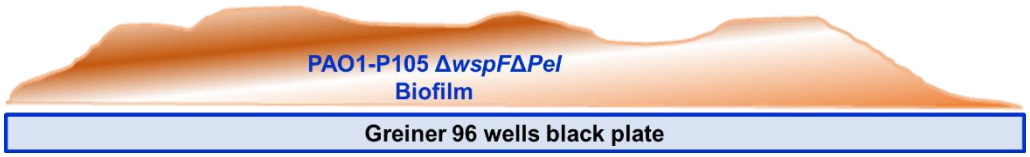
Figure 3.13: Comparison between the binding of Fc-DC-SIGN (R&D) and Fc-DC-SIGN-Ams to PAO1-P biofilms and carbohydrates in two different buffers (TSB and TSM).

Maxisorp plate wells containing 24 h PAO1-P (WT) biofilm (A), 24 h PAO1-PΔ*wspF*Δ*Pel* (PA105) biofilm (B), mannose-PAA (C) or galactose-PAA (D) were incubated for 2 hours at room temperature with (1.25, 2.5, and 5) µg/ml of Fc-DC-SIGN (R&D) diluted in either a buffer (TSB or TSM) or the Fc-DC-SIGN-Ams preparation or Fc-DC-SIGN-Ams. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000 dilution). Absorbance was read at 405 nm. N=2.

3.4.11 Distinct binding patterns of MR-CTLD4-7-Fc and Fc-DC-SIGN to PAO1-P WT biofilms

To confirm the findings in section 3.4.4, confocal microscopy was used to visualize the binding of CTLD4-7-Fc and Fc-DC-SIGN chimeric proteins to both PAO1-P*ΔwspF*Δ*Pel* (PA105) and PAO1-P*ΔwspF*Δ*Psl* (PA106) biofilms. The binding was detected using anti-human IgG Fc-specific conjugated with Alexa fluor-647. After we developed biofilm of PAO1-P*ΔwspF*Δ*Pel* on Greiner plastic black plates, we labelled the biofilm with 10 µg/ml of FM 1-43FX and fixed it with 2% of formaldehyde. We found that both proteins bind to the bacteria (Figure 3.14.I); however, the images were distorted due to the nature of the plate (plastic) and thickness. We next developed, fixed and labelled PAO1-P*ΔwspF*Δ*Pel* (PA105) biofilm on Lab-Tek II chamber glass slides and found that this substrate was suitable for biofilm formation (Figure 3.14.II). Cellview cell culture slide glass bottom plates were then used to visualize the binding of both CTLD4-7-Fc and Fc-DC-SIGN chimeric proteins to both PAO1-P*ΔwspF*Δ*Pel* (PA105) and PAO1-P*ΔwspF*Δ*Psl* (PA106) fixed biofilms. We found that CTLD 4-7-Fc shows weak binding to PAO1-P*ΔwspF*Δ*Pel* (PA105) biofilm, whereas Fc-DC-SIGN strongly binds PAO1-P*ΔwspF*Δ*Pel* (PA105) biofilm (Figure 3.14.III). Lastly, we incubated

both proteins with fixed PAO1-PΔ*wspF*Δ*Psl* (PA106) biofilm, and neither CTLD4-7-Fc nor Fc-DC-SIGN bound to PAO1-PΔ*wspF*Δ*Psl* (PA106) biofilm (Figure 3.14.IV).



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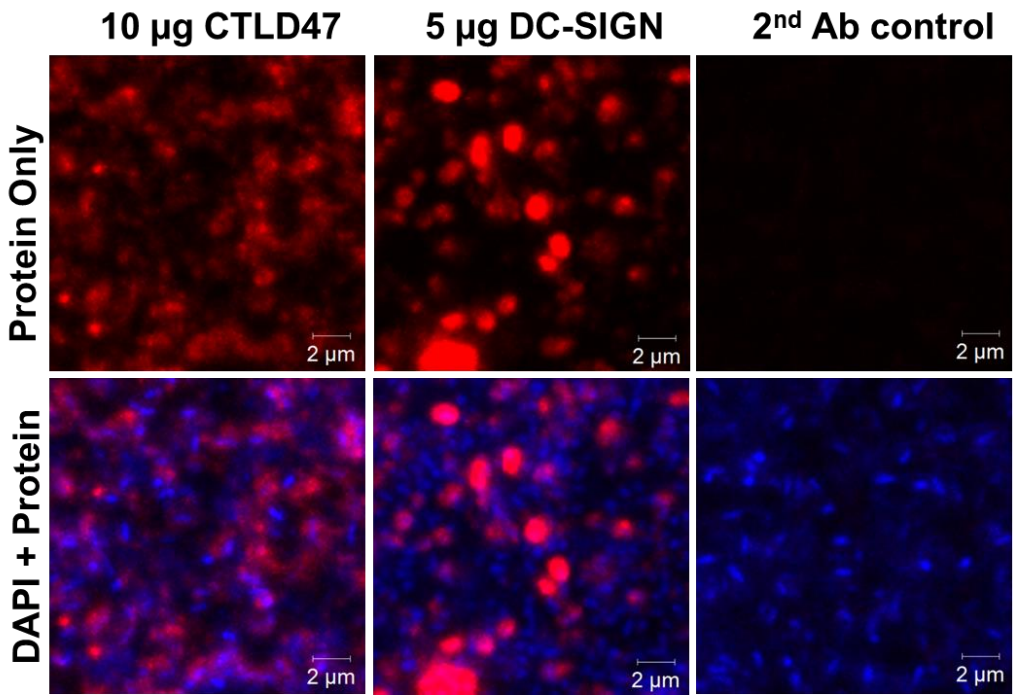
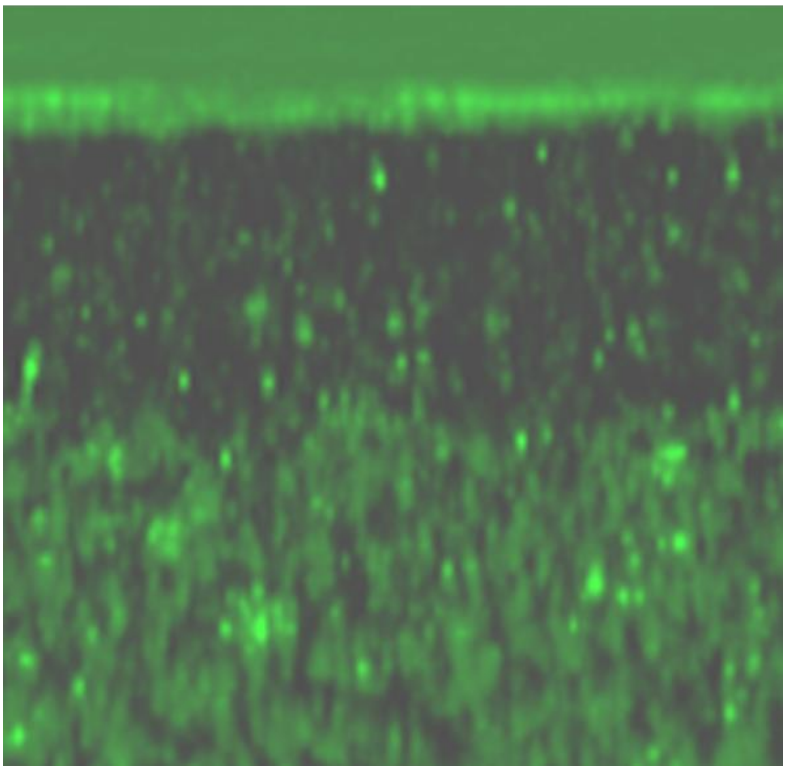


PAO1-P105 Δ*wspF*Δ*Pel*
Biofilm

Lab-Tek II chamber glass slides

II

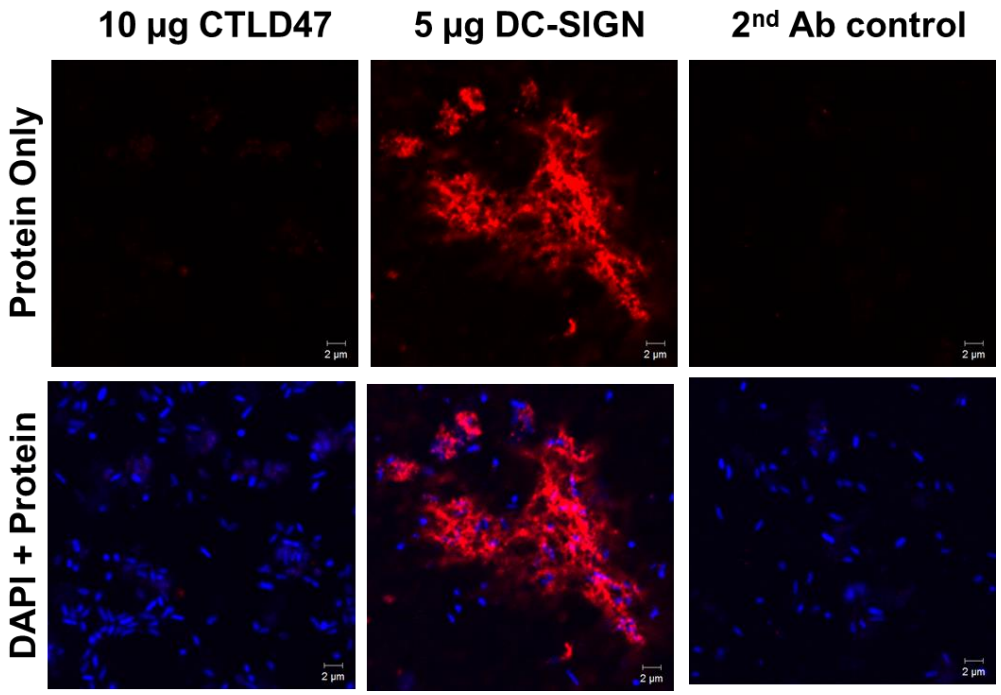
FM 1- 43FX membrane dye



PAO1-P105
Δ*wspF*Δ*Pel* Biofilm

Cellview cell culture slide glass bottom

III


10 µg CTLD47
5 µg DC-SIGN
2nd Ab control
Protein Only
DAPI + Protein
2 µm


PAO1-P106 ΔwspFΔPel
Biofilm
Cellview cell culture slide glass bottom

IV

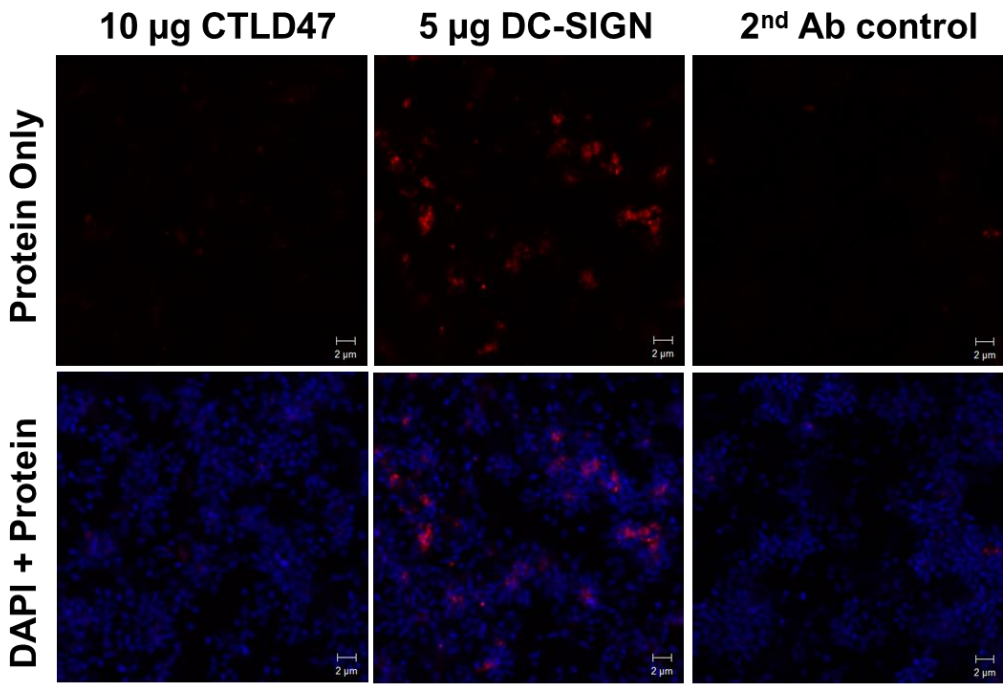


Figure 3.14: Detection of the binding patterns of CTLD4-7-Fc and Fc-DC-SIGN to PAO1-P biofilms using confocal microscopy

Δ*wspF*Δ*Pel* (PAO1-P105) and Δ*wspF*Δ*Psl* (PAO1-P106) biofilms were incubated with 10µg/ml of CTLD4-7-Fc or 5µg/ml of Fc-DC-SIGN chimeric proteins. The binding was detected using anti-human IgG Fc-specific conjugated to Alexa Fluor-647 (red). DNAs was stained with 2 µg/ml of Hoechst (Blue).

3.5 Summary

- PA strains that vary in their ability to express EPS were tested for growth and biofilm formation. PAO1-PΔ*wspF*Δ*Pel* (PA105) and PAO1-PΔ*wspF*Δ*Psl* (PA106) form more biofilm compared to PAO1-PΔ*wspF* (PA176), and PAO1-

PΔ*wspF*Δ*Pel*Δ*Psl* (PA107). This supports the notion that EPS (Psl and/or Pel) is an important scaffolding component of the PA biofilm matrix.

- Differences in growth among different strains appear to relate to their capacity to form biofilm.

- MR and DC-SIGN bind to PA biofilms (laboratory strains, wound isolates, and CF isolates), and DC-SIGN binds significantly better than MR. This indicates that these C-type lectin receptors could play a crucial role in the recognition of PA biofilms.

- Dectin-1 binding to PAO1-P biofilms is weak.

- C-type lectins binding to biofilms is carbohydrate and calcium dependent.

- Binding of Fc-DC-SIGN (R&D) to PA biofilms is influenced by assay conditions being stronger in TSB than in TSM.

- The preparation of Fc-DC-SIGN-Ams (which contains approximately 2 µg protein/ml) failed to bind PA biofilms but bound to mannose-PAA indicating that a component of the supernatant could interfere with biofilm recognition. This is supported by the inhibitory effect of Fc-DC-SIGN-Ams on the binding activity of Fc-DC-SIGN (R&D).

- Fc-DC-SIGN (R&D) can bind galactose-PAA in TSB but not in TSM. This binding has been observed in some instances but, unlike binding to mannose-

PAA, fucose-PAA and, most importantly, PA biofilms, it was not consistent among all experiments.

Chapter 4: Recognition of *Pseudomonas aeruginosa* Psl by MR and DC-SIGN

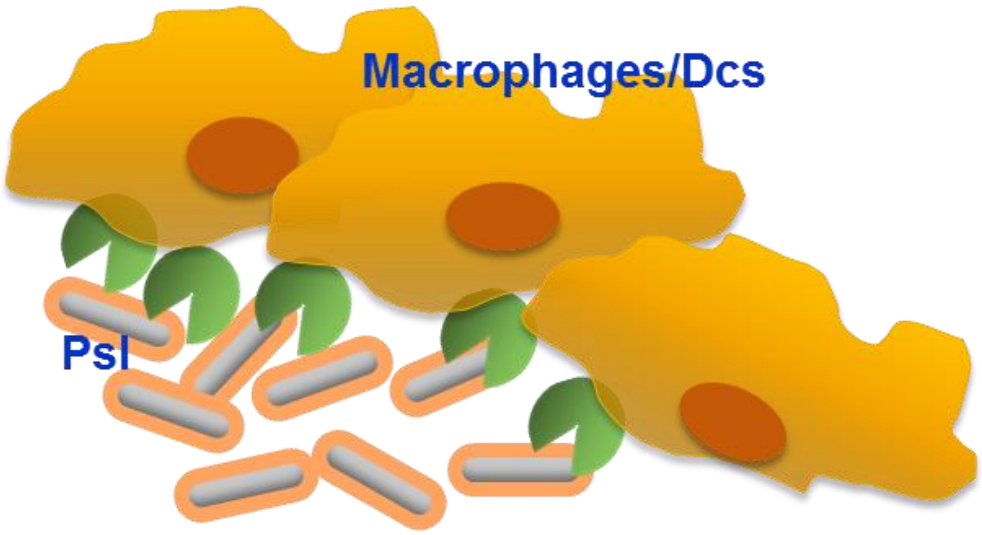
4.1 Introduction

Polysaccharide synthesis locus (*Psl*) controls the production of PA Psl. The proteins involved in Psl production are encoded by fifteen co-transcribed genes (PslA-PslO) (Friedman and Kolter, 2004c, Matsukawa and Greenberg, 2004, Jackson et al., 2004b). PA utilizes Psl or/and Pel to form biofilm (Branda et al., 2005, Colvin et al., 2012b). Psl consists of mannose, glucose, and minor amount of rhamnose and galactose (Laus et al., 2006, Ma et al., 2007b). See section 1.3.3.2 for more details on Psl.

Many factors have been implicated in the regulation of Psl synthesis and biofilm generation. Recently, the universal bacterial second messenger cyclic (c)-di-GMP has been implicated in many functions such as cell cycle regulation, biofilm formation, motility, and virulence factor production. CF patients' airways have been found to contain rugose small-colony variant (RSCV) mucoid PA strains characterised by high levels of c-di-GMP (Romling et al., 2013, Irie et al., 2012b). C-di-GMP positively regulates production of Pel and Psl polysaccharides (Hickman et al., 2005a). Moreover, it has been shown that Psl, itself, elevates c-di-GMP levels, which leads to the generation of a positive feedback loop that results in the continuous stimulation of biofilm formation (Irie et al., 2012a). In contrast, low levels of c-di-GMP promote a planktonic lifestyle (Borlee et al., 2010a).

Psl has been found to support biofilm initiation and formation (Zhao et al., 2013). PslA mutants showed impaired bacteria surface attachment. Psl mediates attachment to a wide range of substrates including biotic and abiotic surfaces (Ma et al., 2006b). Psl genes have been found to promote antibiotic tolerance to the adherent PA (Murakami et al., 2017). Psl can be found as cell-associated and as excreted carbohydrate (Zhao et al., 2013). Mishra et al showed that a *psl* deficient mutant exhibited increased deposition of complement on the bacterial surface and bacterial killing by neutrophils (Mishra et al., 2012b). This suggests that PA uses Psl to facilitate host adaptation and persist during CF infection. Psl has high affinity to ferrous ion; indeed one of PA survival strategies is to use Psl to segregate and store iron in order to utilize it for biofilm formation (Yu et al., 2016). Interestingly, it has been shown that PA deposits a trail of Psl as it moves along surfaces which influences the subsequent movement of the upcoming cells when the Psl trail is encountered. The movement is mediated by Type IV pili twitching motility. (Zhao et al., 2013). As far as we know, no mammalian receptors for Psl have been identified. It has been shown that Anti-Psl antibodies are protective against PA infection (DiGiandomenico et al., 2012). Thus, Psl appears to provide an advantage for PA during the establishment of chronic infection and anti Psl agents could improve the outcome of CF treatment.

4.2 Hypothesis



Chapter 3 shows that the CTLD4-7 region of MR and the extracellular domain of DC-SIGN are able to interact with PA biofilms and that this interaction is Pel-independent. In this chapter we wanted to use a parallel approach to investigate the role of CLRs in PA recognition. We hypothesized that Psl within PA biofilms was the candidate ligand for MR and DC-SIGN.

4.3 Aims

- Establish a protocol to extract Psl from PA biofilms.
- Determine the capability of MR and DC-SIGN to interact with purified PA Psl.
- Evaluate and compare the binding of different DC-SIGN constructs to PA Psl using different techniques.
- Analyse how highly purified Psl preparations modulate the activation of human monocyte-derived DCs.

4.4 Psl purification

In order to test our hypothesis and explore the contribution, if any, of PA Psl to PA pathogenesis, we extracted and purified Psl and Pel from ∆*wspF*∆*Pel* (PAO1-P105), a Pel deficient strain and ∆*wspF*∆*Psl* (PAO1-P106), a Psl deficient strain, respectively. Extraction and purification was performed as described by (Bales et al., 2013).

4.4.1 Growth conditions and Psl extraction

PA from a glycerol stock was plated on an LB agar plate and incubated overnight at 37°C. The following day, a single colony was selected and inoculated into 20 ml of tryptic soy broth (TSB) (22092, Sigma) and incubated overnight at 37°C, shaking at 200-220 rpm. The 20 ml overnight culture was inoculated into 400 ml of TSB in a 1.5 L flask and incubated statically at 37°C for five days in order to promote biofilm formation. After that 2.4 ml of formaldehyde (36.5% solution-33220, Sigma-Aldrich) were added to the culture to achieve a final concentration of 0.02% (v/v) and the culture was incubated at room temperature in a chemical hood for 1 h, shaking at 100 rpm to fix the cells and minimise cell lysis. Then, 160 ml of 1M NaOH (S318-1, Fisher scientific) to achieve a final concentration of 275 mM was added to the flask and incubated at the same conditions for 3 hrs to extract the EPS. The suspension was then centrifuged at 16,000xg for 1 h at 4°C. The dense supernatant was filtered following three different steps, as follows: (1) coffee filter paper, (2) 3 mm chromatography filter paper, and (3) a 0.2 µm 1L Corning filter unit (2832378, Corning). After filtration, the mixture was dialysed/concentrated against HPLC water using VIVAFLOW 200, MWCO 10 kDa (Sartorius Stedim Biotech) to a maximum final volume of 50 ml.

4.4.2 Psl purification

Proteins and nucleic acids within the crude EPS extract were precipitated by adding to 20% (w/v) of tri-chloro-acetic acid (TCA) (3000-50, Fisher scientific) and incubating on ice for 30 min. The solution was then centrifuged at 16,000xg for 1 h at 4 °C. The supernatant was collected into a fresh clean glass bottle and 1.5 volume of cold 95% (v/v) ethanol was added and samples were kept at -20 °C for 24 hrs to precipitate the EPS away from the lipids. This step was done twice to improve purity. The solution was then centrifuged at 16,000xg for 1 hour at 4°C and the pellet was re-suspended in less than 2 ml of HPLC water, dialysed against HPLC water using a 12–14 kDa MWCO membrane (68100, Snakeskin), and lyophilized. The lyophilized powder was then re-suspended in less than 2 ml of PBS (pH 7.4) and fractionated on a HiPrep 26/60, Sephacryl S-200 HR gel filtration column (GE Healthcare) using 971-FP flash purification system (Agilent Technologies), which was calibrated with protein standards (1511901, Bio-rad) to generate a standard curve showing the retention time of known molecular weights. Fractions with approximate MW 15 kDa were collected and tested for the presence of carbohydrate using the phenol-sulphuric acid method as follows: 200 µl of the EPS fraction were mixed with 100 µl of 5% (v/v) phenol (P4557, Sigma) in a glass test tube. Then 500 µl of sulphuric acid (33974, Sigma) were added to the mixture and left in a chemical hood for 10 min at room temperature. After that, tubes were mixed thoroughly for 30 secs and cooled down by placing them in water at room temperature in a chemical hood for 20 min. The absorbance was read at 490 nm using Multiskan FC, Thermo Scientific. The blank was prepared in the same manner, but the EPS was replaced with deionized water. Fractions that represent one peak were pooled, dialysed against HPLC water, and lyophilized. The lyophilized powder was then weighted, re-suspended in HPLC water and stored at -80°C

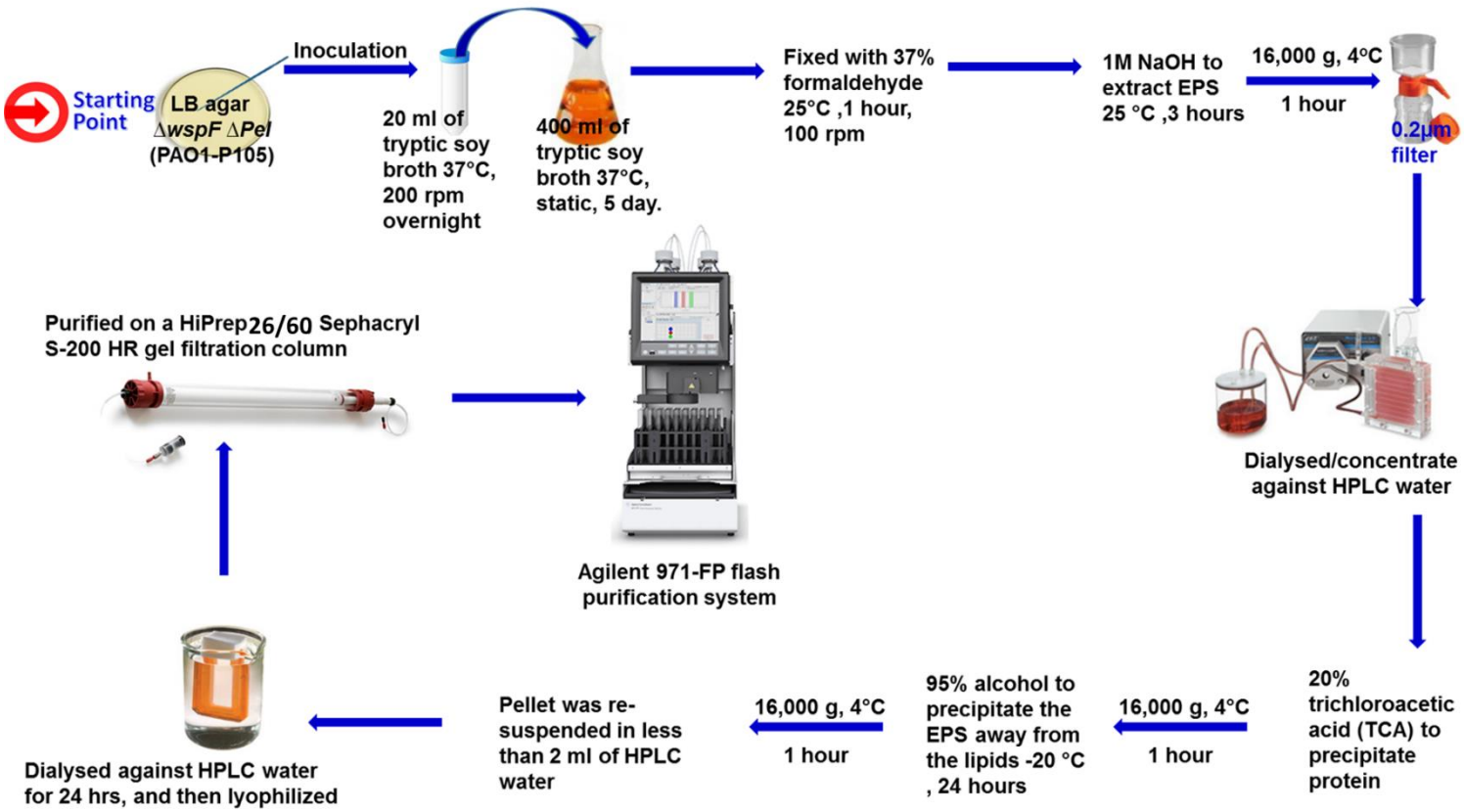


Figure 4.1: Illustration of Psl purification steps.

4.5 Results

4.5.1 Extracted EPS crude from Δ*wspF*Δ*Pel* (PAO1-P105) is weakly recognized by the CTLD4-7 region of MR and the extracellular region of DC-SIGN

Examining the ability of CTLD4-7-Fc to recognize Δ*wspF*Δ*Pel* (PAO1-P105) EPS was initially undertaken by Tamanna Rahman, a former PhD student in Dr Martinez-Pomares' lab. The procedure for extraction that EPS crude was described by (Byrd et al., 2009). Briefly, Δ*wspF*Δ*Pel* (PAO1-P105) culture prepared in M63 minimum media that consists of [(NH4)2SO4, 2g/L, KH2PO4, 13.6 g/L; FeCl3, 0.5 mg/L, 0.5% casaminoacids,1 mM MgCl2, and 0.2% glucose- PH7] was grown in 140-mm diameter Petri dishes at 37°C for 24 hrs. On the following day, the mixture was centrifuged and sonicated; and then the proteins were precipitated by adding 5% (w/v) of Trichloroacetic acid (TCA). Another centrifugation was performed, and supernatant was collected. Finally, the crude was dialyzed, lyophilized and resuspended in HPLC water.

In order to test the binding of CTLD4-7-Fc to Δ*wspF*Δ*Pel* (PAO1-P105) EPS crude extract, Maxisorp plates were coated with 500 or 250 µg/ml of the EPS crude extract overnight at 4ºC. Results showed that CTLD4-7-Fc binds to different concentrations of EPS crude extract (250 and 500 µg/ml) in a dose dependent manner ; while the binding of the negative control, CR-FNII-CTLD1-3-Fc, was low (Figure 4.2). These findings indicate that CTLD4-7-Fc could play a role in PA precognition as a PRR; however, the crude preparations procedure did not include a lipid elimination step which might be a source of contaminants. Additionally, M36 media contains only the minimum growth requirements which might affect the

amount of polysaccharide. Therefore, it was decided to follow the procedure described in section 4.4 followed by fractionation the crude on gel-filtration column.

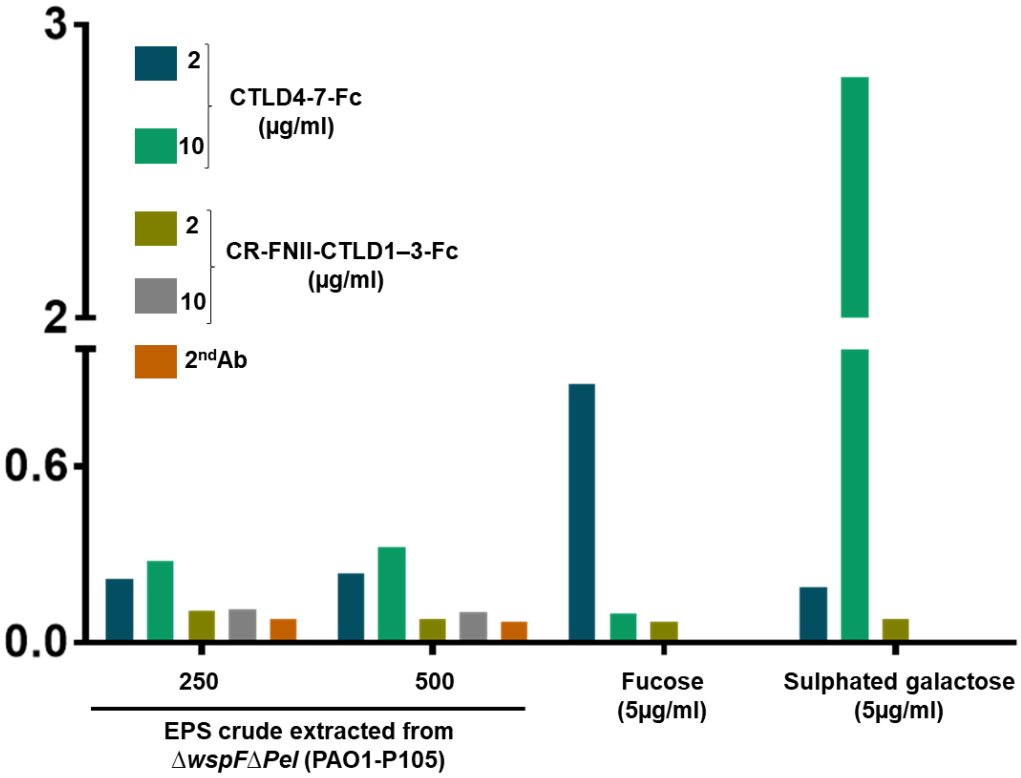
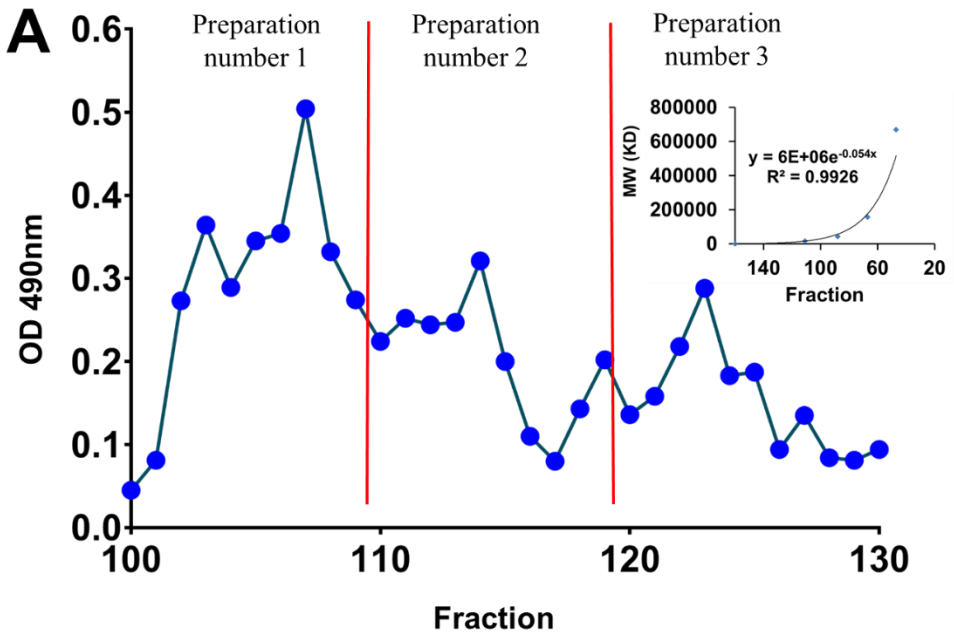


Figure 4.2: CTLD4-7-Fc region of MR binds to Δ*wspF*Δ*Pel* (PAO1-P105) EPS crude extract.

Maxisorp plates were coated with different concentrations of EPS crude extracted from the Pel-deficient strain Δ*wspF*Δ*Pel* (PAO1-P105) diluted in 154mM NaCl overnight at 4°C. CTLD4-7-Fc and CR-FNII-CTLD1-3-Fc chimeric proteins were incubated for 2 hours at room temperature in TSB buffer. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000) dilution. Absorbance was read at 405 nm. N=1.

4.5.2 Analysis of Psl preparations extracted from PAO1-P105

In order to investigate the binding of MR and DC-SIGN to PA Psl, we produced several preparations of purified Psl. One single bacterial culture was used to generate preparations number 1, 2, and 3 following the procedure described above in section 4.4. However, removal of lipid from the crude preparation using ethanol was performed once and also the lyophilized powder was re-suspended in 4 ml of PBS before fractionation. We obtained three heterogeneous peaks which were pooled and constituted the three initial preparations (Figure 4.1-A). Regarding the remaining preparations, we used one independent bacterial culture for each. Also, we performed the lipid removal step twice to reduce the possibility of lipid contamination. To increase the resolution of the column, the lyophilized powder was re-suspended in less than 2 ml of PBS before fractionation. In each preparation (4-7), we obtained a good amount of carbohydrate (Figure 4.3 (B-E)). Protein was quantified in each preparation using the bicinchoninic acid (BCA) method and we found that all the preparations contain very small amount of protein (Table 4.1).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Preparation number 2
Preparation number 3
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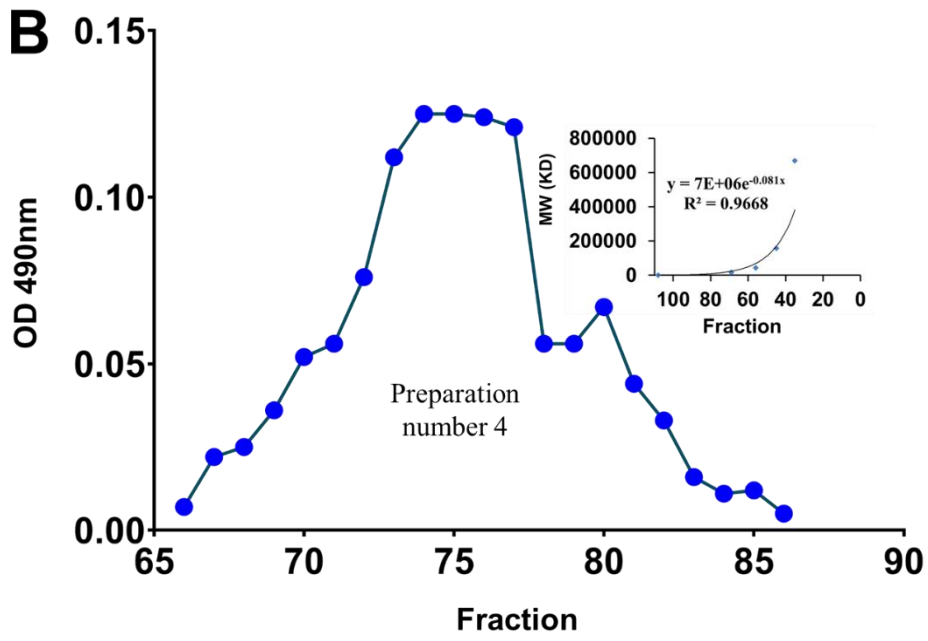
R² = 0.9926
Fraction


B
OD 490nm
Preparation number 4
Fraction
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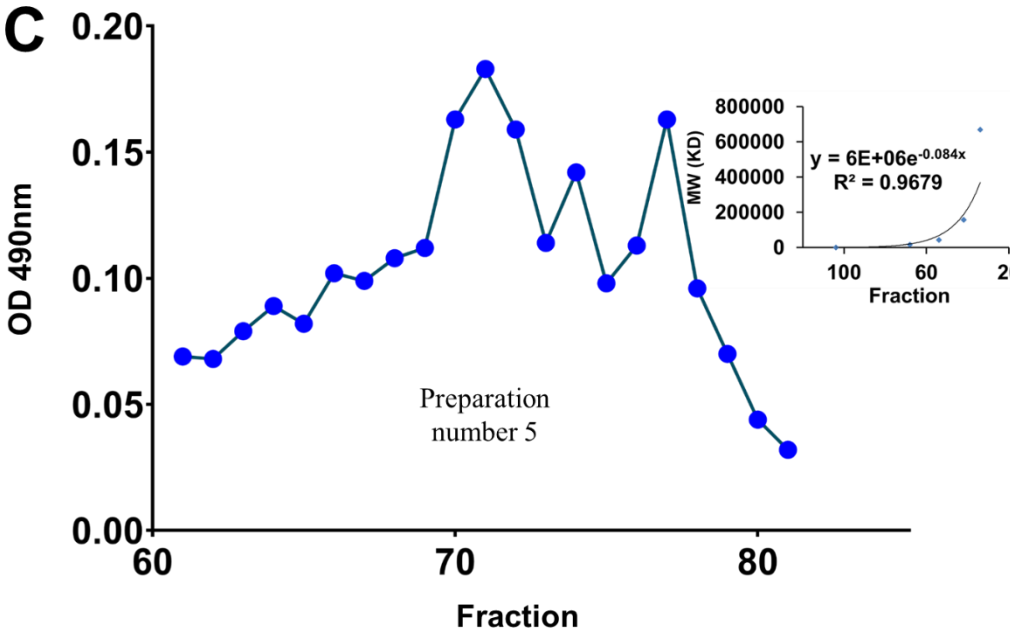
Fraction


C
0.20
0.15
0.10
0.05
0.00
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60
70
80
Fraction
Preparation
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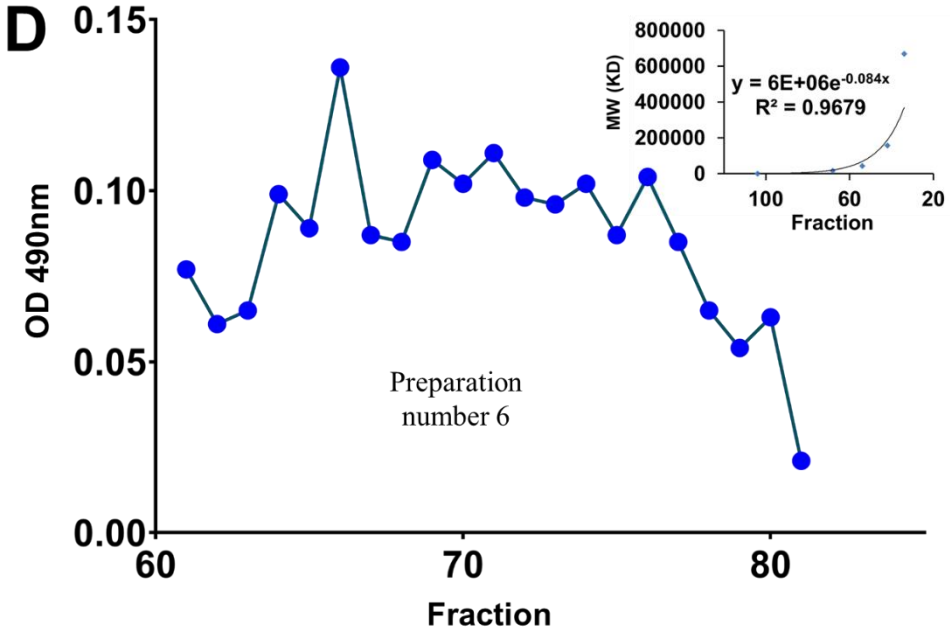
100
60
Fraction


D
0.15
0.10
0.05
0.00
OD 490nm
60
70
80
Fraction
Preparation
number 6
800000
600000
400000
200000
0
MW (KD)
y = 6E+06e-0.084x
R² = 0.9679
100
60
Fraction

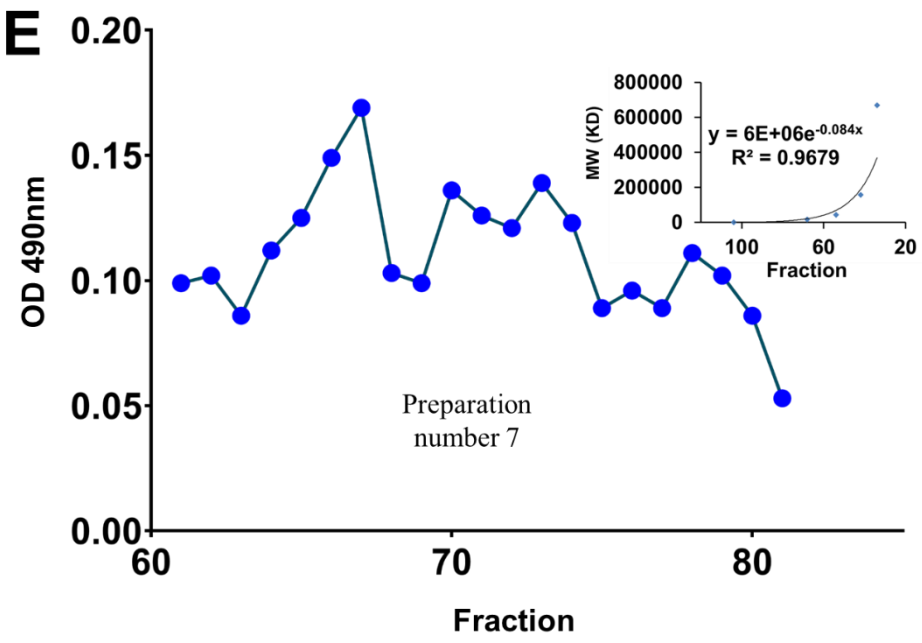


Figure 4.3: Quantification of total carbohydrate content in different preparations of Psl from from ΔwspFΔPel (PAO1-P105).

A HiPrep 26/60, Sephacryl S-200 HR gel filtration column (GE Healthcare) was calibrated with protein standards using a 971-FP flash purification system (Agilent Technologies) to generate a standard curve showing the retention time of known molecular weight markers. Fractions with approximate MW 15 kDa were collected and tested for the presence of carbohydrate using the phenol-sulphuric acid method.

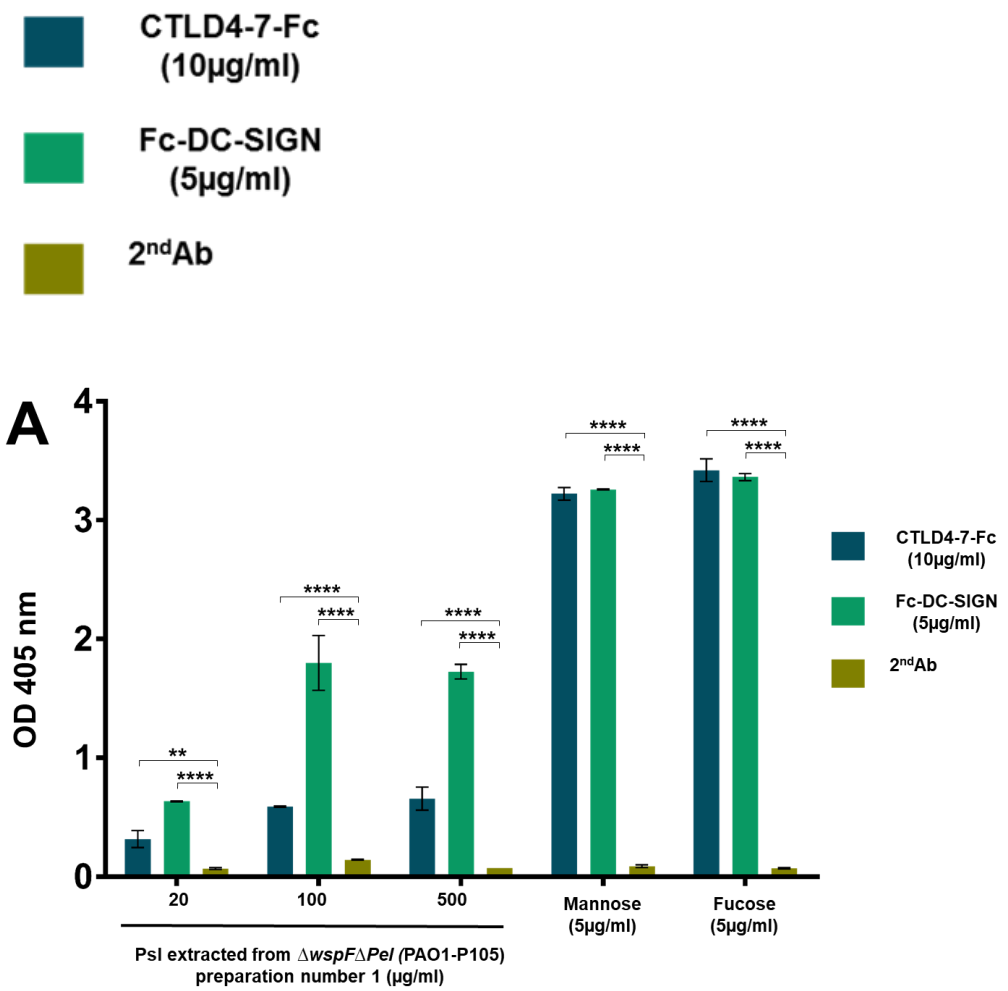
Table 4.1. Psl preparations contain trace amount of protein

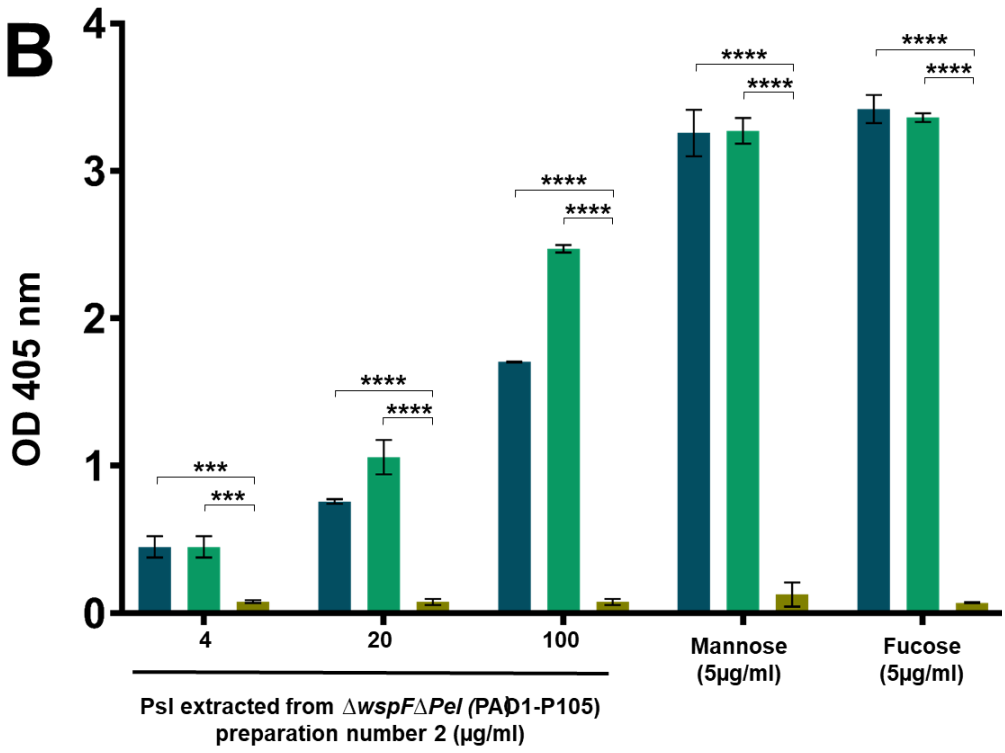
preparation	Protein concentration (µg/ml)	Carbohydrate concentration (µg/ml)	Protein (µg) / (µg) carbohydrate
1	46.4	1400	0.03
2	4.4	1700	0.033
3	103.4	1800	0.06

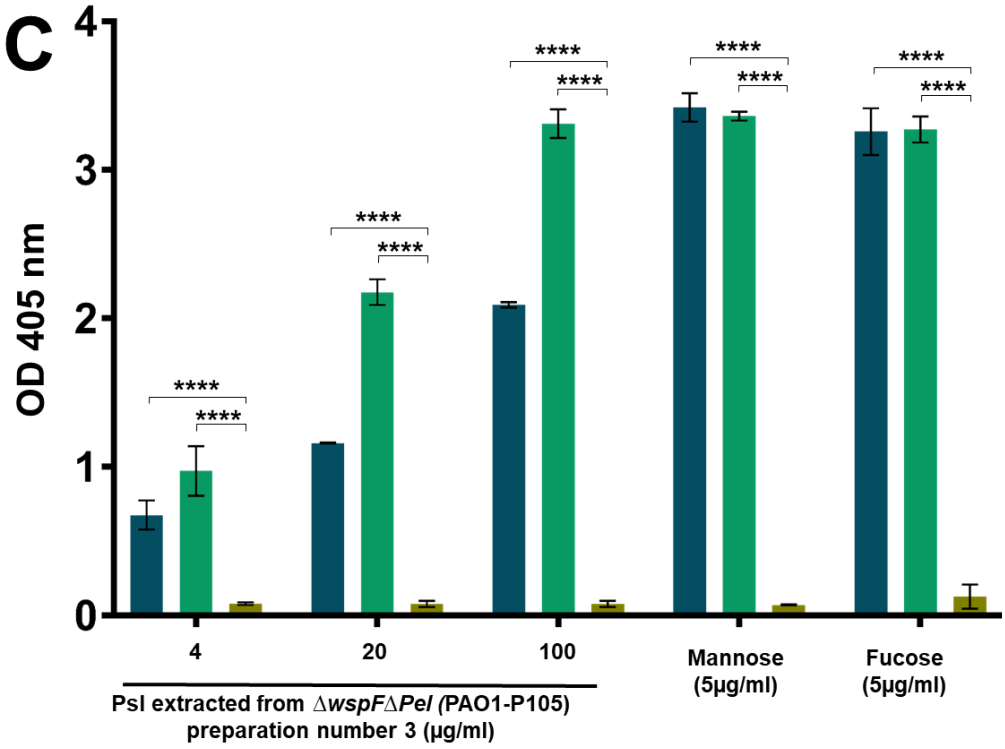
4	**60.4**	**1000**	**0.06**
5	**3.6**	**500**	**0.007**
6	**7.6**	**500**	**0.015**
7	**1.6**	**1000**	**0.002**

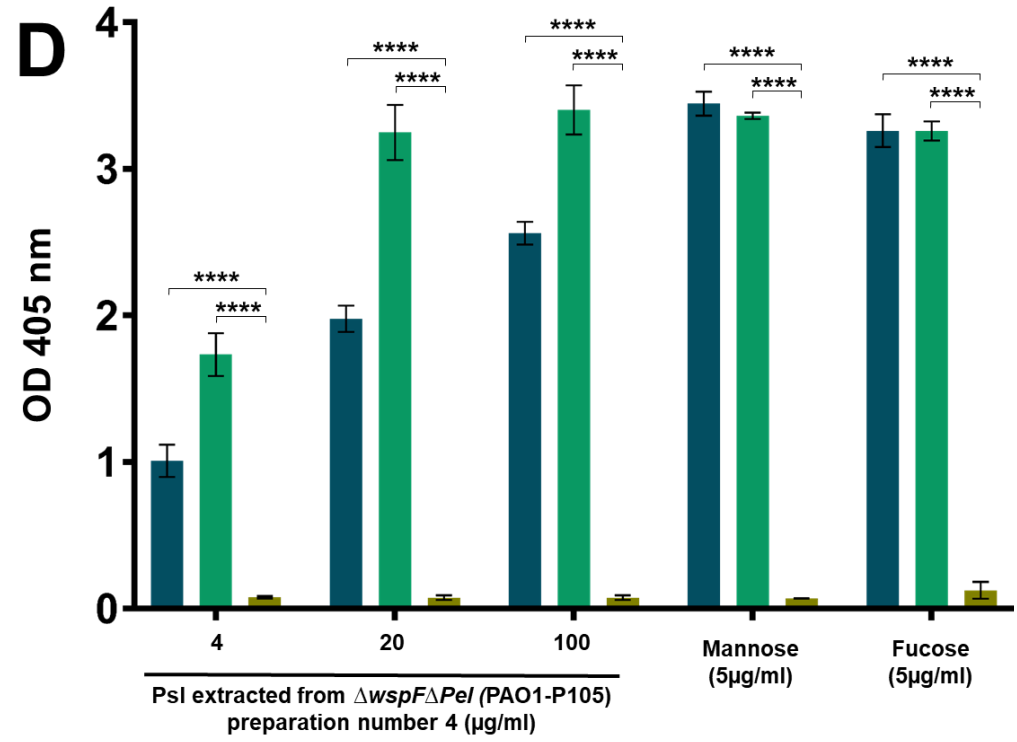
4.5.3 Purified Psl from ∆*wspF*∆*Pel* (PAO1-P105) is recognized by the CTLD4-7 region of MR and the extracellular region of DC-SIGN

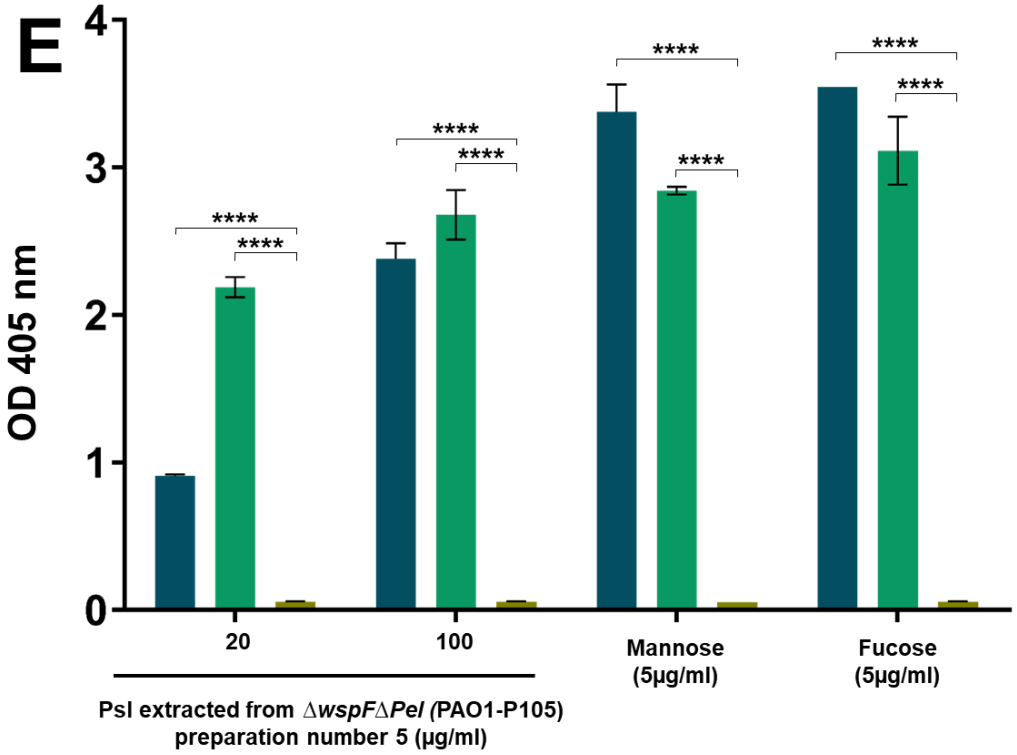
Psl is a major component of EPS within biofilms. Many studies have shown that Psl plays an important role in biofilm formation by promoting cell aggregation and surface attachment. After showing that MR (CTLD4-7-Fc) and DC-SIGN (Fc-DC-SIGN) bound to PA biofilms, we assessed whether these chimeric proteins bound to purified PA Psl. Therefore, we examined 7 independent preparations of Psl which contain different amount of carbohydrate/protein ratios. Results show that CTLD4-7-Fc and Fc-DC-SIGN bind to PA Psl in a dose-dependent manner and that, as seen for total biofilms, DC-SIGN-Fc recognised Psl better than CTLD4-7-Fc in all instances, although differences between both proteins were less obvious (Figure 4.4 A-G). Both CTLD4-7-Fc and Fc-DC-SIGN strongly recognised the commercially available positive controls polymers mannose-PAA and fucose-PAA.

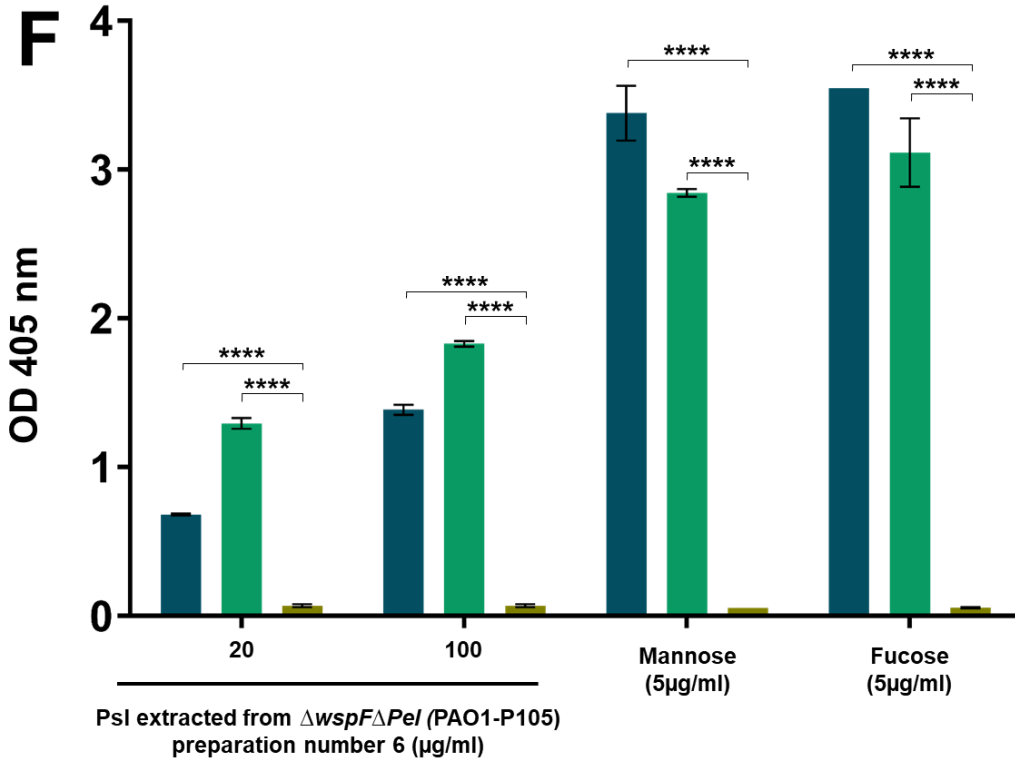

CTLD4-7-Fc
(10µg/ml)
Fc-DC-SIGN
(5µg/ml)
2ndAb
A
OD 405 nm
0
1
2
3
4
**
****
****
****
****
****
****
****
****
20
100
500
Mannose
(5µg/ml)
Fucose
(5µg/ml)
PsI extracted from ΔwspFΔPel (PAO1-P105)
preparation number 1 (µg/ml)
CTLD4-7-Fc
(10µg/ml)
Fc-DC-SIGN
(5µg/ml)
2ndAb


B
OD 405 nm
0
1
2
3
4
***
***
****
****
****
****
****
****
4
20
100
Mannose
(5µg/ml)
Fucose
(5µg/ml)
PsI extracted from ΔwspFΔPel (PAO1-P105)
preparation number 2 (µg/ml)


C
OD 405 nm
0
1
2
3
4
****
****
****
****
****
****
****
****
4
20
100
Mannose
(5µg/ml)
Fucose
(5µg/ml)
PsI extracted from ΔwspFΔPel (PAO1-P105)
preparation number 3 (µg/ml)


D
OD 405 nm
0
1
2
3
4
****
4
20
100
Mannose
(5µg/ml)
Fucose
(5µg/ml)
Psl extracted from ΔwspFΔPel (PAO1-P105)
preparation number 4 (µg/ml)


E
OD 405 nm
0
1
2
3
4
****
20
100
Mannose
(5µg/ml)
Fucose
(5µg/ml)
Psl extracted from ΔwspFΔPel (PAO1-P105)
preparation number 5 (µg/ml)


F
OD 405 nm
0
1
2
3
4
****
****
****
****
****
****
20
100
Mannose
(5µg/ml)
Fucose
(5µg/ml)
Psl extracted from ΔwspFΔPel (PAO1-P105)
preparation number 6 (µg/ml)

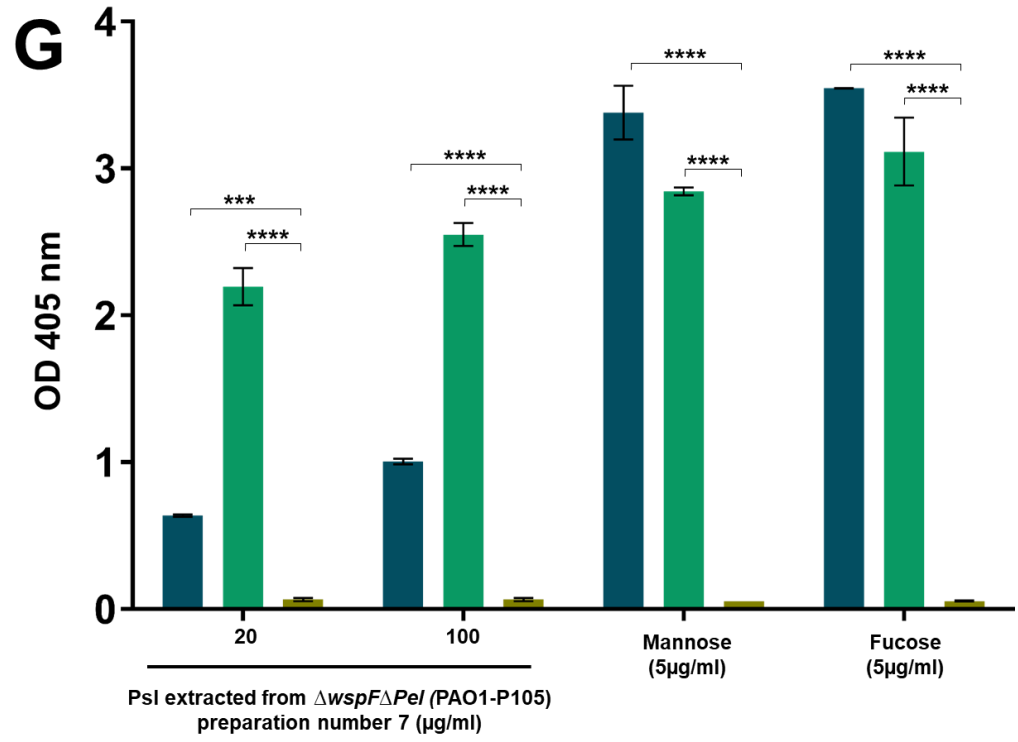

G
OD 405 nm
0
1
2
3
4
***
****
****
****
****
****
20
100
Mannose
(5µg/ml)
Fucose
(5µg/ml)
Psl extracted from ΔwspFΔPel (PAO1-P105)
preparation number 7 (µg/ml)

Figure 4.4: Binding of CTLD4-7-Fc and Fc-DC-SIGN to purified Psl.

Maxisorp plates were coated with different preparations of Psl purified from the Pel-deficient strain ΔwspFΔPel (PAO1-P105) diluted in 154mM NaCl overnight at 37°C. CTLD4-7-Fc and Fc-DC-SIGN chimeric proteins were incubated for 2 hours at room temperature in TSB buffer. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000) dilution. Absorbance was read at 405 nm. Error bars indicate the standard deviation (SD) of three independent repeats. Statistical significance was calculated by two-way ANOVA test. P values are represented as follows: ** = $p \leq 0.01$, *** = $p \leq 0.001$, and **** = $p \leq 0.0001$.

4.5.4 CTLD4-7 region of MR and the extracellular region of DC-SIGN bind to extracted EPS crude from Δ*wspF*Δ*Psl* (PAO1-P106)

After we have shown that CTLD4-7 region of MR and the extracellular region of DC-SIGN bind to different independent preparations of Psl, we wanted to assess the binding of these proteins to the Psl-deficient strain Δ*wspF*Δ*Psl* (PAO1-P106). EPS crude extract from this strain was used the test the binding. Result show that CTLD4-7-Fc and Fc-DC-SIGN bind equally to different dilutions of the EPS crude extract, however, the binding is not dose-dependent (Figure 4.5). The crude extract then was purified and fractionated as described in section 4.4.2 and 160 fractions collected. After that we tested the presence of carbohydrates and none of the fractions contained carbohydrates (data not shown).

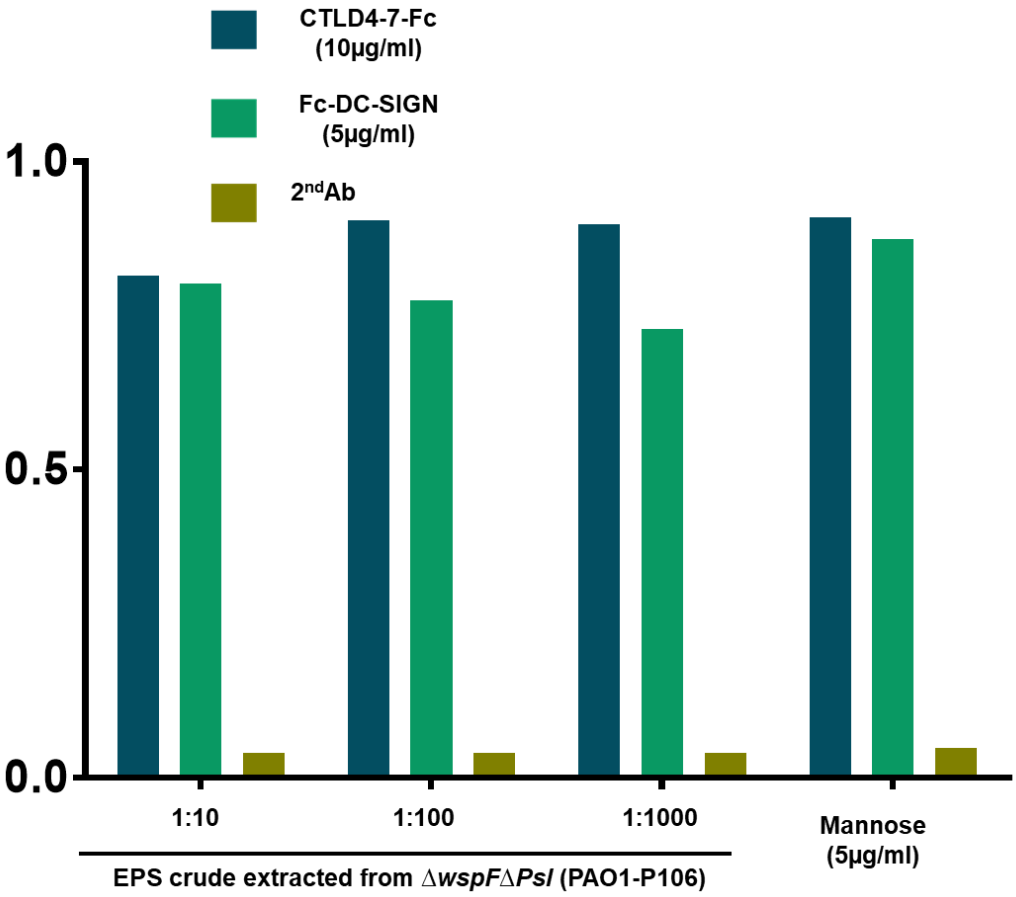
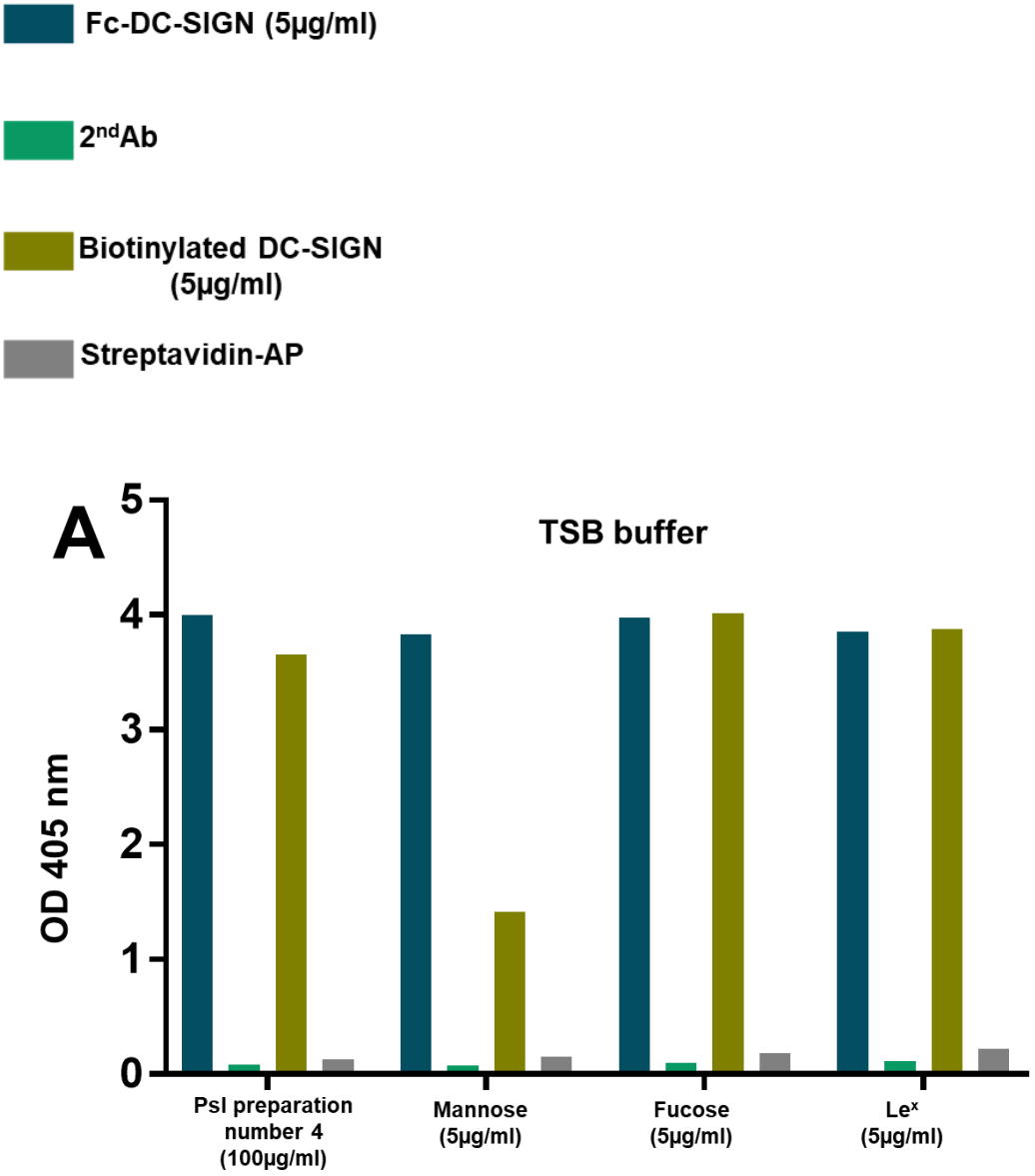


Figure 4.5: CTLD4-7-Fc region of MR and DC-SIGN bind to Δ*wspF*Δ*Psl* (PAO1-P106) EPS crude extract.

Maxisorp plates were coated with different dilutions of EPS crude extracted from the Psl-deficient strain Δ*wspF*Δ*Psl* (PAO1-P106) diluted in 154mM NaCl overnight at 4°C. CTLD4-7-Fc and Fc-DC-SIGN chimeric proteins were incubated for 2 hours at room temperature in TSB buffer. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000) dilution. Absorbance was read at 405 nm. N=1

4.5.5 Biotinylated DC-SIGN binds to purified Psl

After we demonstrated that the binding of Fc-DC-SIGN to purified PA Psl in TSB buffer was highly reproducible, we compared the binding of purified Fc-DC-SIGN (R&D) and biotinylated tetrameric DC-SIGN protein generated in *E. coli* provided by our collaborator Dr. Daniel Mitchell (University of Warwick) (Mitchell et al., 2001). The comparison was performed using two different buffers TSB and TSM as other researchers have used the TSM buffer to investigate DC-SIGN ligand recognition (van Gisbergen et al., 2005, Stax et al., 2011). Results suggest that Fc-DC-SIGN and biotinylated DC-SIGN bound to PA Psl. In both instances binding to Psl and the positive control mannose-PAA was significantly better in TSB than in the TSM buffer suggesting that assay conditions influence the detection of DC-SIGN binding. Fc-DC-SIGN and biotinylated DC-SIGN showed very similar binding to fucose-PAA and Lewis^{x}-PAA in both buffers and binding to mannose-PAA was less pronounced for biotinylated DC-SIGN than for Fc-DC-SIGN (Figure 4.6).


Fc-DC-SIGN (5µg/ml)
2ndAb
Biotinylated DC-SIGN (5µg/ml)
Streptavidin-AP
A
TSB buffer
OD 405 nm
0
1
2
3
4
5
PsI preparation number 4 (100µg/ml)
Mannose (5µg/ml)
Fucose (5µg/ml)
Lex (5µg/ml)

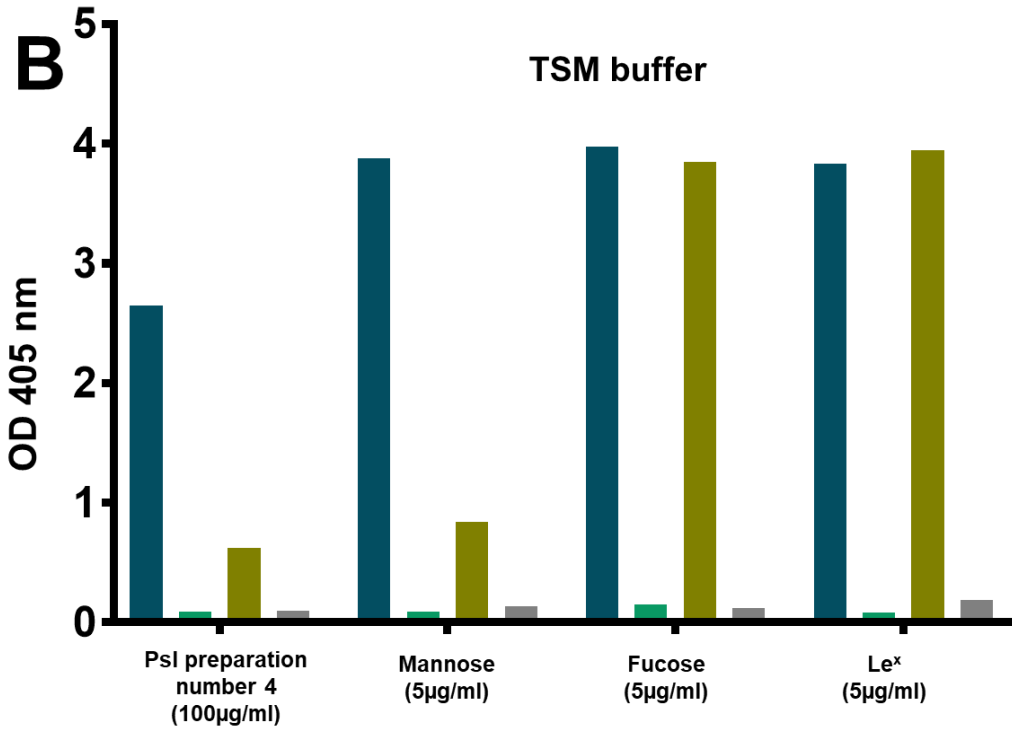


Figure 4.6: Comparison between the binding of biotinylated DC-SIGN and Fc-DC-SIGN to purified Psl in two different buffers (TSB and TSM).

Maxisorp plates were coated with Psl preparation number 4 in 154mM NaCl overnight at 37°C. Biotinylated DC-SIGN or Fc-DC-SIGN diluted in TSB (A) or TSM (B) buffer was incubated for 2 hours at room temperature. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP or Streptavidin conjugated to AP (1:1000 dilution for both antibodies). Absorbance was read at 405 nm. N=2.

4.5.6 Psl binds to immobilised DC-SIGN

After we showed that both Fc-DC-SIGN and biotinylated DC-SIGN bound to PA Psl using ELISA, we wanted to confirm the binding using another approach. Plasmon surface resonance technology was included to measure the interaction affinity between DC-SIGN and PA Psl. The experiment was conducted by a former student in Dr. Martinez-Pomares's lab at Dr Mitchell's laboratory using tetrameric

DC-SIGN produced in *E. coli*. Briefly, DC-SIGN was coupled to a sensor chip and then Psl was flowed over the chip at different concentrations. When the analyte (Psl) binds to a receptor coupled to the chip, the SPR detects signals of changes in the refractive index, and these signals are proportional with the increase in the binding. Finally, the signals are quantified in response units (RU). Data shows that DC-SIGN protein binds to PA Psl (preparation number 4) in a dose dependent manner (Figure 4.7).

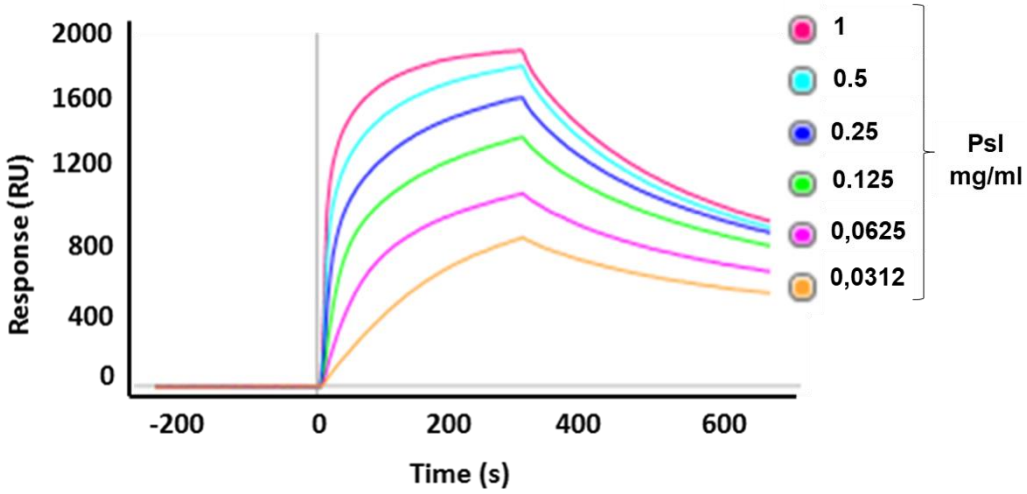
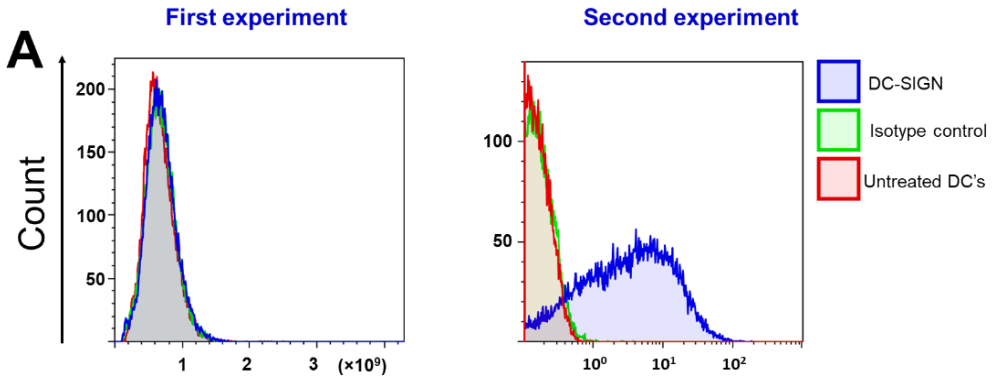


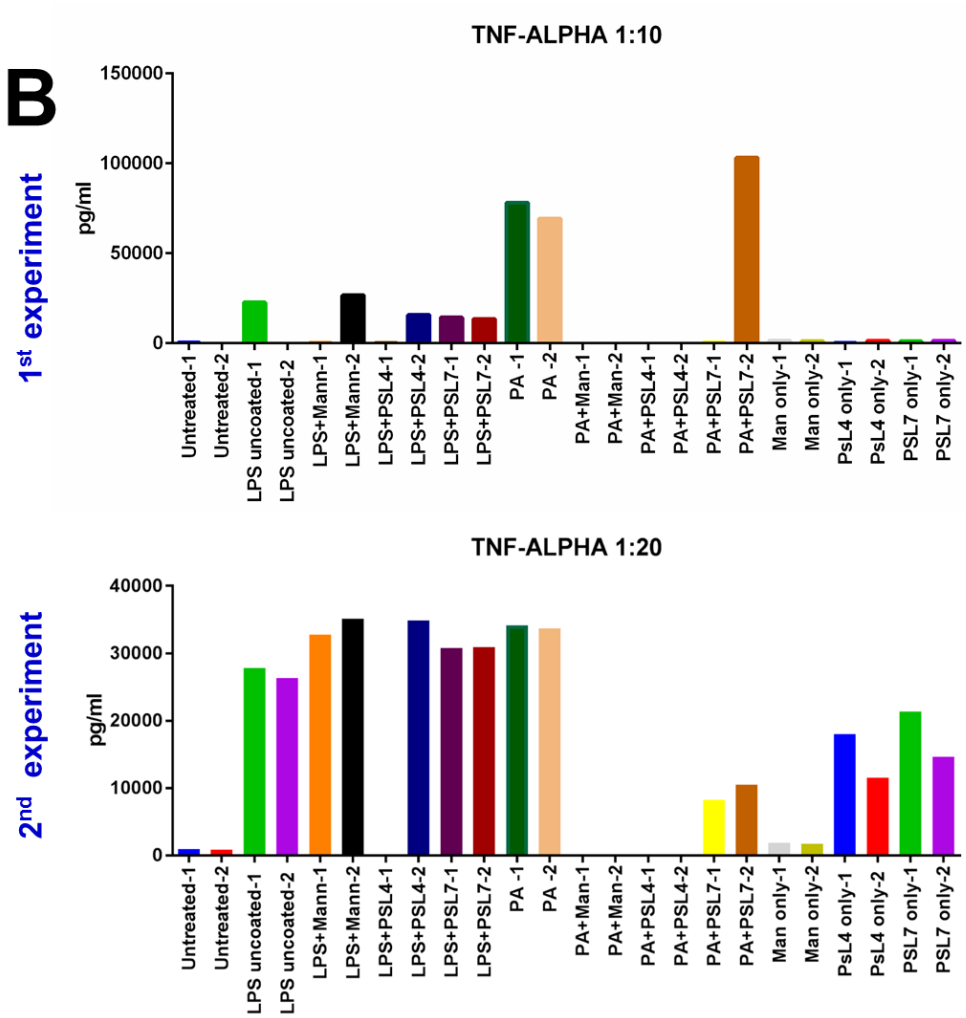
Figure 4.7: Fc-DC-SIGN binds to Psl Using Surface Plasmon Resonance.

Recombinant DC-SIGN was immobilized on a chip and then incubated with different concentrations of Psl extracted from ΔwspFΔPel (PA105O1-P), Pel-deficient. 25 µg/mL of DC-SIGN were immobilized on a sensor chip via amine coupling. Analyte (Psl) was prepared in 25 mM HEPES pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$, and 0.01% Tween-20 at different concentrations (1- 0.03125) mg/ml and then flowed over the channels at a rate of 25 µL/minute at 25˚C for 300 seconds. Dissociation was performed for additional 300 seconds, and regeneration of the sensor chip surfaces was performed using 10 mM EGTA.

4.5.7 Establishing a procedure to assess whether Psl could modulate the activation status of DCs through engagement of DC-SIGN

In order to examine whether Psl has any immunomodulatory action on hu DCs, fresh monocyte-derived hu DCs generated as described in section 2.9 were incubated in wells of Maxisorp plates coated with purified Psl (100µg/ml) or left uncoated, in the absence or presence of LPS (100 ng/ml) or paraformaldehyde-fixed planktonic PAO1-P (MOI 50). Levels of TNF-α, IL-8, IL-β, IL-12(P70), IL-6, and IL-10 were measured by magnetic luminex assay. In some instances, we noticed that Psl (4 and/or 7) induced production of some interleukins such as TNF-α, IL-β, IL-6, and IL-10 when stimulated with fixed PA. While on other occasions, no effect was detectable (Figure 4.8**Figure 4.8**). Due to this inconsistency, we were unable to interpret these findings. Alternative experimental approaches to address the potential immune modulatory properties of Psl will be discussed in Chapter 6.




B
1st experiment
TNF-ALPHA 1:10
pg/ml
150000
100000
50000
0
Untreated-1
Untreated-2
LPS uncoated-1
LPS uncoated-2
LPS+Mann-1
LPS+Mann-2
LPS+PSL4-1
LPS+PSL4-2
LPS+PSL7-1
LPS+PSL7-2
PA -1
PA -2
PA+Man-1
PA+Man-2
PA+PSL4-1
PA+PSL4-2
PA+PSL7-1
PA+PSL7-2
Man only-1
Man only-2
PsL4 only-1
PsL4 only-2
PSL7 only-1
PSL7 only-2
2nd experiment
TNF-ALPHA 1:20
pg/ml
40000
30000
20000
10000
0
Untreated-1
Untreated-2
LPS uncoated-1
LPS uncoated-2
LPS+Mann-1
LPS+Mann-2
LPS+PSL4-1
LPS+PSL4-2
LPS+PSL7-1
LPS+PSL7-2
PA -1
PA -2
PA+Man-1
PA+Man-2
PA+PSL4-1
PA+PSL4-2
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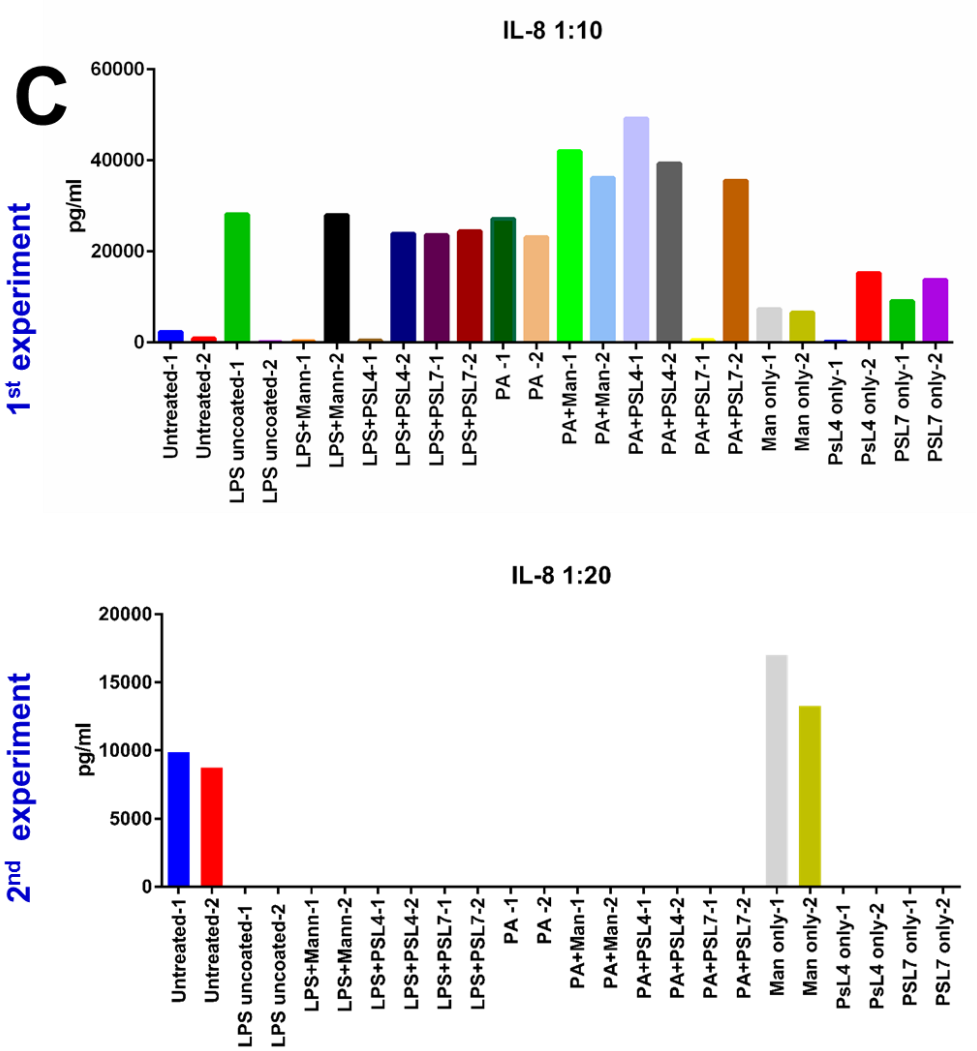
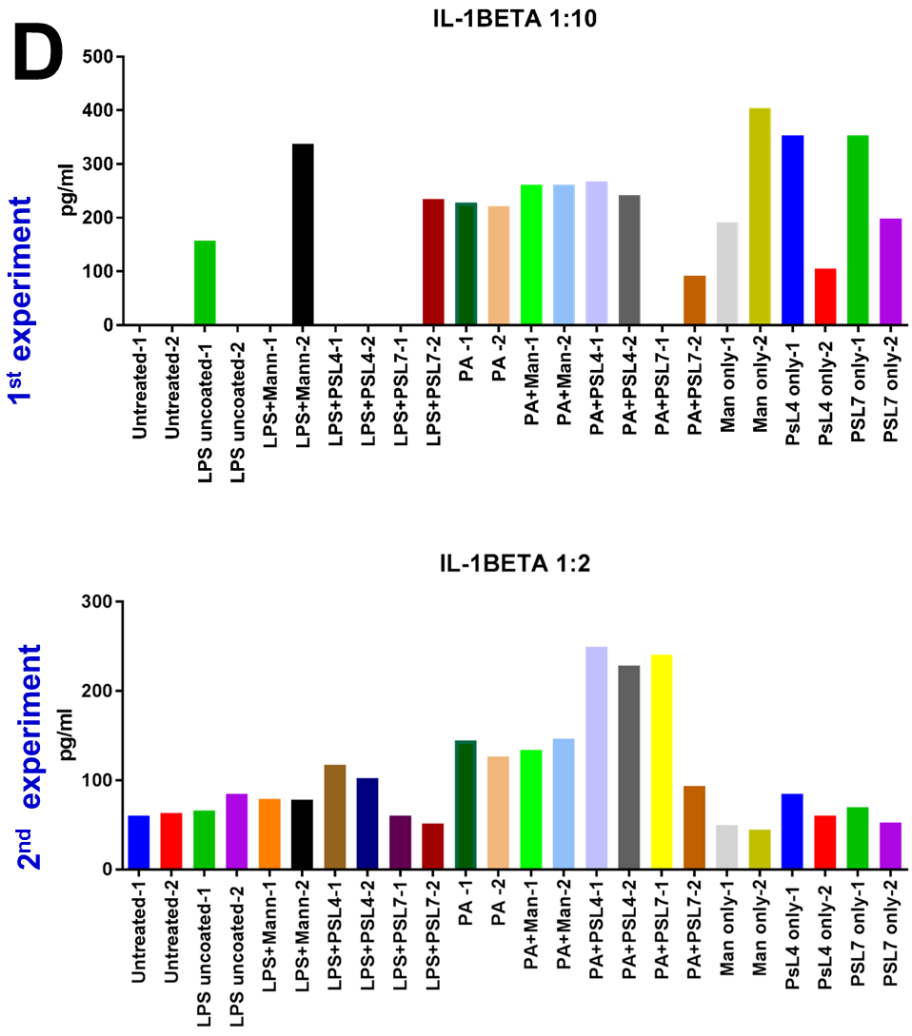
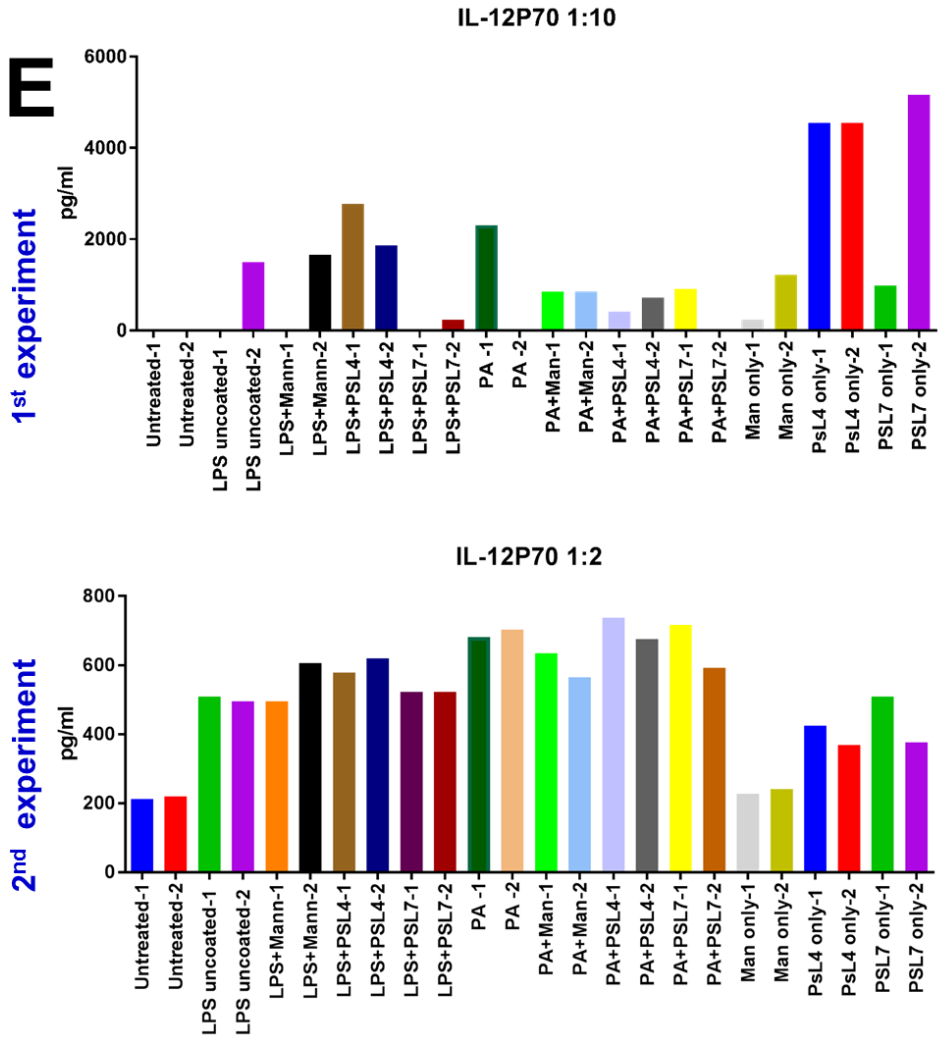
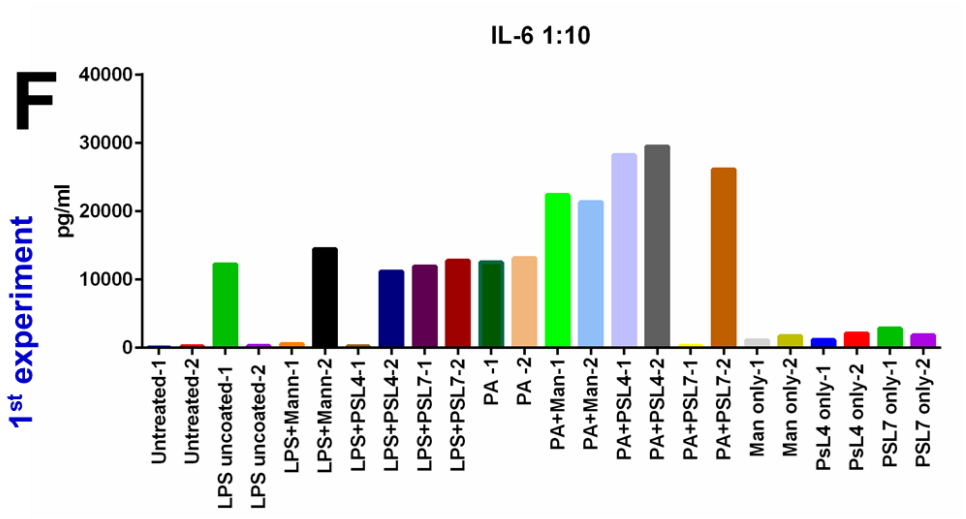
PsL4 only-2
PSL7 only-1
PSL7 only-2


C
IL-8 1:10
1st experiment
pg/ml
60000
40000
20000
0
Untreated-1
Untreated-2
LPS uncoated-1
LPS uncoated-2
LPS+Mann-1
LPS+Mann-2
LPS+PSL4-1
LPS+PSL4-2
LPS+PSL7-1
LPS+PSL7-2
PA -1
PA -2
PA+Man-1
PA+Man-2
PA+PSL4-1
PA+PSL4-2
PA+PSL7-1
PA+PSL7-2
Man only-1
Man only-2
PsL4 only-1
PsL4 only-2
PSL7 only-1
PSL7 only-2
IL-8 1:20
2nd experiment
pg/ml
20000
15000
10000
5000
0
Untreated-1
Untreated-2
LPS uncoated-1
LPS uncoated-2
LPS+Mann-1
LPS+Mann-2
LPS+PSL4-1
LPS+PSL4-2
LPS+PSL7-1
LPS+PSL7-2
PA -1
PA -2
PA+Man-1
PA+Man-2
PA+PSL4-1
PA+PSL4-2
PA+PSL7-1
PA+PSL7-2
Man only-1
Man only-2
PsL4 only-1
PsL4 only-2
PSL7 only-1
PSL7 only-2


D
IL-1BETA 1:10
1st experiment
pg/ml
500
400
300
200
100
0
Untreated-1
Untreated-2
LPS uncoated-1
LPS uncoated-2
LPS+Mann-1
LPS+Mann-2
LPS+PSL4-1
LPS+PSL4-2
LPS+PSL7-1
LPS+PSL7-2
PA -1
PA -2
PA+Man-1
PA+Man-2
PA+PSL4-1
PA+PSL4-2
PA+PSL7-1
PA+PSL7-2
Man only-1
Man only-2
PsL4 only-1
PsL4 only-2
PSL7 only-1
PSL7 only-2
IL-1BETA 1:2
2nd experiment
pg/ml
300
200
100
0
Untreated-1
Untreated-2
LPS uncoated-1
LPS uncoated-2
LPS+Mann-1
LPS+Mann-2
LPS+PSL4-1
LPS+PSL4-2
LPS+PSL7-1
LPS+PSL7-2
PA -1
PA -2
PA+Man-1
PA+Man-2
PA+PSL4-1
PA+PSL4-2
PA+PSL7-1
PA+PSL7-2
Man only-1
Man only-2
PsL4 only-1
PsL4 only-2
PSL7 only-1
PSL7 only-2


E
1st experiment
IL-12P70 1:10
pg/ml
6000
4000
2000
0
Untreated-1
Untreated-2
LPS uncoated-1
LPS uncoated-2
LPS+Mann-1
LPS+Mann-2
LPS+PSL4-1
LPS+PSL4-2
LPS+PSL7-1
LPS+PSL7-2
PA -1
PA -2
PA+Man-1
PA+Man-2
PA+PSL4-1
PA+PSL4-2
PA+PSL7-1
PA+PSL7-2
Man only-1
Man only-2
PsL4 only-1
PsL4 only-2
PSL7 only-1
PSL7 only-2
2nd experiment
IL-12P70 1:2
pg/ml
800
600
400
200
0
Untreated-1
Untreated-2
LPS uncoated-1
LPS uncoated-2
LPS+Mann-1
LPS+Mann-2
LPS+PSL4-1
LPS+PSL4-2
LPS+PSL7-1
LPS+PSL7-2
PA -1
PA -2
PA+Man-1
PA+Man-2
PA+PSL4-1
PA+PSL4-2
PA+PSL7-1
PA+PSL7-2
Man only-1
Man only-2
PsL4 only-1
PsL4 only-2
PSL7 only-1
PSL7 only-2


F
1st experiment
IL-6 1:10
pg/ml
40000
30000
20000
10000
0
Untreated-1
Untreated-2
LPS uncoated-1
LPS uncoated-2
LPS+Mann-1
LPS+Mann-2
LPS+PSL4-1
LPS+PSL4-2
LPS+PSL7-1
LPS+PSL7-2
PA -1
PA -2
PA+Man-1
PA+Man-2
PA+PSL4-1
PA+PSL4-2
PA+PSL7-1
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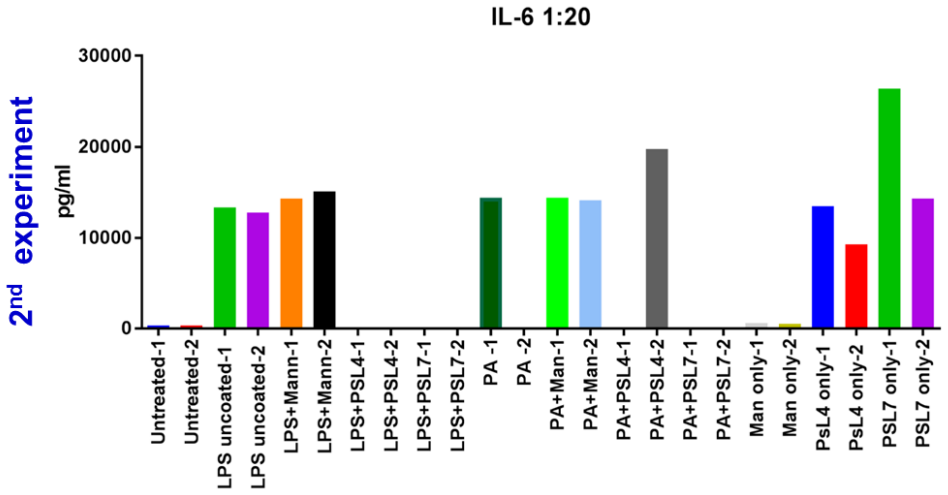
2nd experiment
IL-6 1:20
pg/ml
30000
20000
10000
0
Untreated-1
Untreated-2
LPS uncoated-1
LPS uncoated-2
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LPS+Mann-2
LPS+PSL4-1
LPS+PSL4-2
LPS+PSL7-1
LPS+PSL7-2
PA -1
PA -2
PA+Man-1
PA+Man-2
PA+PSL4-1
PA+PSL4-2
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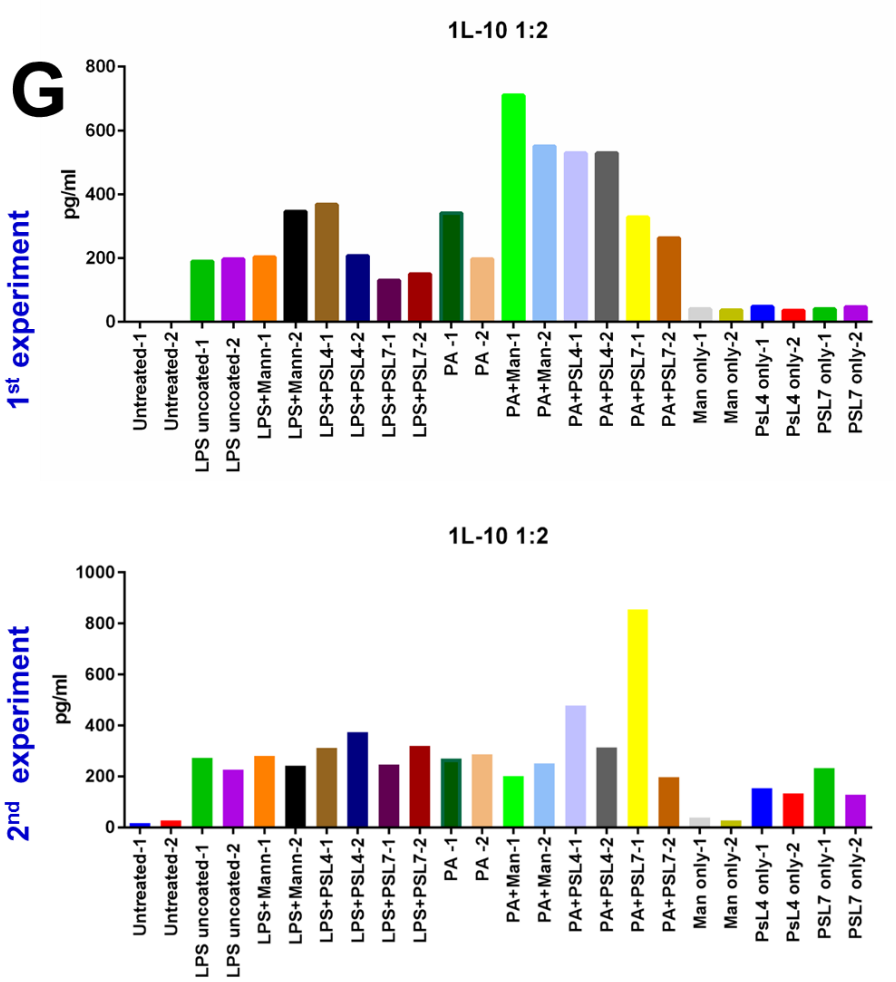
PSL7 only-1
PSL7 only-2


G
1L-10 1:2
1st experiment
pg/ml
800
600
400
200
0
Untreated-1
Untreated-2
LPS uncoated-1
LPS uncoated-2
LPS+Mann-1
LPS+Mann-2
LPS+PSL4-1
LPS+PSL4-2
LPS+PSL7-1
LPS+PSL7-2
PA -1
PA -2
PA+Man-1
PA+Man-2
PA+PSL4-1
PA+PSL4-2
PA+PSL7-1
PA+PSL7-2
Man only-1
Man only-2
PsL4 only-1
PsL4 only-2
PSL7 only-1
PSL7 only-2

1L-10 1:2
2nd experiment
pg/ml
1000
800
600
400
200
0
Untreated-1
Untreated-2
LPS uncoated-1
LPS uncoated-2
LPS+Mann-1
LPS+Mann-2
LPS+PSL4-1
LPS+PSL4-2
LPS+PSL7-1
LPS+PSL7-2
PA -1
PA -2
PA+Man-1
PA+Man-2
PA+PSL4-1
PA+PSL4-2
PA+PSL7-1
PA+PSL7-2
Man only-1
Man only-2
PsL4 only-1
PsL4 only-2
PSL7 only-1
PSL7 only-2

Figure 4.8: Inconsistent cytokine production by hu DC stimulated in the presence or absence of purified Psl.

(A) Flow cytometry analysis of human DCs used in two independent experiments. Histograms denoting expression of DC-SIGN on DCs generated from $CD14^+$ monocytes obtained from buffy coats in RPMI complete medium with 800IU/ml of GM-CSF and 500IU/ml of IL-4. Red lines represent unstained cells, green lines represent cells stained with isotype control antibody, and blue lines represent the specific staining for DC-SIGN.

(B) Wells of sterilised maxisorp plates were coated with 100µg/ml of filtered Psl 4, Psl 7, or mannan prepared in 154mM NaCl and incubated overnight at 37°C, 5%CO_2. DCs (5×10^4 cells/per well) generated from $CD14^+$ cultured in RPMI complete medium with 800IU/ml of GM-CSF and 500IU/ml of IL-4, were resuspended in X-vivo-15 medium and added to each well and incubated for 2 hours at 37°C with 5%CO_2. After checking the viability of the cells by light microscopy, LPS PAO1-10 or fixed PAO1-P WT was added to appropriate wells and incubated for 4 hours at 37°C with 5%CO_2. The cytokines in supernatants were quantified using magnetic luminex screening system according to the manufactures' procedure (B-H). The experiment was done twice.

4.6 Summary

- We were able to extract PA Psl from PA biofilms with reduced protein contamination.
- MR and DC-SIGN bound to PA Psl, and DC-SIGN bound significantly better than MR. This indicates that these C-type lectin receptors could play a crucial role in the recognition of PA.
- MR and DC-SIGN bound to crude extract of a Psl-deficient PA strain.
- Binding of Fc-DC-SIGN (R&D) and biotinylated DC-SIGN to PA Psl is influenced by assay conditions being stronger in TSB than in TSM.

- No Psl immunomodulatory effect on hu DCs was detected due to inconsistent data.

Chapter 5: Recognition of *Pseudomonas aeruginosa* lipopolysaccharide by Fc-DC-SIGN

5.1 Introduction

PA is an opportunistic pathogenic organism that can cause persistent infections in patients with CF, burn, and wound. PA lipopolysaccharide (LPS) plays a crucial role in pathogen virulence and host response (Pier, 2007b). LPS is a glycolipid complex which is the major component of the outer membrane in Gram-negative bacteria (King et al., 2009a). PA LPS plays an important role in the structural integrity of PA biofilms by promoting bacterial adhesion and cell-to-cell adherence (Lau et al., 2009, Wozniak et al., 2003). The impact of LPS on bacterial pathogenicity and host immunity depends on the increased susceptibility of patients to infection and the structure of LPS i.e. it depends particularity on the structural components of lipid A, and O-antigen (Pier, 2007b).

Three LPS main domains have been identified in all Gram-negative bacteria including PA. 1: The lipid A portion, which is attached to the outer membrane. 2: Core Oligosaccharide region. 3: Highly variable O polysaccharide (O antigen) which can facilitate the interaction of the cell to a substrate (Figure 5.1). Lipid A and Core Oligosaccharides are essential for organism viability, but O antigen is not (Kintz and Goldberg, 2008, Kocincova and Lam, 2011) Figure 5.1

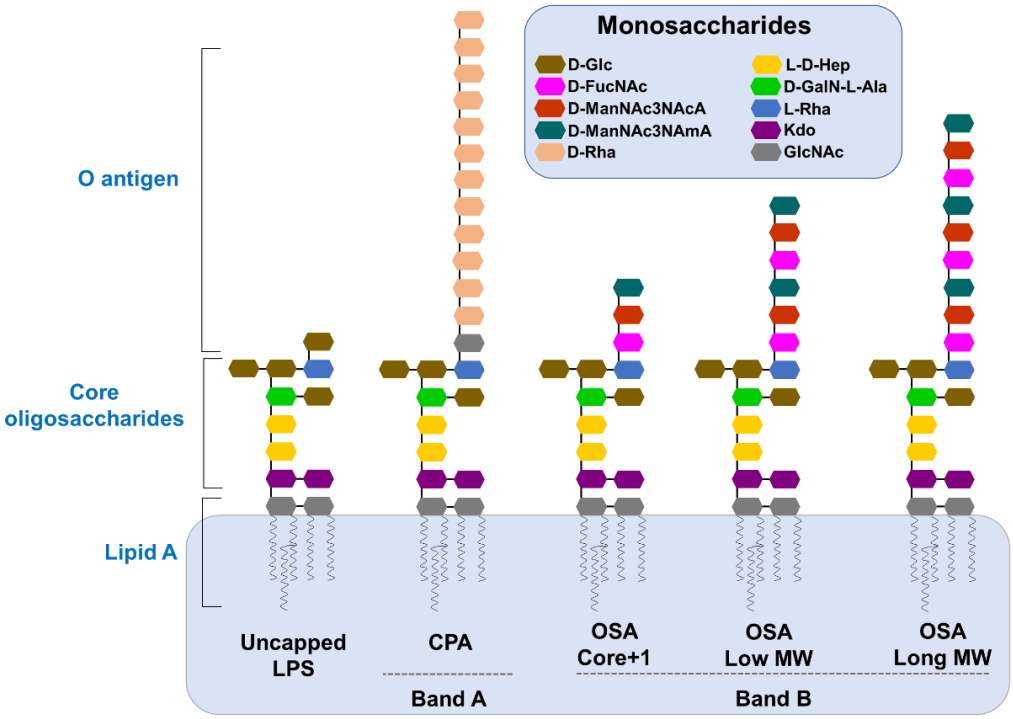


Figure 5.1: Diversity of PA LPS glycol-forms. Adapted from (Lam et al., 2011a)

lipid A is responsible for the toxicity of LPS (Pier, 2007a). Lipid A is composed of an O- and N-acylated di-glucosamine bisphosphate backbone with variable types of fatty acids attached to the backbone. These fatty acids anchor LPS into the outer membrane (Lam et al., 2011a, Al-Wrafy et al., 2017). LPS activates immune cells through the TLR4 receptor complex. Lipid A binds to CD14 to form lipidA-CD14 complex. Lipid A is transferred to MD2 complexed with TLR4 leading to TLR4-TLR4 homo-dimerization. This cascade leads to the activation of NF-κB and IRF3 and production of inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Park and Lee, 2013, Al-Wrafy et al., 2017). In all wild type strains, lipid A is attached to core oligosaccharides (King et al., 2009a). The core oligosaccharide consists of nine or ten sugar units that link the lipid A and the O antigen (King et al., 2009a, Hao et al., 2013).

The O-polysaccharide or O-antigen is a distinctive structure and it is responsible for the different serogroups. O polysaccharide (O antigen) consists of a repetitive carbohydrate polymer attached to the core oligosaccharide (Bystrova et al., 2006, King et al., 2009a). Strains that express O antigen are named as LPS-smooth, while strains that lack the O antigen are named as LPS-rough (Latino et al., 2017).The CF isolates from lung infection are often LPS-rough (Schurek et al., 2012). Most PA strains produce two forms of O antigen.1: A homopolymer of D-rhamnose trisaccharide repeats named as common polysaccharide antigen (CPA) or A band; 2: A heteropolymer that consists of repeating units of three to five distinct sugars named as O-specific antigen (OSA) or B band (Lam et al., 2011a, Wang et al., 2015). Deletion in *rmd*, *wbpM*, or *wbpL* genes produces loss of CPA, OSA, or both, respectively (Murphy et al., 2014b).

OSA is highly immunogenic and able to elicit strong antibody responses. The organization of sugar units varies from strain to strain. The International Antigenic Typing Scheme (IATS) relies on this variation to classify PA strains into 20 serotypes, O1 to O20 (Lam et al., 2011b, Thrane et al., 2015).

As described above CPA is a homopolymer of D-rhamnose and produced by most PA strains. It is not highly immunogenic, and it can elicit a weak antibody response, probably due to the neutral charge of rhamnose. There is evidence that CPA plays a crucial role during PA infections. During chronic lung infection in CF patients, the production of the OSA is reduced while the level of CPA is maintained on the cell surface. It has also been shown that CPA, in vitro, can facilitate the attachment of PA to airway epithelial cells (Hao et al., 2013, Zdorovenko et al., 2015). In vitro, the phenotype of LPS shifts from CPA^{+} OSA^{+} to $CPA^{+}OSA^{-}$ when PAO1 is cultured as biofilms, with this shift being reversible (Murphy et al., 2014b). It has been suggested that this shift promotes persistence of PA in chronic infections. Outer

membrane vesicles (OMVs) are spherical extracellular duds originate from the outer membrane (OM) of Gram-negative bacteria. OMVs have been involved in many activities such as QS molecules release and virulence factors production (Schwechheimer and Kuehn, 2015). It has been shown that OMVs have a role in biofilm formation (Schooling and Beveridge, 2006). A previous study has demonstrated that $CPA^{-}OSA^{+}$ mutant exhibited changes in the biofilm matrix and could not develop vigorous biofilm compared to WT (Murphy et al., 2014b). This underlines the significant role of CPA in biofilm development.

Recent data showed that DC-SIGN can interact with LPS through GlcNAc structures in some Gram-negative bacteria (Geijtenbeek et al., 2009). *H pylori* and some strains of *Klebsiella pneumonia* interact with DC-SIGN through LPS that contains Lewis blood group antigen and mannose, respectively (van Kooyk and Geijtenbeek, 2003a). Specifically, *H. pylori* LPS contains Lewis blood group antigens, Lewis-X and -Y. During infection, *H. pylori* can control the expression of Lewis-X and -Y through LPS phase variation (Lewis-X^{+}/Y^{+} and/or Lewis-X^{+}/Y^{-}). Interestingly, *H. pylori* switches Lewis-X^{+}/Y^{-} and utilizes Lewis-X^{+}/Y^{+} to target DCs through DC-SIGN to increase the level of interleukin-10 and block Th1 responses (Cambi and Figdor, 2005). By contrast, binding of *Neisseria meningitidis* LPS to DC-SIGN skews naïve T cells towards Th1 response (Steeghs et al., 2006). These findings underline the important role for DC-SIGN as a PRR in the recognition of LPS

5.2 Hypothesis

Our early results showed that DC-SIGN binds to biofilm formed by PA106, which is Psl deficient. We hypothesized that DC-SIGN had a candidate ligand, such as LPS, on the outer membrane of PA.

5.3 Aims

- Determine the capability of DC-SIGN to interact with planktonic PA (lab strains and clinical isolates).
- Investigate potential DC-SIGN ligand(s) on the outer membrane of PA.
- Examine the binding of DC-SIGN to PA LPS using LPS mutants, extracted PA LPS, and purified PA LPS.
- Assess the binding of monocyte-derived huDCs to fixed planktonic PA using flow cytometry.

5.4 Results

5.4.1 Biofilm of Δ*wspF*Δ*Psl* (PAO1-PA106) is recognized by the extracellular region of DC-SIGN but not the CTLD4-7 region of MR

After we showed that CTLD4-7-Fc and Fc-DC-SIGN bind to PAO1-P biofilms that contain either Psl and Pel or Psl only (Figure 3.5), we wanted to examine whether these chimeric proteins could recognize Δ*wspF*Δ*Psl* (PAO1-P106) biofilm which contains only Pel. We compared the binding of CTLD4-7-Fc and Fc-DC-SIGN to biofilm from Δ*wspF*Δ*Psl* (PAO1-PA106) and Δ*wspF*Δ*Pel* (PAO1-PA105). We

found that Fc-DC-SIGN bound to PA106 biofilm, Psl deficient, and PA105 biofilm, Pel deficient, while CTLD4-7-Fc could not recognize PA106 biofilm (Figure 5.2).

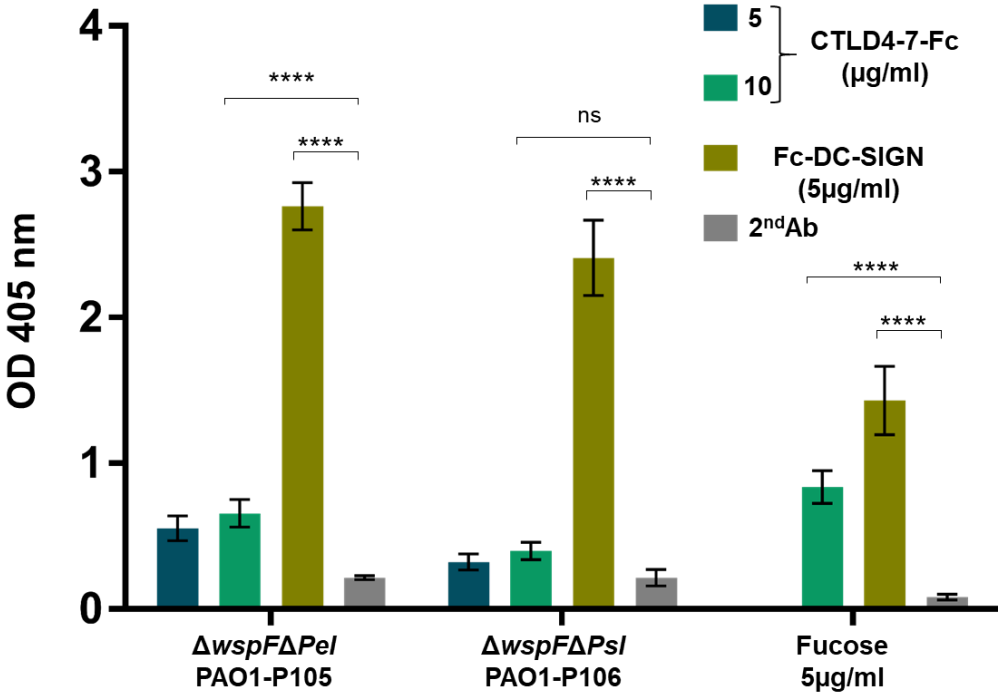


Figure 5.2: Binding of Fc-DC-SIGN to Δ*wspF*Δ*Psl* (PAO1-P106) biofilm

Biofilm from Δ*wspF*Δ*Pel* (PAO1-P105) and Δ*wspF*Δ*Psl* (PAO1-P106), developed over 24 hours using Costar plate, were incubated with 5 and 10 µg/ml of CTLD4-7-Fc and 5 µg/ml Fc-DC-SIGN chimeric proteins for 2 hours at room temperature. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000 dilution). Absorbance was read at 405 nm. Error bars indicate the standard deviation (SD) of three independent repeats. Statistical significance was calculated by two-way ANOVA test. P values are represented as follows: ns = $p > 0.05$ and **** = $P \leq 0.0001$.

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5.4.2 Fc-DC-SIGN binds to planktonic PAO1-P

The fact that DC-SIGN binds to PA biofilm contains Psl or/and Pel, led us to explore another Fc-DC-SIGN ligand within PA cultures other than EPS. We tested the

binding of CTLD4-7-Fc and Fc-DC-SIGN to several fixed whole planktonic PAO1 strains, which have the same level of c-di-GMP but express different types of EPS (Table 2.1). Results show that only Fc-DC-SIGN was able to bind to all the strains regardless their different EPS levels. Also, Fc-DC-SIGN bound to Δ*wspF*Δ*Psl*Δ*Pel* (PAO1-PA107), biofilm deficient (Figure 5.3). This indicates that there is another DC-SIGN ligand, in addition to Psl, within the PA outer cell membrane.

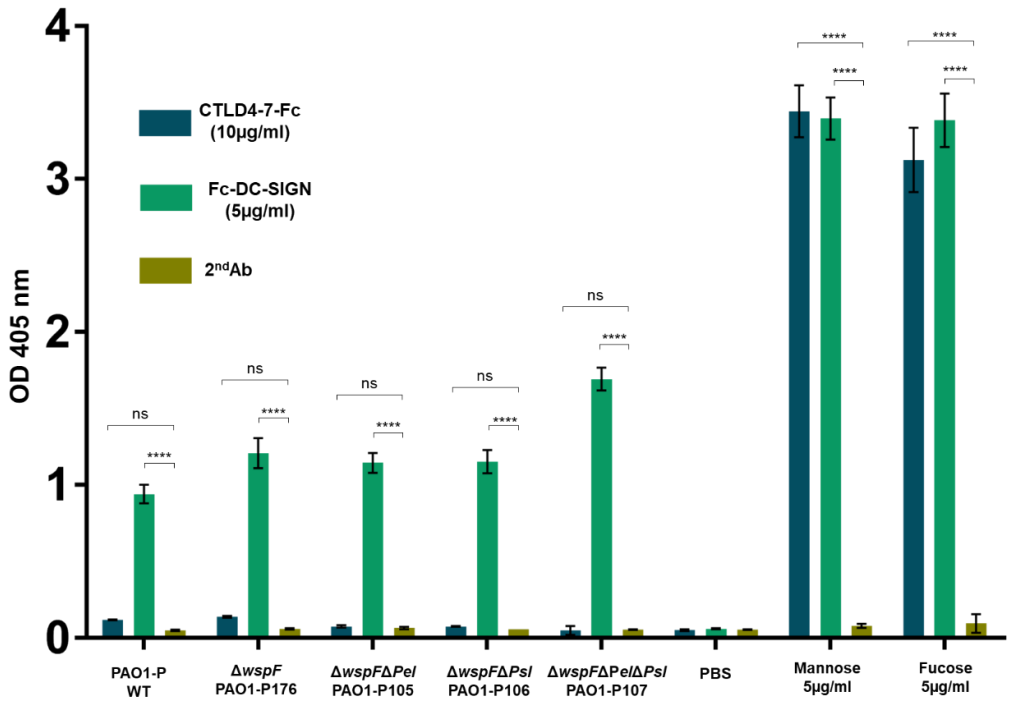


Figure 5.3: Binding of Fc-DC-SIGN to planktonic PAO1-P.

Wells of Costar plates were coated overnight with 100 µl of fixed PAO1-P suspension adjusted to 0.5 OD_{600nm} in PBS overnight at 4 °C. 10µg/ml of CTLD4-7-Fc or 5µg/ml of Fc-DC-SIGN was incubated for 2 h at room temperature to assess the binding to whole bacteria. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000 dilution). Absorbance was read at 405 nm. Error bars indicate the standard deviation (SD) of four independent repeats. Statistical significance was calculated by two-way ANOVA test. P values are represented as follows: ns = $p > 0.05$ and **** = $P \leq 0.0001$.

5.4.3 The extracellular region of DC-SIGN binds to planktonic PA from wound isolates

To confirm that the binding of Fc-DC-SIGN to whole planktonic PA occurred when using strains other than PAO1-P, the binding of Fc-DC-SIGN to clinical wound planktonic PA isolates was tested. We used a combination of PA wound isolates collected from a single patient at different sites (bone, blood, or wound) and PAO1-Nottingham lab strain which has a major deletion in the chromosome. Results show that CTLD4-7Fc displayed very weak binding to all samples while Fc-DC-SIGN bound to all planktonic PA wound isolates as seen for planktonic PAO1-P (Figure 5.4).

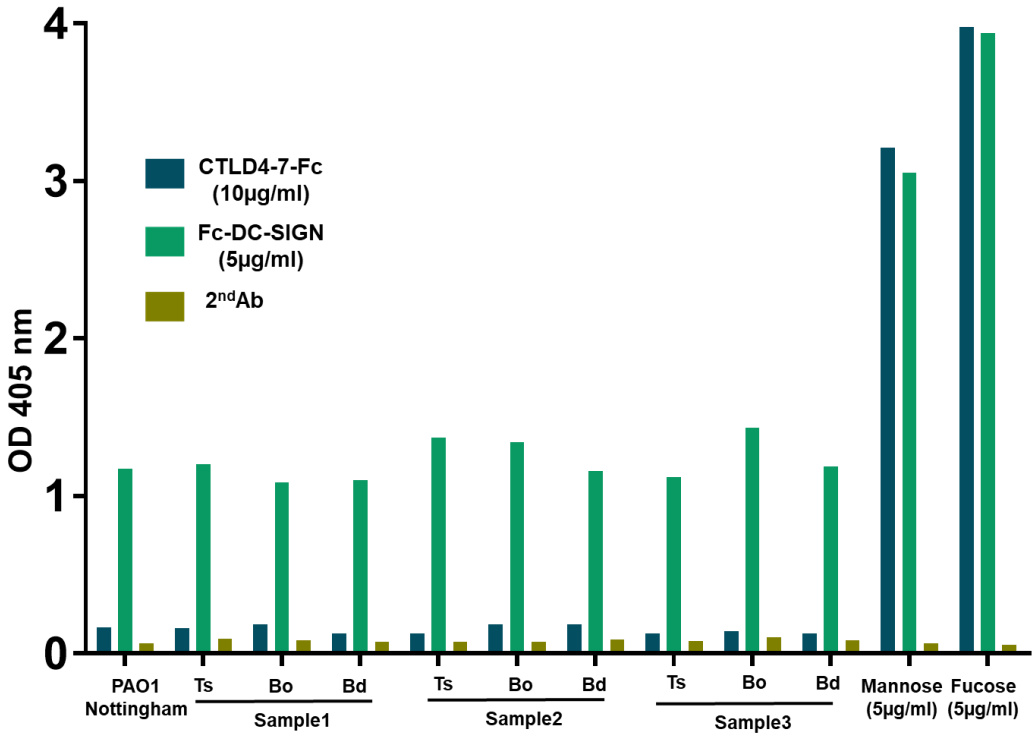


Figure 5.4: Binding of Fc-DC-SIGN to fixed PA wound isolates.

Maxisorp plates were coated overnight with 100 µl of fixed PA wound isolates or the Nottingham laboratory PAO1 strain adjusted to 0.5 OD_{600} in PBS overnight at 4 °C. 10µg/ml of CTLD4-7-Fc or 5µg/ml of Fc-DC-SIGN chimeric protein was incubated for 2 h at room temperature to assess the binding to whole bacteria. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1,000 dilution). Absorbance was read at 405 nm. Ts stands for tissue, Bo stands for bone, Bd stands for blood. N=2.

5.4.4 Mucoid CF isolates form a gel-structure pellet in X-vivo-15 media

In order to test the binding of any chimeric protein to whole planktonic PA, we need to prepare fixed PA as described in Section 2.6. After growing CF mucoid isolates in X-vivo-15 media overnight, the cultures were centrifuged at 16,000xg for 5 min at 4°C. These cultures failed to form an intact pellet and rather formed a gel-structure. This observation led us to compare the growth of these isolates in X-vivo-15 and LB. We were able to recover regular pellet from cultures grown in LB, while cultures grown in X-vivo-15 still formed a gel-structure after centrifugation. The pictures below show the formation of different pellets structures by PA-A002 (CF mucoid isolates). We observed the same phenomenon in all mucoid CF isolates (Figure 5.5). This indicates that the formation of a gel-like pellet from mucoid CF isolates could be attributed to the chemical components of X-vivo-15 media.

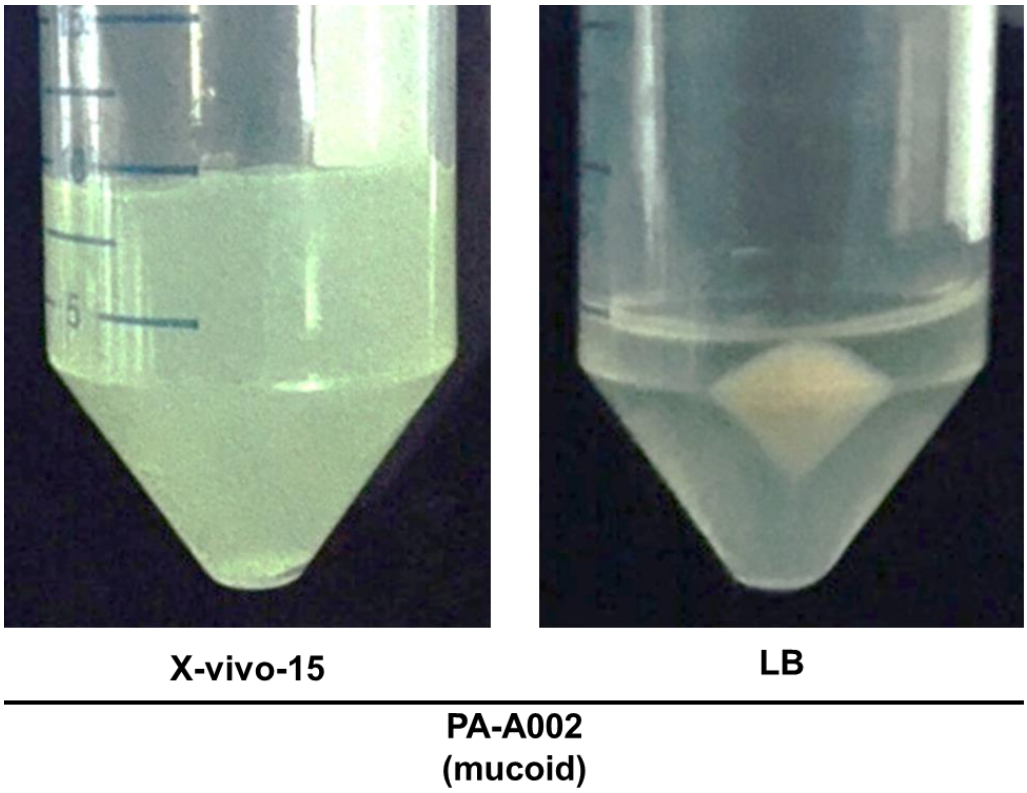


Figure 5.5: Failure of mucoid CF isolates to form pellets after centrifugation when grown in X-vivo-15.

CF mucoid isolates were grown in LB plates and incubated overnight at 37 °C. A single colony was selected and inoculated into 5 ml of X-vivo-15 or LB media and incubated overnight at 37 °C, 200 rpm, 5% CO_2. Cultures were centrifuged at 16,000 x g for 10 min. Images from PA-A002 mucoid CF isolate, but the same procedure was performed with other mucoid CF isolates described in Table 2.1 such as PA-20, PA-37, and PA-PO13 and we observed the same gel-structure pellets in all the CF mucoid isolates.

5.4.5 The extracellular region of DC-SIGN binds to PA CF isolates in planktonic form

To explore the ability of MR (CTLD4-7 region) and Fc-DC-SIGN to bind to planktonic mucoid CF isolates binding assays were performed using bacteria cultures grown in LB. Data suggest that Fc-DC-SIGN bound strongly to both CF isolates in a dose dependent manner while MR showed very weak binding (Figure 5.6).

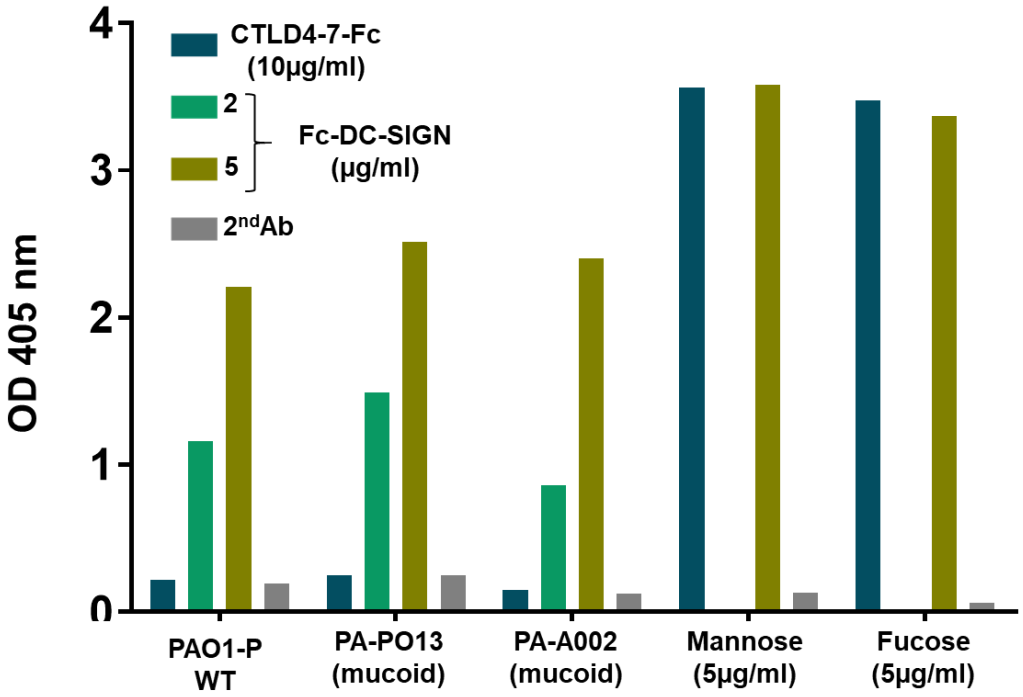


Figure 5.6: Binding of Fc-DC-SIGN to fixed planktonic PACF isolates.

Maxisorp plates were coated overnight with 100 µl of fixed PA CF isolates, grown in LB, adjusted to 0.5 OD_{600nm} in PBS overnight at 4 °C. 10µg/ml of CTLD4-7-Fc or 2 and 5µg/ml of Fc-DC-SIGN chimeric proteins were incubated for 2 h at room temperature to assess the binding to whole bacteria. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000 dilution). Absorbance was read at 405 nm. N=2.

5.4.6 Fc-DC-SIGN binds to planktonic PAO1-C through CPA-LPS

After we found that Fc-DC-SIGN binds to planktonic PA as shown in Figure 5.3, we wanted to further explore the role of DC-SIGN in the recognition of planktonic PA. Thus, we used a combination of PAO1-C lab strains which express different types of PA LPS (Table 2.1). Results show that Fc-DC-SIGN strongly bounds to planktonic PA which express CPA-LPS while binding to strains that only express

OSA-LPS, i.e. CPA deficient, was very weak (Figure 5.7). This indicates that PA CPA-LPS is a candidate ligand of DC-SIGN.

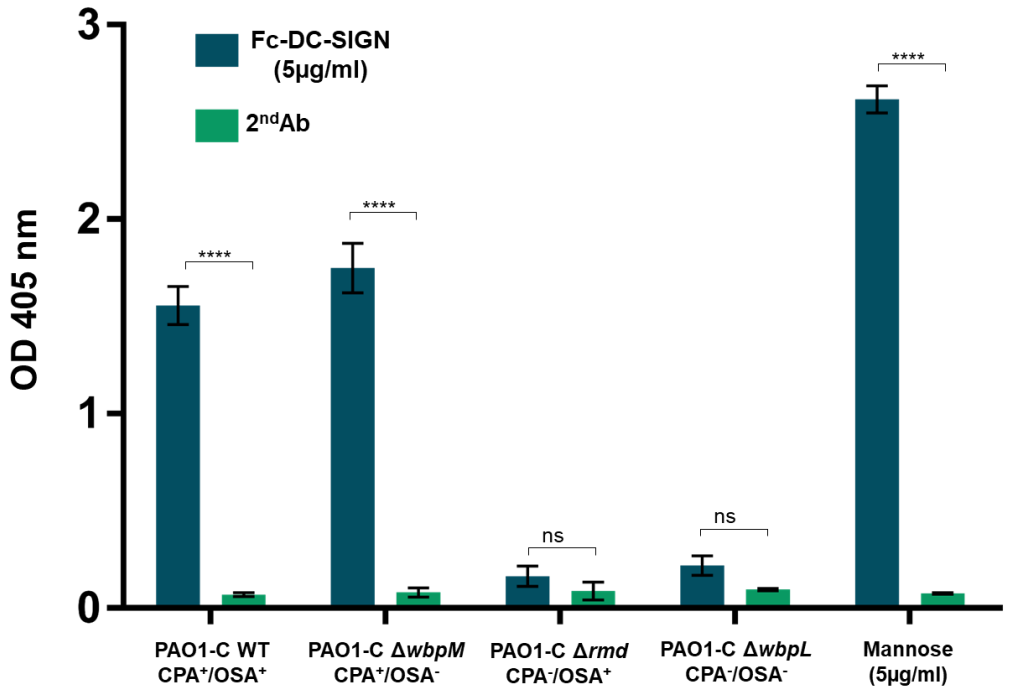
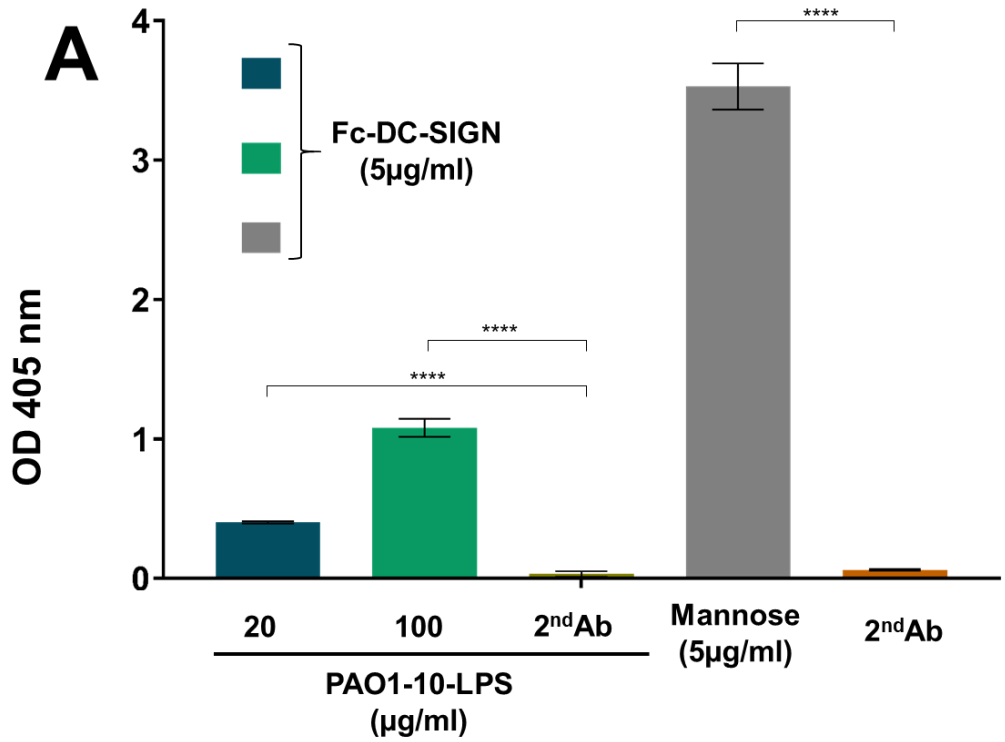


Figure 5.7: Fc-DC-SIGN binds to planktonic PAO1-C and binding depends on the presence of CPA-LPS

OD of fixed PAO1-C strains that express different LPS composition of (CPA and/or OSA) was adjusted to 0.5 OD_{600nm} in PBS. MaxiSorp plates were coated overnight with 100 µl of bacteria suspension at 4 °C, and 5µg/ml of Fc-DC-SIGN was incubated for 2 h at room temperature to assess the binding to whole bacteria. The binding was determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000 dilution). Absorbance was read at 405 nm. Error bars indicate the standard deviation (SD) of six independent repeats. Statistical significance was calculated by two-way ANOVA test. P values are represented as follows: ns = $p > 0.05$ and **** = $P \leq 0.0001$.

5.4.7 PAO1-10 LPS Binds to Fc-DC-SIGN

After we showed that Fc-DC-SIGN bound to PA CPA-LPS, we wanted to assess whether Fc-DC-SIGN bound to both LPS extracted from PAO1-C and purified PA10 LPS (Sigma). Results show that Fc-DC-SIGN binds to purified PA10 LPS in a dose dependant manner as shown in Figure 5.8-A and B. However, Fc-DC-SIGN bound to all extracted PAO1 LPS regardless the type of LPS (Figure 5.8-B) which is inconsistent with the fact that Fc-DC-SIGN only binds to CPA-LPS. This might indicate that the crude extract is contaminated with sugars as the protocol we followed to extract LPS (section 2.5) does not involve polysaccharides elimination.



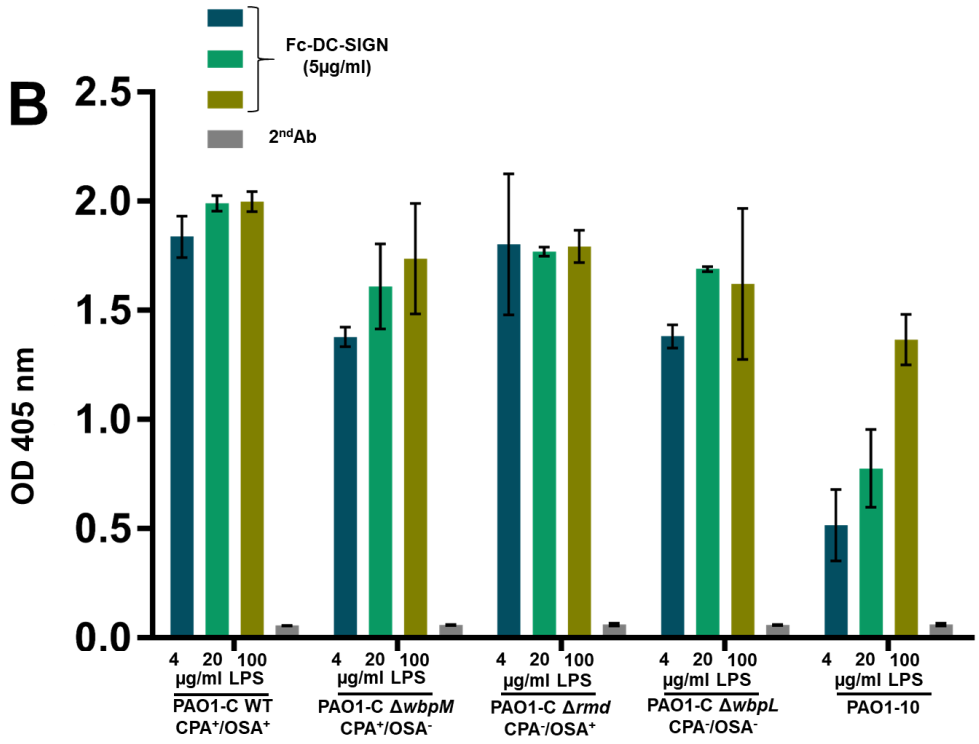


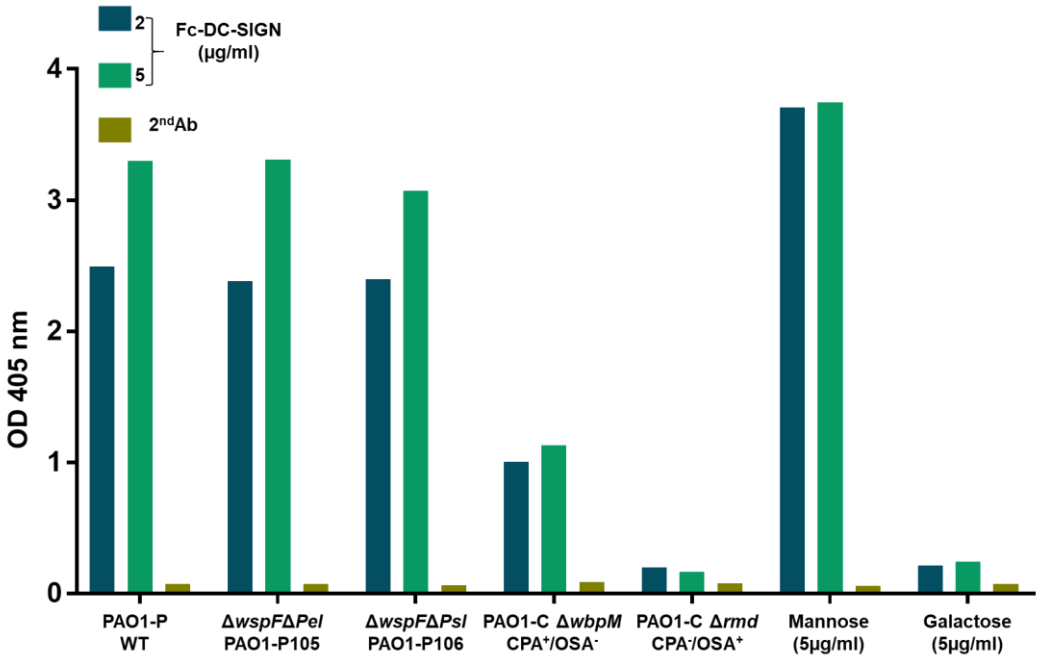
Figure 5.8: Binding of Fc-DC-SIGN to purified PAO1-C LPS and PA10 LPS.

Maxisorp plates were coated with 4, 20 and 100 µg/ml of LPS [purified PA-10- from Sigma (A) and extracted PAO1-C LPS (B)] in 154mM NaCl overnight at 37°C with 5%CO_2. Fc-DC-SIGN chimeric protein was incubated for 2 hours at room temperature. The binding was carried out in TSB buffer and determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000 dilution). Absorbance was read at 405 nm. Error bars indicate the standard error of the mean (SD) of three independent repeats. Statistical significance was calculated by two-way ANOVA test. **** = $p \leq 0.0001$.

5.4.8 Binding of Fc-DC-SIGN to planktonic PA varies among different PAO1 lab isolates

After we demonstrated that the binding of Fc-DC-SIGN to planktonic PAO1-C and PA-LPS in TSB buffer was highly reproducible, we wanted to assess whether the binding occurred using another TSM buffer as other researchers have used the TSM buffer to investigate DC-SIGN ligand recognition. We investigated different

PAO1-P and PAO1-C strains. Results suggest that Fc-DC-SIGN binds to planktonic PAO1-P strains and PAO1-C Δ*wbpM* (CPA^+/OSA^-) in both buffers. In both instances binding to the planktonic strains and the positive control mannose-PAA was significantly better in TSB (Figure 5.9-A) than in the TSM buffer (Figure 9B). Fc-DC-SIGN showed very weak binding to galactose-PAA (Figure 5.9-B). Further, binding of Fc-DC-SIGN to PAO1-C was reduced compared to that of PAO-1P, both (WT and Δ*wspF*-derived mutants).



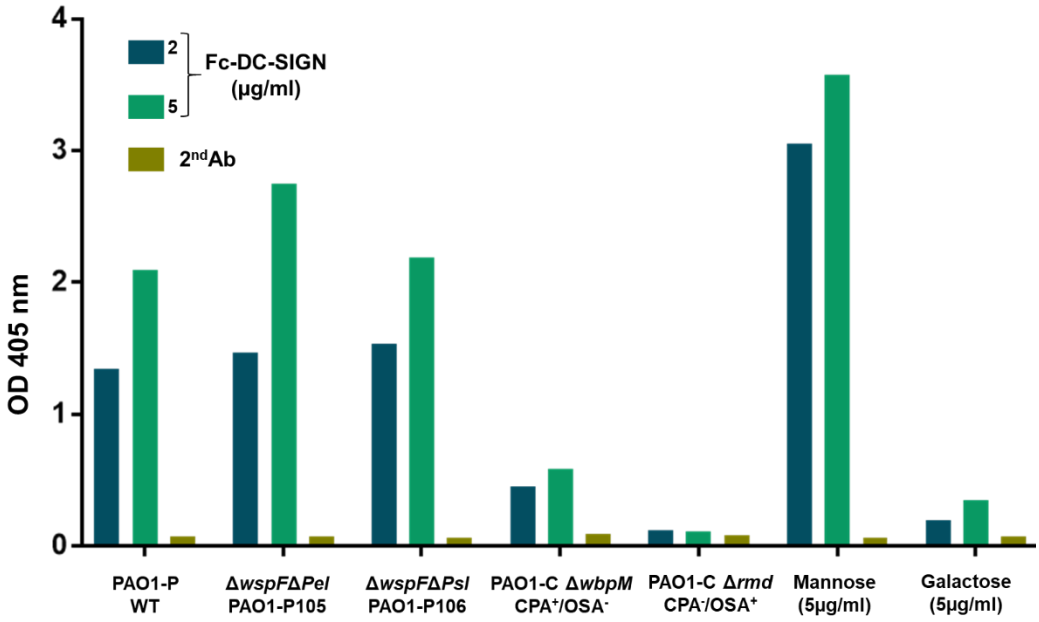


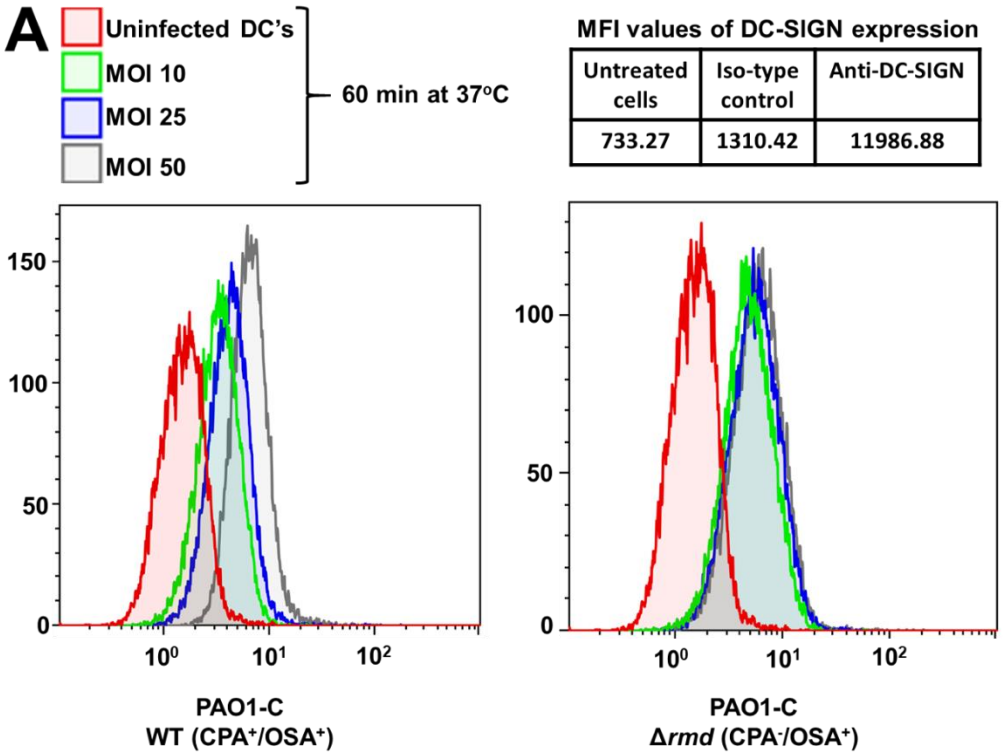
Figure 5.9: Comparison between the binding of Fc-DC-SIGN to a collection of fixed PAO1 (C and P) in two different buffers (TSB and TSM).

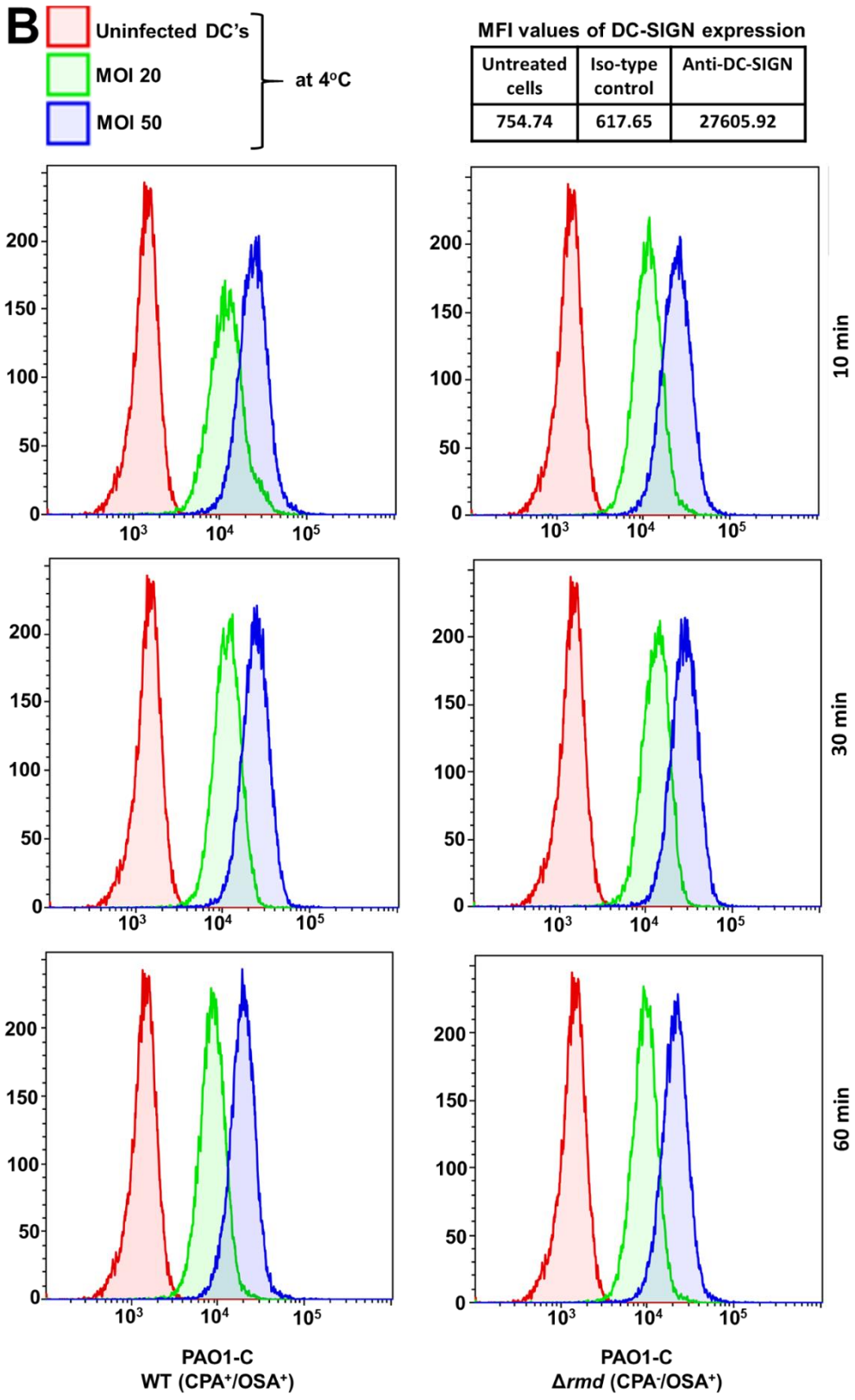
MaxiSorp plates were coated overnight with 100 µl of fixed PAO1 lab isolates (C and P) adjusted to 0.5 OD_{600} in PBS overnight at 4 °C. 5µg/ml of Fc-DC-SIGN was incubated for 2 h at room temperature to assess the binding to whole bacteria. The binding was carried out in TSB (A) or TSM(B) buffer and determined using anti-human IgG Fc-specific antibody conjugated to AP (1:1000 dilution). Absorbance was read at 405 nm. N=2.

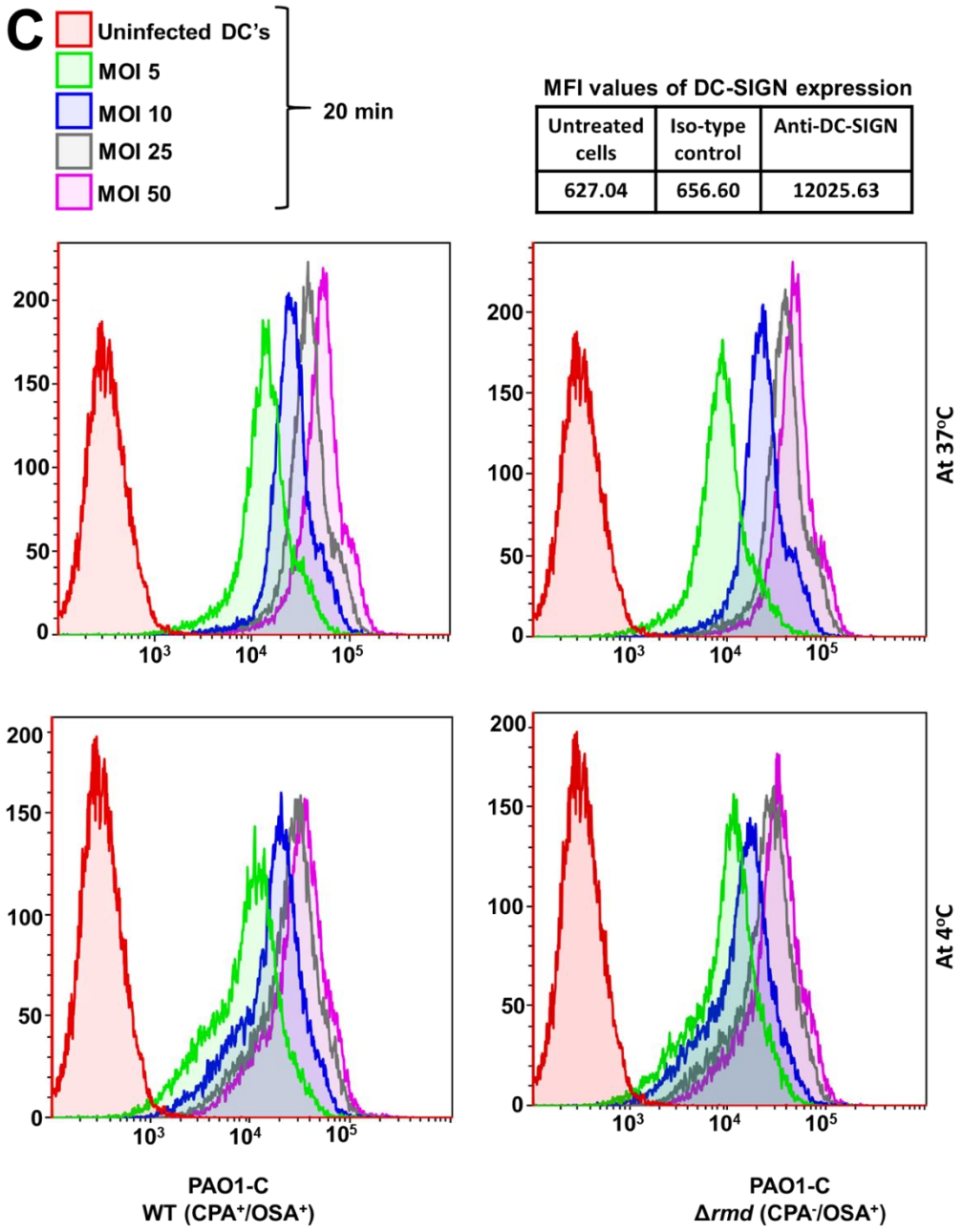
5.4.9 Dendritic cells expressing high level of DC-SIGN bind to WT PAO1(C and P)

To further evaluate if DC-SIGN is a potential receptor of PA in immune cells, we assessed the binding of monocyte-derived hu DCs, generated as described in section 2.9, to fixed planktonic PA. Initially, we confirmed the expression of DC - SIGN receptor on hu DCs in each experiment and the MFI of DC-SIGN receptor is shown within each figure. To assess the binding, we first labelled the bacterial cells with 2-10 µg/ml of FM 1-43 FX membrane dye, then DCs were incubated with fixed planktonic WT PAO1-P, WT PAO1-C, or PAO1-C *Δrmd* (CPA^-/OSA^+) at

different MOI (5-50) and time points (10 -60 min) at 4ºC or 37ºC. We observed a clear shift in fluorescent intensity when incubating DCs with WT PAO1-C (CPA^+/OSA^+) at 37 ºC for 60 min in a dose-dependent manner. However, when incubating the cells with PAO1-C *Δrmd* (CPA^-/OSA^+) at the same conditions, we found that this strain interacted with huDCs but there was no clear differences among the different MOIs (Figure 5.10-A). This indicates that the binding of PAO1-C to DCs at 37 ºC for 60 min is influenced by the presence of CPA and that association to DC's is better in the absence of CPA. Histograms in (Figure 5.10-B) show the binding of DC's to WT PAO1-C or *Δrmd* (CPA^-/OSA^+) for 10, 30, and 60 min at 4 ºC. We found that DCs bound to PA in a MOI-dependent manner. Probably binding occurs at the cell surface as it takes place at 4 ºC but there is no clear differences among the different time points or strains. Another approach was to assess the binding of DCs to PA O1-C for 20 min at either 37 ºC or 4 ºC. We found that there is a clear shift in fluorescent intensity when incubating the cells with either WT PAO1-C or *Δrmd* (CPA^-/OSA^+) but there is no difference in the binding pattern between both strains; however, PAO1-C *Δrmd* at 37ºC, MOI 5 might bind less (Figure 5.10-C). To assess the ability of selected polymers known to bind DC-SIGN or MR or anti-DC-SIGN mAb to inhibit the binding of PA to huDCs, firstly, we pre-incubated DCs cells with the polymers and then incubated the cells with WT PAO1-P at different MOI for 20 min at 4 ºC. DCs bound to WT PAO1-P in a dose dependent manner based on a clear shift in the fluorescent intensity, but the polymers did not inhibit the binding (Figure 5.10-D8).


A
Uninfected DC's
MOI 10
MOI 25
MOI 50
60 min at 37°C
MFI values of DC-SIGN expression
Untreated cells
Iso-type control
Anti-DC-SIGN
733.27
1310.42
11986.88
150
100
50
0
10^0
10^1
10^2
PAO1-C
WT (CPA+/OSA+)
100
50
0
10^0
10^1
10^2
PAO1-C
Δrmd (CPA-/OSA+)


B
Uninfected DC's
MOI 20
MOI 50
at 4°C
MFI values of DC-SIGN expression
Untreated cells
Iso-type control
Anti-DC-SIGN
754.74
617.65
27605.92
10 min
30 min
60 min
PAO1-C
WT (CPA+/OSA+)
PAO1-C
Δrmd (CPA-/OSA+)


C
Uninfected DC's
MOI 5
MOI 10
MOI 25
MOI 50
20 min
MFI values of DC-SIGN expression
Untreated cells
Iso-type control
Anti-DC-SIGN
627.04
656.60
12025.63
200
150
100
50
0
10^3
10^4
10^5
At 37°C
At 4°C
PAO1-C
WT (CPA+/OSA+)
PAO1-C
Δrmd (CPA-/OSA+)

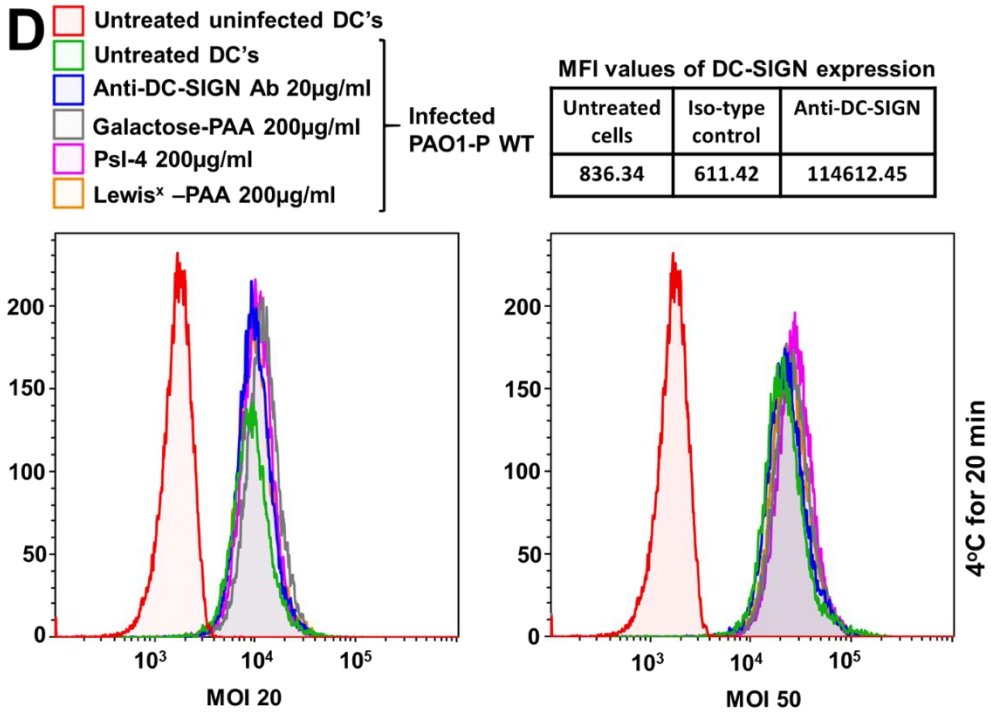


Figure 5.10: The binding of dendritic cells expressing high level of DC-SIGN to PAO1

$CD14^+$ cells were generated from monocyte purified from buffy coats cultured in RPMI complete medium with 800IU/ml of GM-CSF and 500IU/ml of IL-4. Overnight cultures of PAO1 (C and P) were stained with FM 1-43FX membrane dye and then fixed with 4 % formaldehyde in PBS at 4 ºC. PAO1-C (WT and *Δrmd* CPA^-/OSA^+) were added to DCs at different MOI (50-5) and incubated at 37ºC or 4ºC (A-D) for different times at 8 rpm. In some instances, DCs were pre-incubated with different polymers to examine whether they could inhibit the binding of DCs to PAO1-P (E).

5.5 Summary

1. Fc-DCSIGN binds to planktonic PA (lab strains and clinical isolates).
2. Mucoid CF isolates form a gel-like structure which could be induced by culture in X-vivo-15. This phenomenon might relate to the

formation of sticky mucus in CF lung patients which clog the lung and lead to accumulation of infections.

3. Binding of Fc-DC-SIGN to planktonic PA is CPA-LPS dependent. This indicates that CPA is a candidate ligand for DC-SIGN which further support a role of DC-SIGN as PRR in the recognition of PA.
4. PA strongly associates with huDCs in a temperature and MOI-dependent fashion. Absence of CPA-LPS does not seem to affect this interaction
5. Presence of DC-SIGN binding polymers or blocking anti-DC-SIGN mAb do not influence the association of PA with huDCs indicating that DC-SIGN is not the dominant receptor for PA in huDCs.

Chapter 6: Discussion

6.1 Role of EPS in PA biofilm formation

PA is an opportunistic pathogen that can cause serious infections in CF and immunocompromised patients (Falkinham 3rd et al., 2015). Many reports have extensively studied the role of TLRs, a family of PRRs, in the recognition of different PA components (see section 1.5 for more details). For instance, TLR4, which binds LPS, has been shown to be essential to clear PA lung infection (Faure et al., 2004). However, as far as we know, no study yet has determined the role of PRRs, including CLRs, in the recognition of bacterial biofilms. Thus, this project aimed to explore the role of CLRs in the recognition of PA. Persistent chronic lung infection in CF is attributed to the presence of PA biofilms (Gnanadhas et al., 2015) and one of the factors that enable PA to evade the immune system and resist antibiotics is its ability to form biofilm (Wei and Ma, 2013). The resistance of biofilms to environmental challenges likely depends on the presence of extracellular polymeric substance (EPS) within the biofilm (Algburi et al., 2016). Polysaccharides (alginate, Psl, and Pel) have been identified as crucial EPS components (Franklin et al., 2011).

In order to test whether CLRs bind PA biofilms and display selective binding towards particular EPS components, we used different PAO1-P strains that express either Psl and/or Pel. It has been shown that the loss of the *wspF* gene is one of the factors that results in an increased level of c-di-GMP (Hickman et al., 2005a). Therefore, it was decided to use strains with the Δ*wspF* background (Table 2.1). The benefits of using the Δ*wspF* background are 1) increased Psl expression which correlates with c-di-GMP level (Ha and O'Toole, 2015), 2) maintaining the

same level of c-di-GMP in all the strains which will be beneficial for studying the role of immune cells that express CLRs in PA recognition in the future, and 3) as shown by Irie *et al*, in which Psl-induced arabinose stimulates c-di-GMP level, promoting biofilm growth. In that study, Irie and colleagues showed that adding purified Psl increased the level of c-di-GMP two folds, whereas adding Psl pre-treated with specific polyclonal antibodies or cellulase, which has been shown to disrupt Psl, failed to stimulate the level of c-di-GMP, suggesting that extracellular Psl acts as a signal to stimulate biofilm production through increased c-di-GMP synthesis (Irie et al., 2012a).

We first confirmed the Δ*wspF* mutation is present in all strains expect PAO1-P and that the mutations Δ*pel* and Δ*psl* were present in PAO1-PΔ*wspF*Δ*Pel* (PA105) and PAO1-PΔ*wspF*Δ*Psl* (PA106), respectively, while PA107 showed both Δ*pel* and Δ*psl* mutations in addition to ∆*wspF*. In our study we used X-Vivo-15 medium to grow PA both in a planktonic form and as biofilm. X-vivo-15 is a commercial serum-free medium used routinely to culture different cell lines including macrophages and DCs (Buchrieser et al., 2017, Case et al., 1999, Nguyen et al., 2003). This medium was extensively used by a previous student in the laboratory to develop biofilm in the bioflux system (Tamanna Rhamman, PhD thesis) with the final aim of developing a culture system suitable for the formation and maintenance of biofilms and survival of immune cells. In addition, it was considered that components of LB broth, such as yeast extract, could directly activate the immune cells.

We observed that the PAO1-P strain forms smooth colonies on LB agar, whereas other Δ*wspF* mutant strains produce consistently small and rough colonies. This

small colony phenotype called rugose small-colony variant (RSCV); and it is due to the high level of c-di-GMP (Starkey et al., 2009a).

Previous work in our lab has compared the growth of PAO1 in X-vivo-15 medium and LB broth and concluded that the growth rates in terms of log phase, stationary phase, and optical density are almost the same in both media (Singh et al., 2015) . After confirming the mutations, we investigated the growth of these strains in X-vivo-15 medium and the total amount of bacteria, and we found that growth rates of the strains negatively correlate with c-di-GMP level. This observation correlates with previous studies that found high c-di-GMP level promote biofilm formation while strains with low level of c-di-GMP prefer planktonic life style (Ha and O'Toole, 2015).

We tested the PAO1-P strains for biofilm formation using a crystal violet assay (O'Toole, 2011). We found that the overproduction of EPS (Psl or Pel) correlates with biofilm formation. Both PA105 and PA106 form more biofilm than WT PAO1-P and Δ*wspF* (PA176), however, PAO1-PΔ*wspF*Δ*Psl* (PA106) appeared to produce less biofilm than PA105. This supports the importance of Psl as a scaffolding component of the PA matrix (Ma et al., 2009a). PAO1-PΔ*wspF*Δ*Pel*Δ*Psl* (PA107) was confirmed as biofilm-deficient despite the high level of c-di-GMP that resulted from the loss of the *wspF* gene. This is in agreement with the fact that Psl is important for biofilm initiation while Pel plays an important role in biofilm maintenance (Colvin et al., 2013, Colvin et al., 2011b). Our observations are in agreement with a previous report by Colvin *et al* who observed that PAO1∆*psl* and PAO1∆*psl* ∆*pel* formed reduced or no biofilm, respectively, compared to PAO1Δ*pel* mutants which produced distinguishable biofilms. Based

on these observations Colvin and colleagues classified PAO1 as one of the Psl-dominant matrix strains (Colvin et al., 2012c). ELISA was used as an alternative approach to quantify biofilm by different PAO1-P strains. We used polyclonal anti-*Pseudomonas* antibody to analyse bacterial biomass. Anti-PA antibody was raised against PA Boston 41501 strain. The binding specificity was tested and validated by Li and colleagues, and they have found that the anti-PA is 100 % specific (Li et al., 2011). Also, Lepanto *et al* showed that this anti-PA is able to recognise extracellular and intercellular bacteria within PA infected epithelial cell cultures (Lepanto et al., 2014). In agreement with the crystal violet date we found that PAO1-PΔ*wspF*Δ*Pel* (PA105) and PAO1-PΔ*wspF*Δ*Psl* (PA106) formed biofilms more readily than WT PAO1-P and PA176. This supports the essential role of Psl and Pel in biofilm formation.

6.2 Role of the CLRs MR (CD206) and DC-SIGN (CD209) in PA recognition

6.2.1 Involvement of MR and DC-SIGN in the recognition of PA biofilm

The present study demonstrated that biofilms produced by PA strains that rely either on Psl or Pel can be recognized by mammalian C-type lectin proteins. Psl is rich in mannose (Ma et al., 2007b), while Pel is composed of acetylated galactosamine and glucosamine sugars (Jennings et al., 2015). Both MR and DC-SIGN are expressed by selected populations of tissue macrophages and DCs (Geurtsen et al., 2009, Azad et al., 2014). MR consists of multiple CTLDs and binds to D-mannose, L-fucose, and N-Acetyl-glucosamine (GlcNAc) in a calcium-dependent manner (Sorvillo et al., 2012). DC-SIGN contains a single CTLD and has the ability to bind high mannose structures as well as fucose and Lewis antigens in a calcium-dependent manner (Sprokholt et al., 2016). This led us to

hypothesise that mannose-binding lectins could recognize PA biofilms through binding to Psl.

In our study, we used two recombinant C-type lectins, Fc-DC-SIGN and MR-CTLD-4-7-Fc, to test their ability to recognise PA biofilms based on their binding specificities. The MR consists of three main domains: 1) a cysteine-rich (CR) domain, 2) fibronectin type two (FNII) domain, and, 3) multiple C-type lectin-like carbohydrate recognition domains (CTLDs). Both the CR-domain and CTLDs 4-8 have been involved in carbohydrate recognition (Martinez-Pomares, 2012). The binding affinity of each individual CTLD has been previously analysed; CTLDs 4 and 5 are essential for ligand binding however, adding neighbouring CTLDs number 6, 7, and 8 was shown to increase binding affinity. The study concluded that the binding affinity to high mannose ligands of CTLDs (4-7) is similar to that of the whole receptor (Taylor and Drickamer, 1993). Linehan *et al* and Zamze *et al* generated CTLD4-7-Fc by expressing the CTLD4-7 domains of murine MR in a vector that contained Fc portion of human IgG1.The affinity and purity of the protein was tested and validated, and CTLD4-7-Fc was shown to act similarly as whole MR (Linehan et al., 2001, Zamze et al., 2002). Both receptor CTLD4-7-Fc and Fc-DC-SIGN were tested for their ability to bind different carbohydrates, including mannose and fucose (Lee et al., 2010) using an established binding assay (Zamze et al., 2002), and we found that both were functional (data not shown).

When biofilms produced by different lab strains that vary in the amount of Psl were incubated with CTLD4-7-Fc, we found that the binding was dependent on Psl levels and that in general the binding increased when the expression of Psl increased. However, when DC-SIGN was incubated with biofilm produced by WT PAO1-P,

PAO1-PΔ*wspF*, and PAO1-PΔ*wspF*Δ*pel* (Figure 3.5), the binding was strong and significantly better than binding with CTLD4-7-Fc. The fact that DC-SIGN is tetrameric (Feinberg et al., 2005) might explain the high binding affinity; however, no improved binding of CTLD4-7-Fc was observed when using protein crosslinked with the secondary antibody (data not shown). Higher binding was observed at 10 µg/ml compared to 2µg/ml in both proteins indicating that binding is dose dependent.

The fact that MR and DC-SIGN binding was calcium dependent was confirmed by comparing the binding of both proteins to biofilms or control carbohydrate ligands in TSB buffer in the presence and absence of calcium. We found that MR and DC-SIGN strongly bound to PA biofilm, mannose, and fucose in TSB buffer with calcium, and observed a complete elimination of lectin binding in the absence of calcium (Figure 3.8). This confirms the necessity of calcium for the binding of MR and DC-SIGN to PA biofilms and supports the specificity of the interaction.

To further confirm that the binding of MR and DC-SIGN to biofilm is specific and carbohydrate dependent, we carried out inhibition assays in a high salt buffer as high salt conditions make the inhibition assay more robust (McGreal et al., 2006). We first tested the binding of CTLD4-7-Fc and Fc-DC-SIGN to PA biofilms in either TSB with normal salt (0.154 M) or in TSB with high salt concentration (1M). We found that the high salt buffer did not affect binding as MR or DC-SIGN bound biofilms in both TSB buffers. The monosaccharides mannose, fucose, and galactose were tested for their ability to inhibit the binding of MR and DC-SIGN to either PAO1-P biofilm or polysaccharides positive controls mannose-PAA, fucose-PAA, or galactose-PAA. Mannose and fucose monosaccharides were able to

inhibit the binding of MR and DC-SIGN to the biofilms. The inhibition was dose-dependent and complete inhibition was reached at the concentration of 1 mM. Galactose monosaccharide was also able to inhibit the binding of both lectins to biofilms but to a lesser extend compared to mannose and fucose. Regarding polysaccharides positive controls, monosaccharides mannose and fucose were able to inhibit the binding of MR and DC-SIGN to mannose-PAA and fucose-PAA. The inhibition was dose-dependent and complete inhibition was reached at the concentration of 1 mM. MR did not bind galactose-PAA. However, galactose monosaccharide was also able to inhibit the low binding of DC-SIGN to galactose-PAA but to a lesser extend compared to fucose-PAA.

The binding of MR and DC-SIGN to biofilm generated by PA clinical isolates (wound and CF samples) was also tested. Results show that CTLD4-7-Fc and Fc-DC-SIGN bound to PA wound biofilms and that, as seen for PAO1, DC-SIGN-Fc bound significantly better than CTLD4-7-Fc to all samples. In CF samples, Fc-DC-SIGN bounds to all the CF isolates biofilms regardless the pattern of growth (mucoid or non-mucoid) while MR shows only weak binding to the non-mucoid isolate.

The International Antigenic Scheme (IATS) has classified PA into 20 different serotypes based on their LPS O-antigen region (Lam et al., 2011a). Many studies have shown the relation between the PA virulence and different serotypes and found that PA virulence characteristics are different among some serotypes (Lu et al., 2014). For instance, PA serotype 11 exhibits ExoU whereas PA serotype O6 was found to lack ExoU (Le Berre et al., 2011). In our study, we used different PA strains (lab strains and clinical isolates) and the binding of MR and DC-SIGN is

maintained. Thus, these results support the relevance of our findings and show that the binding was not caused due to a particular characteristic of the PAO1 lab strain.

Another approach was employed to test the specificity of Fc-DC-SIGN for PA biofilms. We compared the binding of Fc-DC-SIGN (commercially available from R&D) and Fc-DC-SIGN-Ams [cell supernatants provided by Juan Garcia Vallejo and Yvette van Kooyk (VU University Medical Centre) containing Fc-DC-SIGN]. We also tested two buffers TSB and TSM as TSM was the preferred buffer used in the van Kooyk's laboratory and there had been a previous manuscript showing that DC-SIGN did not bind PA (Appelmelk et al., 2003) . We found that (i) Fc-DC-SIGN (R&D) in TSM binds to PAO1-P and PAO1-PΔ*wspF*Δ*Pel* (PA105) biofilms less than in TSB; (ii) Fc-DC-SIGN (R&D) diluted in DC-SIGN-Ams supernatant binds only to biofilm when the assay was carried out in TSM buffer and binding was reduced compared to when the protein is diluted in TSB buffer; (iii) Fc-DC-SIGN-Ams protein preparation did not bind to PAO1-P biofilm either in TSB or in TSM. Thus, our results support the original observations by Appelmelk et al using the Fc-DC-SIGN supernatants. Even though Fc-DC-SIGN (R&D) seemed to compete with other compounds within the DC-SIGN-Ams supernatants, Fc-DC-SIGN (R&D) still bound PA biofilms under these conditions, which support the specificity of Fc-DC-SIGN binding to PA biofilms. We propose that previous authors failed to detect binding to PA because of the use of cell-supernatants instead of purified proteins. The DC-SIGN-Ams supernatant contained approximately 2 µg/ml of Fc-DC-SIGN and potential inhibitors which could confound the results.

Fc-DC-SIGN human recombinant protein (R&D) is a good tool that has been used in different applications of cell biology research. A previous study used Fc-DC-SIGN (R&D) to determine its ability to inhibit DC-SIGN mediated trans-infection by HIV-1 of $CD4^{+}$T-Lymphocytes. Stax *et al* has shown that Fc-DC-SIGN (R&D) binds to mannan and human lactoferrin, a component of colorectal mucus. They also have shown that mannan completely inhibited DC-SIGN binding to lactoferrin (Stax et al., 2015). Additionally, mannose has been identified as a component of surface Ig (sIg) of follicular lymphoma (FL). Coelho and colleagues have used Fc-DC-SIGN (R&D) to show that Fc-DC-SIGN (R&D) binds both mannosylated sIg and FL cells (Coelho et al., 2010). Therefore, these examples indicate the specificity of Fc-DC-SIGN (R&D) for mannose containing-glycans.

A former PhD student in our lab, Tamanna Rahman, used the fluorescently labelled hippeastrum hybrid amaryllis (HHA) lectin to detect Psl within PA biofilms using confocal microscopy. She found that PAO1-PΔ*wspF*Δ*Pel* (PA105) produces more Psl than WT PAO1-P (Figure 6.1). This observation could explain our finding which showed that binding of Fc-DC-SIGN to PA biofilm correlates with Psl level within the biofilm (Figure 3.5). The binding assays were further confirmed by using confocal microscopy which in addition enable us to determine the location of ligands for CTLD4-7-Fc and Fc-DC-SIGN within biofilms.

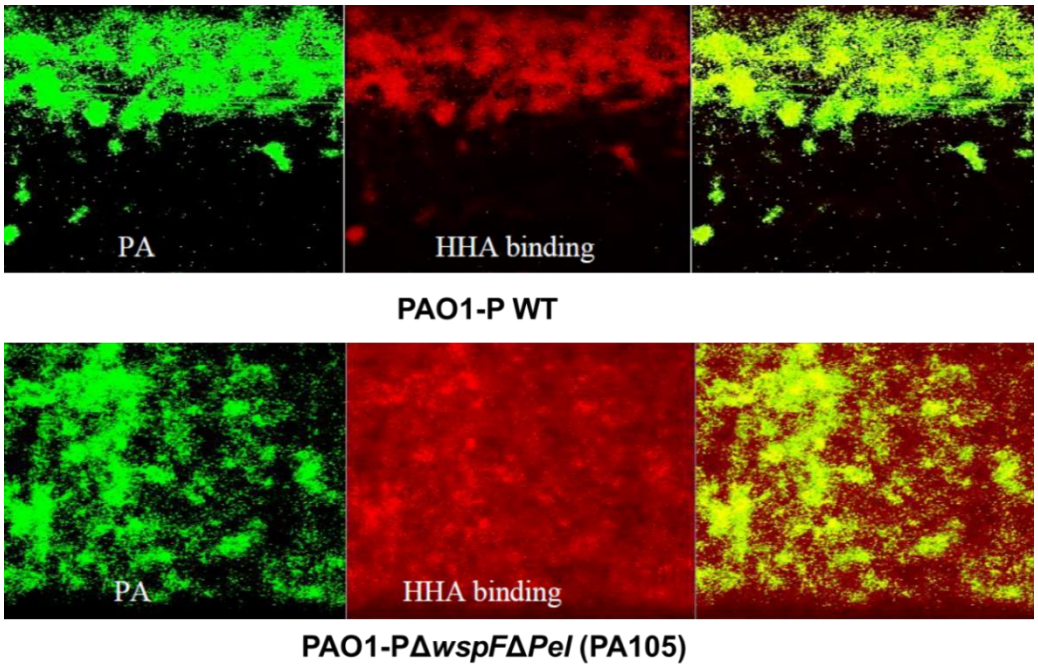


Figure 6.1: Psl Overexpression correlates with HHA binding activity and biofilm formation.

Images taken by confocal microscopy show the binding of HHA lectin to WT PAO1-P and PAO1-PΔ*wspF*Δ*Pel* (PA105) biofilms (red). Psl was detected using 20 µg/ml of HHA lectin. PA biomass was detected using sytox9 stain (green). Images are representative of two independent repeats. (Tamanna Rahman, PhD thesis).

We used confocal microscopy to visualize and localize the binding of CTLD4-7-Fc and Fc-DC-SIGN chimeric proteins to both PAO1-P*ΔwspF*Δ*Pel* (PA105) and PAO1-P*ΔwspF*Δ*Psl* (PA106) biofilms. We developed biofilms on Greiner plastic black plate and found that both proteins bound to the cultures (Figure 3.14); however, the images were distorted due to the use of the plastic plate. We then developed PAO1-P*ΔwspF*Δ*Pel* (PA105) biofilm on Lab-Tek II chamber glass slides and found that glass was the suitable substrate of biofilm (Figure 3.14). Then we visualized the binding of both CTLD4-7-Fc and Fc-DC-SIGN chimeric proteins to both PAO1-P*ΔwspF*Δ*Pel* (PA105) and PAO1-P*ΔwspF*Δ*Psl* (PA106) fixed biofilms developed on Cellview cell culture slide glass bottom. We found that CTLD 4-7-Fc shows weak binding to PAO1-P*ΔwspF*Δ*Pel* (PA105) biofilm and the binding was

on the surface of the bacterial cells, whereas Fc-DC-SIGN strongly bound PAO1-P*ΔwspF*Δ*Pel* (PA105) biofilm (Figure 3.14) and the binding was on the surface of the bacterial cells and between the cells, probably secreted Psl structures. Lastly, we incubated both proteins with fixed PAO1-PΔ*wspF*Δ*Psl* (PA106) biofilm, and neither CTLD4-7-Fc nor Fc-DC-SIGN bounds to PAO1-PΔ*wspF*Δ*Psl* (PA106) biofilm (Figure 3.14).

Information on the interaction of phagocytes and PA biofilms is limited. Phagocytic cells such as neutrophils and macrophages can aggregate at the surface of PA biofilms, but they can't engulf the pathogen. An in vitro study used PA lab strains and CF isolates to show that alginate can protect PA biofilm from phagocytosis by human leukocytes (Leid et al., 2005). This phenomenon is called frustrated phagocytosis and might be due to the presence of PA components that impair the phagocytosis process (Mulcahy et al., 2014). Psl could reduce neutrophil reactive oxygen species (ROS) production and phagocytosis. This is accomplished by reducing complement-mediated opsonisation. Mishra *et al* showed that Psl inhibits the deposition of C3, C5, and C7 on opsonized PA, resulting in inefficient opsonization and reduced killing by phagocytes (Mishra et al., 2012b). In addition, a monoclonal antibody against Psl improved PA killing by phagocytes and limited the attachments of PA to lung cells (Ray et al., 2017, DiGiandomenico et al., 2012). The CTLD4-7 region of MR recognized mannose ligands in pathogens such as C. *albicans*, *Leishmania*, *M. tuberculosis*, HIV, *K. pneumoniae*, (Gazi and Martinez-Pomares, 2009, Martinez-Pomares, 2012). Therefore, MR can act as a PRR in spite of being implicated in important homeostatic processes. Similarly, DC-SIGN has been shown to have a strong binding activity to pathogens containing mannose or fucose such as *M. tuberculosis* and *H. pylori* (Gringhuis et al., 2009a). Intriguingly, accumulation of DCs expressing DC-SIGN positively correlates with

presence of biofilms in chronic rhinosinusitis with nasal polyps (CRSwNP) (Karosi et al., 2013), which correlates with our findings, indicating that DC-SIGN is a candidate receptor for PA biofilm. The potential consequences of DC-SIGN and MR binding to biofilms will be discussed later on.

6.2.2 MR and DC-SIGN recognise PA EPS

Psl is involved in the bacterial attachment to surfaces and maintenance of PA biofilm throughout the different stages (Ryder et al., 2007a, Ma et al., 2009a, Byrd et al., 2010). Psl can be found both attached to the cell surface and secreted (Ma et al., 2009b). As mentioned above, Psl could reduce neutrophil reactive oxygen species (ROS) production and phagocytosis. This is accomplished by reducing complement-mediated opsonisation. An in vitro study demonstrated that Psl monoclonal antibody improved planktonic PA killing by phagocytes and limited the attachments of PA to lung cells (Ray et al., 2017). This suggests that PA uses Psl to facilitate host adaptation and persist during CF infection.

Therefore, to support our observations described in section 6.2.1, we examined the binding of MR and DC-SIGN to either crude extract or purified EPS from Psl or Pel- deficient strains. We first tested the binding of MR and DC-SIGN to a crude extract prepared by Tamanna Rahman from PA105 cultures grown in M63 media. We found that CTLD4-7-Fc bound in a dose-dependent manner to different concentrations of EPS crude extract; while the binding of the negative control, CR-FNII-CTLD1-3-Fc, was low. This further supported a role for MR in binding the EPS component within PA biofilms. However, the procedure to generate these crude preparations did not include a lipid elimination step which might be a source of contaminants. Additionally, M36 media contains only the minimum growth

requirements which might affect the amount of polysaccharide generated. Therefore, it was decided to follow the procedure described in section 4.4, followed by fractionation by gel-filtration, to generate preparations of EPS enriched in carbohydrates.

We were able to produce 7 independent preparation of PA EPS from PA105 biofilms which lack Pel. Hence, we consider these preparations to be largely composed of Psl as PA105 displays non-mucoid phenotype which produces trace amounts of alginate (Hentzer et al., 2001b). All preparations were analysed for carbohydrate presence and protein as well (Table 4.1). We obtained a good yield of carbohydrate (Figure 4.3) with a very small amount of protein contamination in all the preparations (Table 4.1). We examined the binding of MR and DC-SIGN to all the 7 preparations, and we showed that CTLD4-7-Fc and Fc-DC-SIGN bound to all PA Psl stocks in a dose-dependent manner and that, as seen for total biofilms, Fc-DC-SIGN recognised Psl better than CTLD4-7-Fc in all instances. Both CTLD4-7-Fc and Fc-DC-SIGN strongly recognised the commercially available positive control polymers mannose-PAA and fucose-PAA. In order to validate our results, we compared the binding of Fc-DC-SIGN with that of biotinylated tetrameric DC-SIGN (kindly provided by Dr. Daniel Mitchell, University of Warwick) (Pederson et al., 2014) to Psl (preparation number 4), and we found that both bound to Psl (Figure 4.6). In addition to the ELISA technique, we used the surface plasmon resonance technique to test the interaction affinity of DC-SIGN to Psl. We found that tetrameric DC-SIGN bound to PA Psl (preparation number 4) in a dose-dependent manner (Figure 4.7). These observations indicate that Psl could possibly modulate the action of DCs and macrophages against PA through DC-SIGN and/or MR. To explore this possibility further, human DCs preincubated with purified Psl (preparations number 4 and 7) were stimulated with fixed PAO1-P and

levels of TNF-α, IL-8, IL-β, IL-12(P70), IL-6, and IL-10 were measured. We observed that Psl induced some cytokines such as TNF-α, IL-β, IL-6, and IL-10 when DCs were stimulated with fixed PA. While, on other occasions, no effect was detectable (Figure 4.8). Due to this inconsistency, we were unable to interpret these findings. The reason behind that might be due to the long incubation of DCs in non-tissue culture plates and also variability in cellular characteristics. To address these issues, we could use tissue culture plates and treat the cells with Psl instead of incubating the cells with pre-coated Psl wells. If we could re-produce the findings observed in the first attempt that might indicate that the engagement of DC-SIGN makes the disease worse by induction of proinflammatory cytokines. Other aspects could be considered in the future such as 1) using EPS preparations >15 kDa and characterise them for carbohydrate composition and size, 2) examining T cell activation by incubating them with DCs treated with PA (biofilm or planktonic) , and 3) testing the adhesion activity of DCs and u937 cells expressing DC-SIGN which might include knocking down expression of DC-SIGN or other receptors using siRNA to confirm involvement in the responses.

6.2.3 Involvement of DC-SIGN in the recognition of PA LPS

PA lipopolysaccharide (LPS) plays a crucial role in pathogen virulence and host response (Pier, 2007b). PA LPS plays an important role in the structural integrity of PA biofilms by promoting bacterial adhesion and cell-to-cell adherence (Lau et al., 2009, Wozniak et al., 2003). LPS activates immune cells through the TLR4 receptor complex. This leads to the activation of NF-κB and IRF3 and production of inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Park and Lee, 2013, Al-Wrafy et al., 2017). LPS is the major component of PA outer membrane. PA has two unique types of LPS, CPA and OSA (Lam et al., 2011c). There is evidence that

CPA, which is common among all serotypes, plays a crucial role during PA infections. During chronic lung infection in CF patients, the production of the OSA is reduced while the level of CPA is maintained on the cell surface. It has also been shown that CPA can promote PA attachment to epithelial cells in vitro *(Hao et al., 2013,* Zdorovenko *et al., 2015).* In vitro, the phenotype of LPS shifts from CPA^+ OSA^+ to CPA^+OSA^- when PAO1 is cultured as biofilms, with this shift being reversible, suggesting that this shift promotes persistence of PA in chronic infections (Murphy et al., 2014b).

The fact that we detected binding of Fc-DC-SIGN to PA106 biofilms, which are Psl deficient, led us to investigate the presence of another DC-SIGN ligand within PA biofilms. We tested the binding of Fc-DC-SIGN to different fixed planktonic PAO1-P strains that express different types of EPS. We found that Fc-DC-SIGN bound to all strains, including Δ*wspF*Δ*Psl*Δ*Pel* (PAO1-PA107) which is biofilm-deficient. This indicates that DC-SIGN has a ligand on the PA outer membrane. Similarly, we found the same results when testing clinical isolates [wound and CF (mucoid and non-mucoid)] (Figure 5.4 and Figure 5.6). While testing the binding of Fc-DC-SIGN to mucoid CF isolates we could not recover regular pellets from cultures grown in X-vivo-15 as they adopted a gel-like consistency. The formation of a gel-like pellet from mucoid CF isolates could be attributed to the chemical components, probably proteins, of X-vivo-15 media as it was not observed in cultures grown in LB. We speculate that these components might function similarly in vivo (i.e. in the CF lung), inducing the sticky glue appearance reported in CF lung patients as a result of alginate overproduction (Lim et al., 2011).

We tested the binding of Fc-DC-SIGN to planktonic PA that differ in the LPS composition, and we found that Fc-DC-SIGN strongly bound to planktonic PA which express CPA-LPS while binding to strains that only express OSA-LPS, i.e. CPA deficient, was very weak. This indicates that PA CPA-LPS is a candidate ligand of DC-SIGN. To further explore the role of CPA as a DC-SIGN ligand, we tested purified LPS (CPA^+ and/or CPA^-), and we found that Fc-DC-SIGN bound to all extracted PAO1 LPS regardless the type of LPS, which is inconsistent with the fact that Fc-DC-SIGN only binds to strains expressing CPA-LPS. However, Fc-DC-DC-SIGN bound to purified PA10 LPS and positive controls which indicates that binding of Fc-DC-SIGN to the PA OSA deficient mutant is probably caused by recognition of LPS. This might indicate that the crude LPS extract could be contaminated with sugars as the protocol we followed to extract LPS does not involve polysaccharides elimination. Binding of DC-SIGN to fixed PA through CPA suggests a role for DC-SIGN in acute PA infection.

Our findings are consistent with previous finding by our collaborator Dr. Daniel Mitchell, University of Warwick. He found that tetrameric DC-SIGN, a different recombinant DC-SIGN, bound to different fixed PA strains by ELISA-style micro-well plate assay (Figure 6.2). Also, he found that Fc-DC-SIGN (R&D) binds to different concentrations of purified LPS PA-10 using surface plasmon resonance technology (Figure 6.3).

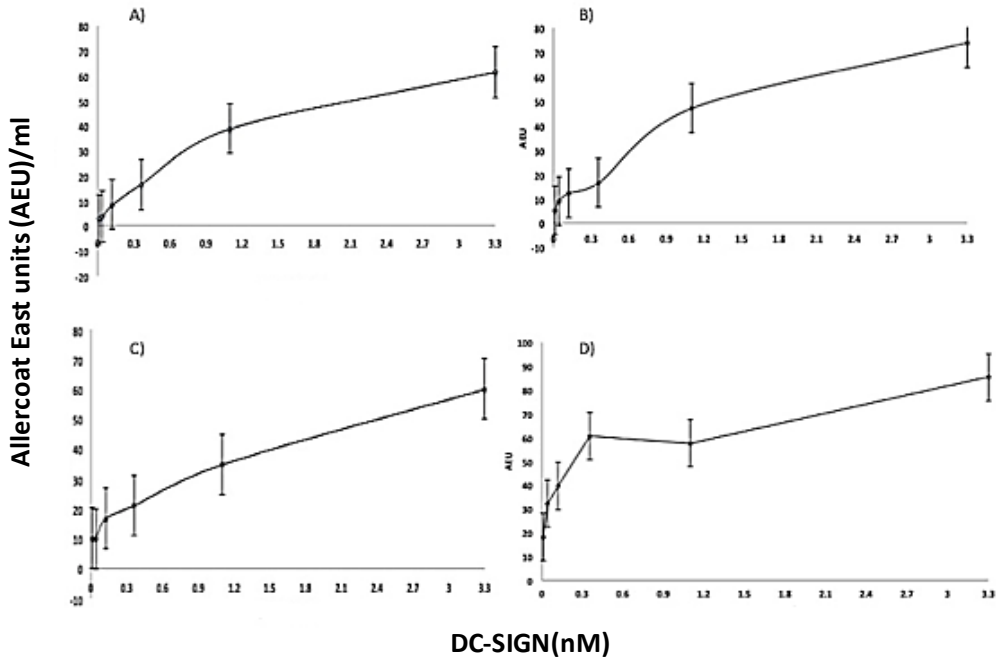


Figure 6.2: The binding of Biotinylated DC-SIGN binding to heat-killed PA.

Heat-killed PA strains were immobilized via ELISA-style micro-well plate assay. Panel A: PA strain 0005; Panel B: PA strain 0032; Panel C: PA 0048; Panel D: PA LPS-10.

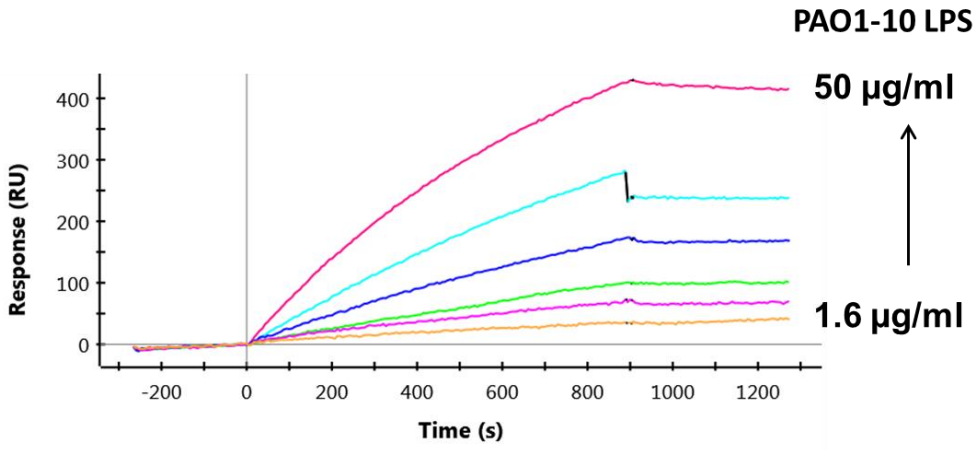


Figure 6.3: Fc-DC-SIGN binds to purified LPS PA-10 using surface plasmon resonance.

Recombinant Fc-DC-SIGN was immobilized on a chip and then incubated with different concentrations of purified LPS PA-10 (1.6 -50 µg/ml)

To further investigate our findings, we evaluated whether DC-SIGN is a potential receptor for planktonic PA in immune cells. We assessed the binding of monocyte-derived huDCs to fixed planktonic PA. Initially, DCs that express DC-SIGN were incubated with fixed planktonic WT PAO1-P, WT PAO1-C, or PAO1-C *Δrmd* (CPA^-/OSA^+) at different MOI (5-50) and time points (10 -60 min) at 4ºC or 37ºC. We observed a clear shift in fluorescent intensity when incubating DCs with WT PAO1-C (CPA^+/OSA^+) at 37 ºC for 60 min in a dose-dependent manner (Figure 5.10-A). However, when incubating the cells with PAO1-C *Δrmd* (CPA^-/OSA^+) under the same conditions, we found that this strain interacted with huDCs but there were no clear differences among the different MOIs (Figure 5.10-B). This indicates that the binding of PAO1-C to DCs at 37 ºC for 60 min is influenced by the presence of CPA and that association to DCs is better in the absence of CPA. We also found that DCs bound to WT PAO1-C or *Δrmd* (CPA^-/OSA^+) after incubating for 10, 30, and 60 min at 4 ºC in a MOI-dependent manner. Probably binding occurs at the cell surface as it takes place at 4 ºC but there are no clear differences among the different time points or strains. Another approach was to assess the binding of DCs to PAO1-C for 20 min at either 37 ºC or 4 ºC. We found that there is a clear shift in fluorescent intensity when incubating the cells with either WT PAO1-C or *Δrmd* (CPA^-/OSA^+) but there is no difference in the binding pattern between both strains. However, PAO1-C *Δrmd* at 37ºC, MOI 5 bound less than PAO1-C Figure 5.10-C). We also assessed the ability of selected polymers known to bind DC-SIGN or MR or anti-DC-SIGN mAb to inhibit the binding of PA to huDCs, firstly, we pre-

incubated DCs cell with the polymers and then incubated the cells with WT PAO1-P at different MOI for 20 min at 4 ^{o}C. We used PAO1-P for these assays because it was found to bind PAO1-P Fc-DC-SIGN better than PAO1-C. DCs bound to WT PAO1-P in a dose dependent manner based on a clear shift in the fluorescent intensity, but the polymers did not inhibit the binding (Figure 5.10-D). These findings suggest that LPS CPA could be a candidate ligand of one or more receptor of DCs. The reason why we do not see clear differences in the absence of CPA could be due to the ability of both OSA and CPA to bind unknown receptor of DCs.

6.3 Outcome of mannose-binding lectins engagement during PA infection

In this present study we showed a pivotal role of two important CLRs in the recognition of PA, particularity DC-SIGN. Our data strongly indicate that the CTLD 4-7 region of MR and Fc-DC-SIGN recognize PA biofilms through binding to a mannose-rich structure, Psl. Also, we showed that PA CPA-LPS is a candidate ligand of DC-SIGN. The engagement of CLRs to PAMPs and cross talk between CLRs and other PRRs are important processes that trigger appropriate immune response against invading pathogens (Jang et al., 2015). Pathogen recognition might lead to two contradictory immunological outcomes which are:1) complete elimination of pathogen, resulting in infection clearance, or 2) persistence of pathogen through targeting CLRs and exploiting mechanisms within the immune system that regulate inflammation by promoting resolution, in order to reduce damage which could result in chronic infection. Both MR and DC-SIGN are expressed by DCs, which play a remarkable role in linking innate and adaptive immunity and shaping appropriate immune response (Steinman, 2006).

DCs control the appropriate immune response against invading pathogens by activating naïve T cells (Sallusto and Lanzavecchia, 2002). The outcome ultimately depends on the pathogen that is recognized by DCs. As a result, T cells can be

differentiated into Th1,Th2, or Th17 (Gringhuis et al., 2014b). DCs also contribute to generation of an essential T cell subset named regulatory T cells (Tregs). These cells play a crucial role in protecting against self-antigens and suppressing excessive harmful immune responses. The impairment of Treg leads to autoimmunity and severe inflammatory response (Sakaguchi et al., 2008).

MR has been reported to play a role in pathogen recognition (Martinez-Pomares, 2012). MR promotes IL-17 production in response to *C. albicans* (van de Veerdonk et al., 2009). Also, MR has been implicated in Th-2 response against *S. mansoni* (Everts et al., 2012). MR has also been implicated in induction of allergy. It was found to be responsible for uptake of Der P 1, a major house dust mite allergen, by human monocyte-derived DCs (tan Deslée et al., 2002). Royer and colleagues found that MR binds to a wide range of allergens such as Der p 1 and Der p 2 in mite, Ara h 1 in peanut, Bla g 2 in cockroach, and Can f 1 in dog. In that study, they showed the involvement of MR in inducing Th2 response as MR-deficient DCs exposed to Der p 1 promoted Th1 responses (Royer et al., 2010). Interestingly, $CD11b^{+}$ cells in PA-infected CF samples have high expression of MR and high level of IL-4 and IL-13 accompanied by low level of IFN-γ, indicating a Th-2 response (Murphy et al., 2010a). Moreover, CF patients have been shown to demonstrate Th2 and Th17-biased immune responses. The high concentrations of Th2 and Th17 cytokines and chemokines in CF lung suggest that abundant Th17 response and /or lack of Th1 response could promote chronic PA infection (Singh et al., 2015). MR is involved in uptake of mannosylated particles through phagocytosis (Fernández et al., 2005, Astarie-Dequeker et al., 1999). For example, *mannosylated components of M. tuberculosis* have been found to bind MR, allowing survival of *M. tuberculosis* within the host (Sasindran and Torrelles, 2011). The association of FcRγ-chain and growth factor receptor-bound protein (Grb2)

with MR has been shown to be required for MR-mediated phagocytosis of *M. tuberculosis*. This is followed by recruitment of Src homology region 2 domain-containing phosphatase-1 (SHP-1) to *M. tuberculosis*-containing phagosome, which limits phosphatidylinositol-3-phosphate PI(3)P generation at the phagosome and subsequently promotes growth of *M. tuberculosis* (Rajaram et al., 2017a). In addition MR can regulate TLR 4 signalling as a mean to modify or regulate proinflammatory cytokines favouring Th2 responses (Salazar et al., 2016b). Therefore, MR is involved in Th2 induction or at least it is not involved in induction of Th1 responses. Thus, binding of CTLD4-7-Fc to Psl, mannose rich, could be an advantage of PA allowing it to evade the immune system and establish chronic infection.

One of DC-SIGN features is that it can bind to high mannose saccharides and fucosylated Lewis blood-group antigens (Le^x, Le^y, Le^a and Le^b)(Appelmelk et al., 2003). Thus, it is important to explore the role of DC-SIGN in recognition of PA as we have detected two novel DC-SIGN ligands within PA (Psl and CPA-LPS). DC-SIGN expressed by DCs binds to ICAM3 on T cells, facilitating T-cell proliferation and immune response initiation (Geijtenbeek et al., 2000b) Some pathogens targets DC-SIGN in order to manipulate the T cells differentiation, allowing them to escape the immune response and survive within the host (Geijtenbeek and van Kooyk, 2003a). For example , DC-SIGN binds to *H. pylori* LPS and increases the level of IL-10 and blocks Th1 responses (Cambi and Figdor, 2005). However, binding of DC-SIGN to *Neisseria meningitidis* LPS favours Th1 response (Steeghs et al., 2006). Generally, binding of DC-SIGN to mannose – containing pathogens such as *Mycobacterium tuberculosis* and HIV-1 recruits signalling proteins that activate Raf-1. Then Raf-1 modulates TLR-induced NF- κB activation enhancing cytokines expression, including IL-10 which leads to altered DC maturation and

unbiased T cell differentiation. In contrast, binding of DC-SIGN to fucose–containing pathogens such as *H. pylori* and *Schistosoma mansoni* drives T cell differentiation to Th-2 response (Gringhuis et al., 2014b).

L-SIGN or DC-SIGNR is a homologue of human DC-SIGN (Celerino da Silva et al., 2011). L-SIGN is expressed by liver sinusoidal cells and the lymph nodes. L-SIGN has high ability to bind mannose-containing pathogens in a calcium dependent manner such as HIV gp120-binding protein, Ebola virus, and hepatitis C virus (Gardner et al., 2003). It has been demonstrated that both DC-SIGN and L-SIGN can capture HCV from blood and deliver it to HCV primary target, hepatocytes (Ludwig et al., 2004). Mice express eight different DC-SIGN homologues called SIGNR (1-8) (Takahara et al., 2011). Most of SIGNR receptors can bind mannosylated and fucosylated glycans (Takahara et al., 2004, Powlesland et al., 2006, Galustian et al., 2004). However, some of them can not bind human DC-SIGN ligands. For example, SIGNR 2, 7, and 8, can bind sulfated and sialylated glycans (Powlesland et al., 2006). SIGNR5 particularly binds mannose but it does not have endocytic activity. Therefore, according to glycan specificity, SIGNR1 and 3 are the closest receptor that mirror the specificity of hDC-SIGN (Garcia-Vallejo and van Kooyk, 2013).

Many studies have shown that some pathogens target DC-SIGN as a means to escape immune response and cause prolonged persistence of infection. It has been shown that the binding of DC-SIGN to CMV confers protection of the virus from degradation action by DCs (Halary et al., 2002). Therefore, DC-SIGN might play a role in virus propagation and immune response alteration. Ebola virus is one of the viruses that cause a form of haemorrhagic fever, it has been reported Ebola virus binds DC-SIGN. Using particles pseudotyped with Ebola envelope glycoproteins, DCs were able to capture these particles through DC-SIGN and

transmit them to recipient cells (van Kooyk and Geijtenbeek, 2003b). *Mycobacteria*, *Schistosoma*, *Leishmania*, and *Helicobacter* can cause a life-time chronic diseases (Monack, 2013). One of the strategies to achieve that is manipulating Th1/Th2 balance. These pathogens have been demonstrated to bind DC-SIGN not only to skew Th1/Th2 balance but also to establish persistent chronic infection (Geijtenbeek and Van Kooyk, 2003b). These findings underlie the role of DC-SIGN in immune response impairment and chronic disease establishment.

6.4 Previous information regarding CLR-PA interaction

One of the extracellular PA constituents is slime glycol-lipoprotein (GLP) (Lagoumintzis et al., 2003) which mainly contains mannose and glucose (Evans and Linker, 1973). GLP has been shown to activate immune response through binding to MR (Cigana et al., 2011). Both mucoid and non-mucoid PA can produce GLP *in vivo* (Cigana et al., 2011) and GLP induces the production of TNF-α by human and mouse macrophages (Dimitracopoulos and Bartell, 1980). This suggests that TNF-α plays an essential role during PA infection. In 2003, previous work tested the binding of Fc-DC-SIGN to PA and no binding was detected (Appelmelk et al., 2003). Findings in this thesis contradict this original result. In this project we have shown that: (1) CTLD4-7, a region of MR, and DC-SIGN binds to PA biofilms with DC-SIGN binding significantly better than MR, (2) Both lectins also recognised several independent preparations of EPS lacking Pel, (3) DC-SIGN requires the presence of the common polysaccharide antigen (CPA) which is shared among all PA serotypes to bind planktonic cells. For these studies we have used two independent purified DC-SIGN preparations (Fc-DC-SIGN and biotinylated tetrameric DC-SIGN) and in all instances binding was calcium and sugar dependent. Further, we reproduced the original observations as the Fc-DC-SIGN preparation provided by Dr Garcia Vallejo and Prof van Kooyk, which

consists of supernatants from transfected cells containing Fc-DC-SIGN at a concentration of 2 µg/ml, failed to bind to PA biofilms. In addition, when commercial Fc-DC-SIGN was diluted into this preparation binding to biofilms was lost. We hypothesise that components within the supernatants block binding of Fc-DC-SIGN to PA.

6.5 Other lectins might contribute to PA recognition

MR and DC-SIGN are not the only lectins that could be involved in PA recognition. The CLR family of receptors (Figure 1.6) comprise multiple members with sugar specificity consistent with potential binding to Psl, such as Dectin-2.

Dectin-2

Dectin-2 is expressed by macrophages, Langerhans cells, neutrophils and DCs (Kerscher et al., 2013, Taylor et al., 2005b). Dectin-2 detects fungal cells containing high mannose, but with 10-fold lower affinity compared to MR and DC-SIGN (Hollmig et al., 2009). Dectin-2 consists of a single extracellular CTLD domain, stalk region, a transmembrane domain, and cytoplasmic domain (Tang et al., 2018). It performs an important role in activation of pro-inflammatory cytokines leading to the production of various cytokines and chemokines including TNF, IL-2, IL-10, IL-23, IL-1β, IL-6 and IL-12 (Lee et al., 2016, Saijo and Iwakura, 2011). Dectin-2 associates with the FcγR common chain that contains an immunoreceptor tyrosine-based activation motif (ITAM). Phosphorylation of the ITAM region leads to recruitment of spleen tyrosine kinase (Syk), which in turn activates NF-κB signalling pathway that mediates signalling. Dectin-2 has been shown to regulate the respiratory burst and promotes Th17 *in vitro* differentiation (Marakalala and Ndlovu, 2017, Kimura et al., 2016, Kerscher et al., 2013, Robinson et al., 2009). IL-17A-deficient mice are susceptible to *C. albicans* infection, indicating the protective role of IL-17A against this fungus (Saijo et al., 2010). Dectin-2 is able to

recognize multiple microbes such as *C. albicans, Saccharomyces cerevisiae, Aspergillus fumigatus, Mycobacterium tuberculosis* and *Schistosoma mansoni*, and house dust mite allergens (Kerscher et al., 2013). Deficiency of Dectin-2 in mice infected with *C. albicans* was correlated with reduced survival rates (Ifrim et al., 2014).

Macrophage inducible C-type lectin (Mincle)

Mincle is comprised of a CTLD domain containing an EPN motif (Yamasaki et al., 2009). The EPN motif, as mentioned in Chapter 1, is involved in recognition of mannose and fucose-type sugars (Alenton et al., 2017); hence, it could be involved in Psl recognition. Mincle is expressed by multiple innate immune cells including monocytes, B cells, macrophages, and neutrophils (Behler et al., 2015). Mincle also associates with the FcγR common chain leading to Syk activation (Patin et al., 2017) and induction of several cytokines and chemokines, including TNF, macrophage inflammatory protein 2 (MIP-2; CXCL2), keratinocyte-derived chemokine (KC; CXCL1) and IL-6 (Kerscher et al., 2013).

L-SIGN or DC-SIGNR (CD209L)

L-SIGN is a type II transmembrane receptor (da Silva et al., 2011). L-SIGN shares about 70% of DC-SIGN amino acids sequence (Gardner et al., 2003). While DC-SIGN is mainly expressed by DCs and some populations of macrophages, L-SIGN is expressed by lymph nodes and liver sinusoidal cells (Knolle and Wohlleber, 2016). Similar to DC-SIGN, L-SIGN has high binding affinity to multiple ligands including ICAM-3, HIV gp120-binding protein, Ebolavirus, hepatitis C virus, and phleboviruses (Yamasaki et al., 2009). L-SIGN binds both Man9GlcNAc2 and

mannose, however, the binding affinity to Man9GlcNAc2 is 17-fold more than mannose (Mitchell et al., 2001). Future work in the lab will investigate the ability of L-SIGN to bind Psl. This binding might lead to association of biofilms to sinusoidal cells.

6.6 Lectin receptors could be involved recognition of biofilms produced by different ESKAPE organisms.

The glycosyl analysis of carbohydrates produced by ESKAPE organisms revealed that these can be classified as follows (Bales et al., 2013) :

- Mannose-rich such as PA and *Acinetobacter baumannii*
- Mannose-Rhamnose-Glucose-Galactose-rich such as *Klebsiella pneumoniae* (Strain-dependent)
- Mannose-Glucose-rich such as *S. epidermidis*
- Fucose-Glucose-N-acetyl-galactosamine-rich such as *Escherichia coli*

Based on these results it is likely that biofilms generated by these organisms will engage a different set of lectin receptors. Thus, it is likely that there are several ways for biofilms to modulate immunity; differences in carbohydrates along with other biofilm components will determine how immunity will be influenced by these structures. Furthermore, because in many instances biofilms are polymicrobial (Peters et al., 2012), it is conceivable that new carbohydrate structures will be present under these conditions further extending the range of lectins being involved.

6.7 General conclusions and future work

6.7.1 Examining whether carbohydrate associated to PA biofilms affect the activation of human myeloid populations.

To test if the presence of Psl and/or Pel in PA biofilms affect the activation of immune cells, biofilms from WT, Δ*psl*, and Δ*pel*, could be incubated with human myeloid cells. It should be considered that bacterial cultures might affect receptors expression and signalling in myeloid cells, thus different populations of human monocyte ($CD14^+$) can be included such as : 1) M-CSF- macrophages (MR^+/DC-$SIGN^-$, M2-like cells), 2) GM-CSF-macrophages (MR^+/DC-$SIGN^-$, M1-like cells), 3) M-CSF/IL4-macrophages (MR^+/DC-$SIGN^+$, tolerogenic cells), and 4) GM-CSF/IL4-macrophages (MR^+/DC-$SIGN^+$, immunogenic cells). Assessing the cellular activation can be achieved by quantifying pro-inflammatory cytokines (TNF-α, IL-12,1L-6, and IL-1β), anti-inflammatory cytokines (IL-10 and TGF-β), and MHC-II expression as well as activation markers (CD80, CD83, and CD86). This could provide a better understanding of the role of biofilm EPS (if any) in myeloid cells activation.

To further test the activity of biofilm-treated myeloid cells on T cells, myeloid cells, which have been incubated with biofilms, could be collected and co-cultured with human $CD4^+$ T cells and effect on proliferation could be tested using flow cytometry. Also, supernatants can be collected from the co-culture and used to treat $CD4^+$ T cells stimulated with CD3/CD28 beads. The generation of Th-1, Th2, Th17, and T-reg could be investigated by measuring the level of IFN- γ, IL-4, IL-17A, and IL-10, respectively, by flow cytometry. If a Th2 bias is observed in the presence of Psl, this would be in agreement with Th2/Th17 response reported in CF, and MR and/or DC-SIGN could be key players in the establishment of chronic infection in CF.

6.7.2 Examining whether carbohydrate of PA biofilm affects the activation of human myeloid cells.

Different Psl preparations should be purified and tested for carbohydrate composition, linkage, and ability to activate both surface and endosomal TLRs. Any preparations with none or minimal protein and lipid contamination and that do not activate TLRs can be used to stimulate the four types of cell mentioned above in presence and absence of TLRs agonist or PA. This can be followed by assessing the activation and proliferation of T as above.

6.7.3 Examining whether the ability of planktonic PA to interact with DC-SIGN affects its interaction with myeloid cells.

Fixed Planktonic PA could be labelled with DC-SIGN and analysed using super resolution microscopy. PA LPS preparations from PA WT, CPA-deficient, OSA-deficient, or CPA/OSA-deficient can be incubated with DC-SIGN and analysed for binding. Planktonic PA WT, CPA-deficient, OSA-deficient, or CPA/OSA-deficient can be used to infect cultures of M-CSF/IL4-macrophages (MR^+/$DC\text{-}SIGN^+$, tolerogenic cells), and GM-CSF/IL4-macrophages (MR^+/$DC\text{-}SIGN^+$, immunogenic cells) in order to assess cytokines production and T cell proliferation as above.

Summary

To conclude, we have shown that CTLD4-7, a region of MR, and DC-SIGN binds to PA biofilms with DC-SIGN binding significantly better than MR. Both lectins also recognised several independent preparations of EPS lacking Pel. We also found that DC-SIGN also binds to planktonic PA in the absence of Psl and Pel which indicates that DC-SIGN could recognize non-EPS carbohydrate-containing ligands in the bacteria. Further investigation unveiled that DC-SIGN requires the presence of the common polysaccharide antigen (CPA) which is shared among all PA serotypes to bind planktonic cells. These results indicate that CPA is a candidate ligand for DC-SIGN in PA.

These findings shed light on the potential impact of PA Psl and CPA-LPS on the recognition of PA by immune cells expressing CLRs and might open new avenues for therapeutic approaches.

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